HANDBOOK OF

Analysis of Edible Animal By-Products

Edited by
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FIDEL TOLDRÁ

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Preface

Offal or edible and inedible animal by-products comprise a wide variety of products like the skin, blood, bones, meat trimmings and mechanically separated meat, fatty tissues, horns, hoofs, feet, skull, and entrails and internal organs of a butchered animal. Depending on cultures and countries, edible by-products may be considered as waste material being thrown away or as delicacies commanding high prices. Offal not used directly for human or pet food is often processed as material that is used for animal feed, fertilizer, or fuel.

This book contains 23 chapters classified into 4 parts: Part I (Chemistry and Biochemistry— Chapters 1 through 5), Part II (Technological Quality— Chapter 6), Part III: (Nutritional Quality— Chapters 7 through 11), and Part IV (Safety— Chapters 12 through 23).

Chapter 1 introduces readers to the topic of the book. Chapters 2 through 5 focus on the analysis of chemical and biochemical compounds of animal by-products. The usage and detection of food-grade proteins and analysis of rendered fats and cholesterol are detailed. One chapter discusses oxidation in edible animal by-products. Chapter 6 describes the measurement methods of color in these types of products.

Chapters 7 through 11 deal with the analysis of composition and nutrients in animal by-products, such as essential amino acids, fatty acids, vitamins, minerals, and trace elements.

Chapters 12 through 23 deal with safety parameters, especially analytical tools for the detection of pathogens, toxins, and chemical toxic compounds usually found in muscle foods. Some chapters discuss tissues typically found in animal by-products, such as neuronal tissues, non-muscle tissues, and bone fragments.

This unique handbook is intended to provide readers with a full overview of the analytical tools available for the analysis of animal by-products and the role of these techniques and methodologies for the analysis of technological, nutritional, and sensory quality, as well as for safety aspects. In short, this book deals with the main types of analytical techniques and methodologies available worldwide for the analysis of animal by-products.

It was not an easy task to find authors for such chapters. We would like to thank all the contributing authors for their excellent efforts and hard work.

> It is better to keep your mouth closed and let people think you are a fool than to open it and remove all doubt.
>
> **Mark Twain**

Leo M.L. Nollet
Fidel Toldrá

Editors

Dr. Leo M.L. Nollet has been the editor and associate editor of numerous books. He edited for Marcel Dekker, New York (now CRC Press of the Taylor & Francis Group), the first and second editions of *Food Analysis by HPLC* (2000) and the *Handbook of Food Analysis* (2004). The last edition is a three-volume book. He also edited the *Handbook of Water Analysis, Chromatographic Analysis of the Environment*, third edition (CRC Press, 2005), and the second edition of the *Handbook of Water Analysis* (CRC Press, 2007).

With F. Toldra, he has coedited two books: *Advanced Technologies for Meat Processing* (CRC Press, 2006) and *Advances in Food Diagnostics* (Blackwell, 2007).

With M. Pöschl, he coedited *Radionuclide Concentrations in Foods and the Environment* (CRC Press, 2006).

He has coedited several books with Y. H. Hui and other colleagues: the *Handbook of Food Product Manufacturing* (Wiley, 2007); the *Handbook of Food Science, Technology, and Engineering* (CRC Press, 2005); *Food Biochemistry and Food Processing* (Blackwell, 2005); and the *Handbook of Flavors from Fruits and Vegetables* (Wiley, 2010).

Finally, he edited the *Handbook of Meat, Poultry and Seafood Quality* (Blackwell, 2007) and *Analysis of Endocrine Compounds in Foods* (Blackwell-Wiley, 2010).

With F. Toldra, he prepared or is preparing six books on meat analysis methodologies:

- *Handbook of Muscle Foods Analysis*
- *Handbook of Processed Meats and Poultry Analysis*
- *Handbook of Seafood and Seafood Products Analysis*
- *Handbook of Dairy Foods Analysis*
- *Handbook of Analysis of Edible Animal By-Products*
- *Handbook of Analysis of Active Compounds in Functional Foods*

With H. Rathore, he worked or is working on two books related to pesticide analysis: the *Handbook of Pesticides: Methods of Pesticides Residues Analysis* (CRC Press, 2009) and *Pesticides: Evaluation of Environmental Pollution.*

Dr. Nollet received his MS (1973) and PhD (1978) degrees in biology from the Katholieke Universiteit Leuven, Belgium. He is a professor at University College Ghent (Hogeschool Gent), a member of Ghent University Association, Faculty of Applied Engineering Sciences, Ghent, Belgium.

Dr. Fidel Toldrá, PhD, is a research professor at the Department of Food Science, Instituto de Agroquímica y Tecnología de Alimentos (CSIC), and serves as European editor of *Trends in Food Science & Technology*, editor in chief of *Current Nutrition & Food Science*, and member of CEF Panel at the European Food Safety Authority. He is a member of the editorial board of eight journals, including *Food Chemistry*, *Meat Science,* and *Food Analytical Methods.* He has acted as editor or associate editor of several books in recent years. He was the editor of *Research Advances in the Quality of Meat and Meat Products* (Research Signpost, 2002) and associate editor of the *Handbook of Food and Beverage Fermentation Technology* and the *Handbook of Food Science, Technology, and Engineering* published in 2004 and 2006, respectively, by CRC Press. In collaboration with L. Nollet, he coedited two books published in 2006: *Advanced Technologies for Meat Processing* (CRC Press) and *Advances in Food Diagnostics* (Blackwell Publishing). Both were also associate editors of the *Handbook of Food Product Manufacturing* published by John Wiley & Sons in 2007. Professor Toldrá edited the books *Meat Biotechnology* (2008, Springer) and *Safety of*

Meat and Processed Meat (2009, Springer) and also authored the book *Dry-Cured Meat Products* published by Food & Nutrition Press (now Blackwell) in 2002.

With F. Toldra, he prepared or is preparing five books on meat analysis methodologies:

- *Handbook of Muscle Foods Analysis*
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- *Handbook of Dairy Foods Analysis*
- *Handbook of Analysis of Edible Animal By-Products*

Both are also preparing the *Handbook of Analysis of Active Compounds in Functional Foods*, also for CRC Press.

Dr. Toldrá was awarded the 2002 International Prize for meat science and technology by the International Meat Secretariat and the Distinguished Research Award in 2010 by the American Meat Science Association. He was elected in 2008 as Fellow of the International Academy of Food Science & Technology (IAFOST) and in 2009 as Fellow of the Institute of Food Technologists.

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Part I

Chemistry and Biochemistry

1 Introduction—Offal Meat: Definitions, Regions, Cultures, and Generalities

Leo M.L. Nollet and Fidel Toldrá

CONTENTS

1.1 INTRODUCTION

Offal or edible and inedible animal by-products comprise a wide variety of products like the skin, blood, bones, meat trimmings and mechanically separated meat, fatty tissues, horns, hoofs, feet and skull, and entrails and internal organs of a butchered animal [1,2]. Depending on cultures and countries, edible by-products may be considered as waste material being thrown away, or as delicacies commanding high prices. Offal not used directly for human or pet food is often processed as material that is used for animal feed, fertilizer, or fuel [3]. The yield of edible by-products depends on species, sex, age, live weight, and others. This yield varies from 10% to 30% for beef, pork and lamb and from 5% to 6% for chicken. Yields of different by-products for different species are shown in Table 1.1.

Consumption of meat has increased in recent years; however, the use of its by-products for human consumption has decreased. Most consumed animal by-products are liver, heart, kidney, tongue, thymus or sweetbreads, brain, and tripe.

In some parts of Europe, scrotum, brain, chitterlings (the large intestine of a pig), trotters (feet), heart, head (of pigs, calves, sheep and lamb), kidney, liver, lights (lung), sweetbreads (thymus), fries (testicles), tongue, snout (nose), and tripe (stomach) from various mammals are common menu items.

TABLE 1.1
Weight of By-Products

	By-Product: Percentage of Live Weight			
	Beef	**Hog or Pig**	**Lamb**	**Chicken**
Blood	2.4–6	2–6	4–9	1.4–2.3 kg
Brain	0.08–0.12	0.08–0.1	0.26	0.2–0.3
Chitterlings	0.06			
Ears	0.02			
Feet	1.9–2.1	1.5–2.2	2.0	
Gizzard				1.9–2.3
Gullet	0.03	0.1		
Head		5.2	6.7	
Heart	0.3–0.5	0.15–0.35	0.3–1.1	0.3–0.8
Intestines		1.8	3.3	
Kidney	0.07–0.24	0.2–0.4	0.3–0.6	
Liver	1.0–4.5	1.1–2.4	0.9–2.2	1.6–2.3
Lungs	0.4–0.8	0.4–0.85	0.7–2.2	0.7
Pancreas	0.06	0.1	0.2	
Penis	0.18			
Spleen	0.1–0.27	0.1–0.16	0.1–0.4	0.15
Tail	0.1–0.25	0.1		
Tongue	0.25–0.5	0.3–0.4		
Tripe	0.75	0.6–0.7	2.9–4.6	

Source: Ockerman, H.W. and Basu, L., By-products/edible, for human consumption, in: Devine, C., Dikeman, M., and Jensen, W.K. (Eds.), *Encyclopedia of Meat Sciences*, Academic Press, New York, 2004, pp. 104–112. With permission.

Mammalian offal is slightly more popular in the southern parts of the United States, where some recipes include chitterlings, chicken gizzards and livers, and pig stomach (hog maw). Scrapple, sometimes made from pork offal, is more common in the northeast United States. Traditional recipes for turkey gravy include giblets of the bird.

In Australia, offal is most commonly consumed in meat pies, or in ethnic dishes. Addition of offal to food and labeling is regulated.

In China, different organs and other parts of animals are used for food or traditional Chinese medicine. Pork is the most consumed meat in China. Popular pork offal dishes include stir-fried cleaned pork kidneys or a spicy stew with pork intestine slices and pork blood cubes.

1.2 NUTRIENT CONTENT

Edible animal by-products are significant sources of nutrients. Examples of typical protein, fat, mineral, and vitamin contents of different organs of beef and pork are shown in Tables 1.2 and 1.3. In general, they have a good nutritional value due to the high protein and low fat levels as well as good content in vitamins and minerals. Liver contains the largest amounts of nutrients, especially B group vitamins, copper, and manganese. In the case of cholesterol, the brain is the organ with substantial larger amounts, while the contents in liver and kidneys are also relatively high [4].

TABLE 1.2
Components per 100 g of Beef Offal

	Protein (g)	Fat (g)	Ca (mg)	P (mg)	Fe (mg)	Na (mg)	K (mg)	Mg (mg)	Zn (mg)	Cu (mg)	Mn (mg)
Brain	10.4–11.5	8.6	10	312	2.1–2.4	125	219	13	1.22	0.20	0.04
Heart	14.9–28.5	3.6–20.0	5	195–230	4.0–4.9	86–95	193–320	23	2.38	0.36	0.04
Kidney	15.3–24.7	2.6–6.7	10–11	219–230	5.7–7.4	176–180	225–230	17	1.85	0.47	0.10
Liver	19.0–22.9	3.8–7.8	6–8	352–360	6.5–7.0	81–136	281–320	19	3.92	2.76	0.26
Pancreas	17.6–27.1	7.3	8	216–330	2.8–8.4	67	276	18	2.58	0.06	0.15
Tongue	15.3–22.2	10.4–14.6	6–8	170–182	2.1–2.9	73	197–250	16	2.47	0.17	0.03

	Thiamin (mg)	Riboflavin (mg)	Niacin (mg)	Vitamin B6 (mg)	Pantothenate (mg)	Biotin (µg)	Folacin (µg)	Vitamin B12 (µg)	Vitamin A (IU)	Ascorbic Acid (mg)	Cholesterol (mg)
Brain	0.07–0.23	0.22–0.26	3.0–4.7	0.10–0.26	2.5	2.0–6.1	4–12	7–4.7–10.9	Nihil	16.6–23.0	—
Heart	0.19–0.68	0.23–0.43	6.3–9.5	0.23–0.43	1.2–2.3	2.0–7.3	2–110	8.0–13.7	Traces-3.0	2.0–7.6	140
Kidney	0.28–0.38	0.32–0.44	5.4–7.9	0.32–0.44	3.4	24.0–92.0	41–77	8.5–31.0	264–880	8.9–15.0	285
Liver	0.23–0.28	0.74–0.94	12.8–21.0	0.74–0.94	5.5–8.3	33.0–100.0	81–330	65.0–110.0	12709–105032	2.6–31.0	354
Pancreas	0.14	0.20	3.1–5.8	0.20	3.8	14.0	—	4.8–5.0	Nihil	13.7–14.0	—
Tongue	0.12–0.17	0.13–0.31	3.9–4.9	0.13–0.31	2.0	1.0–3.3	4–7	3.8–7.0	Nihil	31–7.0	87

Sources: Ockerman, H.W. and Basu, L., By-products/edible, for human consumption, in: Devine, C., Dikeman, M., and Jensen, W.K. (Eds.), *Encyclopedia of Meat Sciences*, Academic Press, New York, 2004, pp. 104–112; Anderson, B.A., Composition and nutritional value of edible meat by-products, in: Pearson, A.M. and Dutson, T.R. (Eds.), *Edible Meat By-Products. Advances in Meat Research*, Elsevier Applied Science, London, U.K., 1988, vol. 5, pp. 15–45.

TABLE 1.3
Components per 100 g of Pork Offal

	Protein (g)	Fat (g)	Ca (mg)	P (mg)	Fe (mg)	Na (mg)	K (mg)	Mg (mg)	Zn (mg)	Cu (mg)	Mn (mg)
Brain	10.3–122	8.6–9.2	10	312	1.6–2.4	125	219	14	1.27	0.24	0.09
Heart	16.8–23.5	2.7–4.4	3–6	131–220	3.3–4.8	54–80	106–300	19	2.80	0.41	0.06
Kidney	15.4–25.4	2.7–3.6	8–11	218–270	5.0–6.7	115–190	178–290	17	2.75	0.62	0.12
Liver	18.9–21.6	2.4–6.8	6–10	356–370	19.2–21.0	73–87	271–320	18	5.76	0.68	0.34
Pancreas	28.5	4.0–15.0	—	—	18.9	—	—	17	2.62	0.09	0.16

	Thiamin (mg)	Riboflavin (mg)	Niacin (mg)	Vitamin B6 (mg)	Pantothenate (mg)	Biotin (µg)	Folacin (mg)	Vitamin B12 (µg)	Vitamin A (IU)	Ascorbic Acid (mg)	Cholesterol (mg)
Brain	0.16–0.23	0.26–0.28	4.3–4.4	0.19	2.8	—	6.0	2.2–2.8	Nihil	13.5–18.0	2195
Heart	0.13–0.16	0.81–1.24	6.6–9.6	0.29–0.39	2.5	4.0–18.0	2–4	2.4–8.0	Traces-106	3.0–5.3	131
Kidney	0.26–0.58	1.70–1.90	7.5–9.8	0.55	3.1	32.0–130	42	6.6–14.0	130–230	14.0–14.2	319
Liver	0.28–0.31	3.00	14.8–16.4	0.68–0.69	0.9	27.0	110–212	25.0–26.0	Nihil–10900	13.0–25.3	301
Pancreas	0.11	0.46	3.5	—	4.6	—	—	6.5–7.0	Nihil	15.0–15.3	—

Sources: Ockerman, H.W. and Basu, L., By-products/edible, for human consumption, in: Devine, C., Dikeman, M., and Jensen, W.K. (Eds.), *Encyclopedia of Meat Sciences*, Academic Press, New York, 2004, pp. 104–112; Anderson, B.A., Composition and nutritional value of edible meat by-products, in: Pearson, A.M. and Dutson, T.R. (Eds.), *Edible Meat By-Products. Advances in Meat Research*, Elsevier Applied Science, London, U.K., 1988, vol. 5, pp. 15–45.

1.3 MAIN EDIBLE ANIMAL BY-PRODUCTS AND ITS CONSUMPTION

1.3.1 Liver

The liver of beef, veal, lamb, and pork weights in average 5, 1.5, 1.4, and 1.4 kg, respectively. Liver is mostly thinly sliced and cooked. Further on it may be minced and incorporated in many preparations, e.g., braunschweiger, liver paste, and liverwurst.

Liver is one of the most nutritious parts of by-products and constitutes a rich source of vitamins B_{12} and A.

In the United Kingdom, Midlands faggots are made from ground or minced pig offal (mainly liver and cheek), bread, herbs, and onion wrapped in pig's caul. A similar dish, almôndega or meatball, is traditional in Portugal.

Ground chicken livers, mixed with chicken fat and onions, called chopped liver, is a popular Jewish dish.

1.3.2 Heart

The heart of beef, veal, pork, and lamb averages 1.4 kg, 227 g, 227 g, and 113 g, respectively. Hearts must be cooked for longer periods. So, they are diced and added to stews or other meat to add protein and color. In Perú, cow heart is used for anticuchos, a sort of brochettes. Anticuchos can be made of any type of meat, the most popular are made of beef heart (anticuchos de corazón). In Brazil, churrasco often includes chicken hearts, roasted on a big skewer.

1.3.3 Tongue

The tongue of beef, veal, pork, and lamb weighs ±2, 0.7, 0.3, and 0.2 kg, respectively. After blanching, the outer membrane is removed.

In some countries of South America, the tongue is usually boiled, sliced, and marinated with a mixture of oil, vinegar, salt, chopped peppers, and garlic.

1.3.4 Kidney

The pair of beef and veal kidneys are lobed and weigh ±0.5 kg each for beef or 340 g for veal. Sheep and pork kidneys have one lobe and weigh ±57 and 110 g, respectively. Kidneys may be added to meat casseroles, stews, and pies. Steak and kidney pie, typically with veal or beef kidneys, is widely known and enjoyed in the United Kingdom.

1.3.5 Sweetbreads

Sweetbreads are gathered from calves or lambs. Thymus or neck sweetbread (throat sweetbread) and heart sweetbread, degenerates in adult animals. Pancreas is called gut bread or stomach sweetbread. Thymus sweetbread is very likely to spoil.

1.3.6 Tripe

Beef tripe consists of the first and second stomachs of cattle. Stomachs of sheep and pork are also used for tripe. Beef first and second stomachs weigh ±3.9 kg; sheep stomach ±1 kg and pork stomach ±1.2 kg.

The traditional Scottish haggis consists of sheep stomach stuffed with a boiled mix of liver, heart, lungs, rolled oats, and other ingredients. Most modern commercial haggis is prepared in a casing rather than an actual stomach. Sometimes haggis is sold in tins, which can simply be microwaved or ovenbaked. Some supermarket haggis is largely made from pig, rather than sheep, offal.

Drob is in Romania a dish similar to haggis. It is served on Easter.

In Bulgaria, Republic of Macedonia and Turkey, Shkembe chorba is a widespread soup variety made from tripe.

Tripes are extensively used in Spain as casings for stuffing in the manufacture of traditional semidry and dry-fermented sausages. In some Latin American countries, tripe is used to make menudo and mondongo. The soup menudo is a traditional Mexican dish: a spicy soup made with tripe. Sopa de mondongo is a hearty traditional soup of Latin America and the Caribbean. It is made from slow-cooked diced tripe.

In the Chinese mainland, beef tripe is used as a cold appetizer.

Cooked buffalo tripe rolls were prepared from a combination of buffalo tripe (75%) and buffalo meat (25%) by using mincing and blade tenderization and their quality was then evaluated [5]. They were stored at 4°C ± 1°C and studied for various physicochemical, sensory and microbial qualities.

1.3.7 Chitterlings

Chitterlings are the intestines and rectum of a pig that have been prepared as food. As pigs are a common source of meat throughout the world, the dish known as chitterlings can be found in most pork-eating cultures. Chitterlings are popular in most parts of Europe, where pig intestines are also used as casing for sausages. In England, chitterlings remain especially popular in Yorkshire.

They are eaten as a dish in East Asian cuisines. In America, chitterlings are an African-American culinary tradition and a Southern culinary tradition sometimes called soul food cooking. In America, chitterlings are sometimes battered and fried after the stewing process.

Chitterlings are carefully cleaned and rinsed several times before they are boiled or stewed for several hours. Pajata is a traditional dish from Rome, Italy. It refers to the intestines of an unweaned calf, i.e., only fed on its mother's milk. The calf is killed soon after nursing. The intestines are cleaned, but the milk is left inside. When cooked, the combination of heat and the enzyme rennet in the intestines coagulates the milk and creates a sort of thick, creamy, cheese-like sauce.

In France, chitterlings sausage, a delicacy, is called andouillette.

Care must be taken when preparing chitterlings, due to the possibility of diseases. These diseases are caused by bacteria including *E. coli* and *Yersinia enterocolitica*, or *Salmonella*.

1.3.8 Brains

Beef brains weigh ±450 g; pork brains ±120 g and lamb brains ±130 g. Cattle brains belong to risk material and is thus forbidden because of its relation to bovine spongiform encephalopathy (BSE) or "mad cow" disease. Brains of other animals are edible but very perishable.

Fried-brain sandwiches are a specialty in the Ohio River Valley. Cow's brains, sesos, are used to make ravioli stuffing in some countries of South America. Sesos also constitute a typical and popular dish in Spain.

1.3.9 Testicles

Testicles of a bull or a ram weight ±0.25 kg; the testicles of a boar weigh ±130 g. Rocky Mountain oysters are a delicacy eaten in some cattle-raising parts of the western United States and Canada. Rocky Mountain oysters or prairie oysters is a North American culinary name for edible offal, specifically buffalo, boar, or bull testicles.

1.3.10 Blood

Beef contains 10–12 L of blood and a sheep ±1.5 L. Pork blood is used in Spain as an ingredient in a kind of typical cooked sausages called "morcilla." It is used in other cooked meat products due to

its good binding of fat and water. Plasma is the most interesting part of blood due to its color and functional properties. Albumin is the main component of the plasma fraction of blood and is the main responsible for plasma gel firmness upon heating [6]. Blood proteins also have an excellent foaming capacity.

1.3.11 Lard and Tallow

Lard is considered as the fat rendered from edible pork tissues while tallow is defined as the hard fat rendered from fatty tissues in cattle [7]. The fatty acid composition of these fats, especially lard, depends on the feed given to the animal before slaughter. Tallow may also contain some trans-fatty acids, including the conjugated linoleic acid (CLA), due to the action of rumen. There are many traditional uses for lard and tallow like deep-fat frying, use in margarine and shortenings, cover of dry-cured hams, etc.

1.3.12 Other Edible By-Products

Other parts suitable for human consumption are spleen, oxtail, bones for stock, trimmings such as beef diaphragm muscle, gullet or beef cheek papillae, pork jowl, pig tail, pigs' feet (trotters) and ears and poultry giblets.

In the United States, the giblets of chickens, turkeys and ducks are commonly consumed.

Brawn is a British English term for head cheese or the collection of meat and tissue found on an animal's skull (typically a pig) that is chilled and set in gelatin.

Iceland has its own version of both haggis and brawn. The Icelandic haggis, slátur, is made in two versions. Blóðmör or bloodlard is a stomach of a sheep stuffed with a mixture of sheep's blood, rolled oats and slices of sheep's fat, and lifrarpylsa or liver sausage consisting of sheep stomach stuffed with a mixture of ground lamb's liver, rolled oats, and cut up bits of sheep. The Icelandic brawn, svið, is made from singed sheep heads.

Romanian peasants make a kind of traditional sausages from pork offal, called caltabos. In Greece and in Turkey, splinantero consists of liver, spleen, and small intestine, roasted over an open fire. A festive variety is kokoretsi, made of pieces of lamb offal (liver, heart, lungs, spleen, kidney, and fat). These pieces are pierced on a spit and covered by washed small intestine wound around in a tube-like fashion. It is a traditional dish for Easter. Another traditional Easter food is mageiritsa: a soup made with lamb offal and lettuce in a white sauce.

An Armenian traditional dish is khash. The main ingredient in khash is pig's or cow's feet, although other animal parts, such as the ears and tripe may also be used. It is rich in cartilage and other connective tissues.

In Italy, consumption of entrails and internal organs is quite widespread, among the most popular preparations are fried or stewed brain, boiled intestines (trippa), lampredotto (the fourth stomach of the cow), liver, kidneys, heart and coronaries (coratella or animelle), head, eyes, testicles of pig; several preparations are based on chicken entrails.

In Sicily, many enjoy a type of sandwich pani ca meusa, or bread with spleen and caciocavallo cheese. In New York, it is named vastedda.

In Spain, the organs of the entrails are used in many traditional dishes: callos or cow tripe in Madrid and Asturias, liver, kidneys, criadillas or bull's testicles, cow's tongue, pork's head and feet in Catalonia and pork's ears in Galicia.

In the French city of Marseille lamb's trotters and a package of lamb tripe are a traditional food, pieds et paquets.

Feijoada is a stew of beans with beef and pork meats, ears, feet and tail, which is a typical Portuguese dish, also typical in Brazil, Angola and other former Portuguese colonies.

Lungen stew is a traditional dish among American Jews. In Argentina, Chile and Uruguay, the traditional asado is often made along with several offal types, achuras, like chinchulines and tripa gorda (chitterlings), mollejas (sweetbread) and riñón (kidney of a cow).

Pork tongue slices with salt and sesame oil is a common dish, especially in Sichuan province of China. Braised pork ear strips are available as street merchant food or in some supermarkets. Cleaned pork stomach roasted primarily in sugar and soy sauce then sliced is a popular food in Hong Kong. Pork liver slices served stir fried with onions or in soups is another hawker food. Pork blood soup is at least 1000 years old.

The offal of cattle, duck, and chicken is also used in traditional Chinese cooking. The Cantonese dish lou mei is made by simmering the organs and off-cuts of these animals in a soy-based sauce.

In Korea, offal usage is very similar to mainland China but less frequent. In Singapore, soup with organs of pigs is common for hawkers.

In Indonesia, goat's organs are very popular for soups and almost all of the parts of the animal are eaten. Babat or the stomach of cows and iso (intestines of cows) are popular in Javanese cuisine.

In Japan, chicken offal is often skewered and grilled over charcoal as yakitori. Offal originating from cattle is also an ingredient in certain dishes. However, Japanese culture mostly declines the use of offal from large animals due to the traditional Japanese preference for cleanliness, derived from Shinto purity beliefs.

In the Philippines, people eat practically every part of the pig, including snout, intestines, ears, and innards. Dinuguan is a particular type of blood-stew using pig intestines, pork meat and sometimes ears and cheeks. Bopis is a spicy dish with pork lungs and heart. Isaw is another course in the Philippines. It is a kebab made with pieces of the large intestine of a pork barbecued.

In India, Pakistan, Nepal, and Bangladesh, different parts of the goat, brain, feet, head, stomach, tongue, liver, kidney, udder, and testicles are eaten. The heart and liver of chickens are also enjoyed. One popular dish, Kata-Kat, is a combination of spices, brains, liver, kidneys and other organs.

Rakhti is a combination of heavily spiced porcine offal and cartilaginous tissue, consumed by the local Christian community in southern India.

In Lebanon, lamb brain is used in *nikhaat* dishes and sometimes as a sandwich filling. Another popular dish is korouch, rice-stuffed sheep intestine.

In Iran, sheep liver, heart, and kidneys are used as certain types of kebab and are frequently eaten, as well as sheep intestines and stomach. Sheep brains and tongue are eaten with traditional bread.

1.4 FOOD SAFETY ISSUES

Offal of certain animals may be unsafe to consume. Some animal intestines are very high in coliform bacteria and need to be washed and cooked thoroughly to be safe for eating. Wong et al. [8] discussed the presence of the pathogens *Salmonella* and *Escherichia coli* O157:H7, and *E. coli* biotype 1 on 100 New Zealand-produced pig carcasses and 110 imported pig meat samples.

Bhandare et al. [9] investigated the microbial contamination (*Staphylococcus—Bacillus—Enterococcus—Clostridium—Enterobacteriaceae—Pseudomonaceae—Faecal Coliforms—Salmonella*) on sheep/goat carcasses in a modern Indian abattoir and traditional meat shops.

Nervous system tissue can be contaminated with transmissible spongiform encephalopathies (TSE) prions, which cause BSE or mad cow disease. Some tissues are classified as specified risk materials and are subject to special regulations. So, certain tissues like tonsils, intestine, brain, and spinal cord from cattle, sheep, and goats were controlled since 1989 in the United Kingdom and prevented to enter the human food chain and in 2000 were harmonized through the EU [10]. The production of mechanically recovered meat from all ruminant bones was also prohibited in the EU [10]. Adequate detection methodologies for the detection of BSE in meat and meat products, based on the detection of markers from the lipids, proteins, or nucleic acids fractions were developed [11]. Offal may contain high quantities of purines able to provoke an acute attack of gout.

Heterocyclic amines (HAs) are potent mutagens formed during intense heat-processing of proteinaceous food. PhIP (2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine) is the most ubiquitous and abundant mutagenic HA. In the study of Khan et al. [12], several offal products (beef liver, lamb

kidney and beef tongue) were thermally processed and analyzed for HAs. Norharman and harman were the amines most abundant, found at concentrations below 2 ng g^{-1}.

The practice of feeding raw offal to dogs on farms and ranches can spread echinococcosis, a potentially fatal parasitic disease of many animals, including wildlife, commercial livestock, and humans. The disease results from infection by tapeworm larvae of the genus *Echinococcus* (*E. granulosus*, *E. multilocularis*, *E. vogeli*, and *E. oligarthrus*).

Offal of bovine, ovine, and porcine may accumulate potentially toxic heavy metals, such as Cd and Pb, posing a risk for human health and making necessary the development of new methodologies like SF-ICP-MS for its quantification [13].

In summary, there is a wide variety of animal edible by-products with traditional consumption, sometimes high, in many countries worldwide. This book provides a full overview of the analytical tools available for the analysis of animal by-products and the role of these methodologies in the analysis of technological, nutritional, and sensory quality, as well as for safety aspects.

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2 Food-Grade Proteins from Animal By-Products: Their Usage and Detection Methods

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CONTENTS

2.1 INTRODUCTION

The parts of an animal that are not intended for direct use as human food are referred to as animal by-products. In the United States, the meat industry considers everything produced by or from an animal, with the exception of dressed meat, to be a by-product. These by-products include bones, skin, trimmed fat and connective tissues, feet, abdominal and intestinal contents, and blood. Biologically, however, most of the non-carcass material obtained after slaughtering the animal is edible once it has been properly cleansed, handled, and processed. Globally, the use of these non-carcass materials in the human food chain varies from region to region depending on such factors as religion and custom, with organs such as the liver, heart, kidney, and tongue being the most commonly used. Other edible parts include poultry feet, pig's feet, the brain, blood, and so on [1].

Animal by-products fall into two categories, namely edible and inedible, although the distinction is not always clear cut and may vary from situation to situation. For example, bovine liver is a beneficial by-product when passed as suitable for human consumption, but if it is infected with fascioliasis it is inedible [2]. Other animal by-products that are initially considered to be unfit for

human consumption when produced at the slaughter house may, after further processing, become fit for human consumption. For example, bones, connective tissues, hides, and skins can be processed to make gelatin and collagen, while sheep intestines can be turned into casings for use in sausage type products. Such products are referred to as edible co-products and must not be confused with edible by-products, which include parts of animals that are perfectly fit for human consumption but are not meant for such [3]. Animal by-products that are absolutely unfit for human consumption are used in animal feedstuffs such as meat-and-bone-meal, pet food, and in other technical products, such as glue, leathers, soaps, and fertilizers. The alternative is their destruction, often by incineration. This classification may change over time; whey, the watery part of milk that separates from the curd when milk is curdled in the production of cheese, used to be considered a by-product of the cheese industry and was disposed of as such, but is now widely used in the food industry. Whey is thus an animal by-product that for the purposes of this chapter will be included as a food grade protein source.

The combined livestock and poultry industry represents the largest agricultural businesses in the United States. According to the United States Department of Agriculture (USDA), it was forecast that 57,038 metric tons of beef and veal (carcass weight equivalent), 100,171 metric tons of pork (carcass weight equivalent), and 76,227 metric tons of broiler and turkey (ready to cook equivalent) meat would be consumed in 2009 [4]. As reported by the Food and Fertilizer Technology Center for the Asian and Pacific Region publication, by-products constitute 52%, 66%, and 68% of the live weight of pigs, cattle, and sheep, respectively; this implies that billions of kilograms of animal by-products are produced annually and a valuable source of revenue will be lost if these products are not well utilized. Also, disposing of unwanted by-products is expensive and continues to rise [1]; even worthless by-products must be disposed of in an environmentally responsible manner, which adds to the cost of meat production. As the selling price of the carcass (dressed meat) alone cannot compensate for the high cost of rearing the live animal, a great deal of effort has been devoted to finding ways to fully utilize these animal by-products, not only to increase profits and hence the viability of the meat industry, but also to address issues of environmental pollution associated with their inefficient disposal. However, despite the strenuous efforts that have already been made to fully utilize animal by-products, more than 2% of the weight of the carcass is still lost to effluent and there is therefore more room for improvement in this area [5].

This chapter will examine four food grade protein materials derived from major animal by-products, namely, blood, collagen, gelatin, and whey. Their use as ingredients in food production will be discussed, along with possible concerns associated with their use in food product formulation and the analytical methods for their detection that have been developed to address these concerns.

2.2 BLOOD

For many years, blood, which is the first by-product obtained after slaughter of an animal, used to be discarded as an unwanted by-product by slaughter houses in the United States. Blood is made up of two fractions, namely the cellular fraction which comprises the red blood cells, white blood cells and platelets, and the plasma fraction, with the former suspended in the latter. Plasma accounts for 65%–70% of the total volume of blood, with the cellular fraction accounting for the remainder [6]. Animal blood contains about 18% of protein, with hemoglobin, which is present in the red blood cells, accounting for more than half of the proteins present [7]. The typical nutrient composition of bovine blood consists of 80.9% water, 17.3% protein, 0.23% lipid, 0.07% carbohydrate, and 0.62% minerals. [8]. Plasma contains about 7.9% protein, consisting principally of immunoglobulins (4.2%), albumins (3.3%), and fibrinogen (0.4%) [9]. More than one hundred proteins have been well characterized from plasma. Selected specific proteins are listed in Table 2.1.

Of all the unwanted animal by-products, blood causes the most problems as a result of the huge volume produced and its high pollutant load. It is estimated that the annual blood waste in the United States is around 1.6 million tons. Given the large quantities of blood produced and its high solids

TABLE 2.1
Selected Well Characterized Plasma Proteins

Protein	Molecular Weight (kDa)	Amount in Serum (mg%)
Albumin	66	3500–5500
Antithrombin III	65	29
α_1-Acid glycoprotein	44.1	90
α_1-Antitripsin	54	290
α_1-Antichymotrypsin	68	45
α_1B-Glycoprotein	50	22
α_2-HS-Glycoprotein	49	60
α_2-Macroglobulin	820	240–290
β_2-Glycoprotein I	40	20
β_2-Glycoprotein III	35	10
C1 inactivator	104	24
C2	117	3
C3	185	110
C3 activator (β2II)	60	18
Ceruloplasmin	132	35
Cold insoluble globulin	350	33
Fibrinogen	341	300
Gc globulin	50.8	40
Hemopexin	57	80
Haptoglobulin 1-1	100	170–235
Histidine-rich 3.8 S α2-glycoprotein	58.5	9
Immunoglobulin A	158–162	90–450
Immunoglobulin M	800–950	60–250
Immunoglobulin D	175–180	<15
Immunoglobulin E	185–190	<0.06
Inter-α-trypsin inhibitor	160	45
Lipoproteins		
LDL	2500	350
HDL_2		40–90
HDL_3		225
Plasminogen	81	12
Prealbumin (thysoxine-binding)	54.98	25
Pregnancy-specific β1-glycoprotein	90	5–20
Prothrombin	72	6
Retinol-binding protein	21	4.5
Steroid-binding β-globulin	65	0.4–0.8
Thyrpxon-binding prealbumin	54.98	25
Thyroxine-binding globulin	60.7	1.5
Transcortin	55.7	4
Transferrin	80	295
Vitamin D-binding protein	52.8	0.5
Zn-α2-glycoprotein	41	5

Source: Selected from Heide, K. et al., Plasma protein fractionation, in: Putman, F.W. (Ed.), *The Plasma Proteins: Structure, Function, and Genetic Control*, 2nd edn., vol. III, Academic Press, Inc., New York, pp. 558–561, Table V. With permission.

content (18%), and chemical oxygen demand (COD) (500,000 mg O_2/L), the environmental problems caused by its disposal are enormous [10]. Hence efforts to fully utilize blood through the recovery of its proteins are both necessary and justified. The food industry currently uses about 30% of the blood products produced [11], mainly in meat products as a gelling agent and natural colorant. Other applications for blood products are the pet food industry (to increase the palatability of pet food), animal farming (as a feed ingredient), agriculture (as fertilizer), pharmaceutical diagnostic agents (especially bovine serum albumin [BSA] and immunoglobulins), and the paper industry (as glue). This practice of utilizing blood for a wide range of other applications avoids the cost of the environmental compatible disposal of these animal by-products, hence increasing the value of the animal to the farmer [12]. Here, we will focus on the use of blood as an ingredient in the food industry.

2.2.1 Use of Blood and Blood Products as Food Ingredients

Animal blood has long been used in Europe to make blood sausages, biscuits, bread, and blood pudding. It is also used widely in Asia in food products such as blood curd, blood cake, and blood pudding. Although the above-mentioned blood products are not common in the United States, blood finds its way into the human food chain in various forms. The U.S. Meat Inspection Act approves the use of blood in food provided that it is obtained by bleeding an animal that has been inspected and passed for use as meat (9CFR 310.20). Its uses include in sausage products to enhance the color and as an extender in meat products, with the primary purpose of lowering costs [13]. Blood from bovine and porcine origin is most commonly used in the formulation of meat products. The amount of whole blood that is used in meat products is very low however, as increasing the proportion has a detrimental effect on sensory qualities, particularly color and flavor [14]. In sausage products, the use of blood is restricted to 0.5%–2% of the sausage content, as levels above this have a negative impact on the sensory attributes of the final product. Restricting the addition of blood to within this range improves the overall perception of color and meat taste compared to reference samples with no blood added [15]. The adverse effect on sensory qualities associated with increased amounts of blood in meat products and food products in general is attributed to the presence of hemoglobin, which has an objectionable color and odor as a result of the heme component of the protein [7]. Plasma, which has a neutral taste and is devoid of the dark color normally associated with blood, is therefore preferred over whole blood and is more widely used in meat products [16]. Plasma proteins are good emulsifiers and are thus used in emulsified meat systems, where they serve as a source of large quantities of nutritionally beneficial protein [17]. They are also used as protein supplements and fat replacers in meat products such as sausages. Major muscle proteins such as myosin have the ability to cross-link with plasma proteins, enhancing resistance to endogenous protease degradation. Thus, dried plasma is used as an inhibitor for endogenous proteases in surimi-type products made from certain species of fish in order to inhibit degradation by endogenous proteases [18,19]. The predominant use of plasma in the meat industry, however, is as a binder because of its ability to form gels upon heating.

Plasma is produced by removing the blood cells from blood, but unfortunately this also removes the majority of the blood proteins present. This has led to a great deal of research into better ways of incorporating blood cells into food products to take advantage of the high protein content, while at the same time avoiding the undesirable sensory effects of hemoglobin. One approach has been to recover the proteins in the blood cells through decolorizing blood by removing the heme group from hemoglobin to produce what is known commercially as globin or decolorized blood, a more useful product in food formulations [8,20]. In addition to whole blood, plasma and decolorized blood, blood serum concentrates, and other proteins isolated from blood are now used as ingredients in food products. Examples of commercial food grade proteins produced from whole blood and blood fractions for use by the food industry are shown in Table 2.2.

Blood and blood products are used widely in the meat industry, but potential applications in other areas of the food industry have also been suggested by a number of studies for specific situations. For example, spray-dried plasma can be used as an egg substitute in bakery products because

TABLE 2.2
Examples of Food Grade Proteins Produced from Blood and Blood Fractions

Product	Company	Description	Usage
ImmunoLin	Proliant Inc., USA	Serum concentrate	Dietary supplement to be added to bars and drinks to boost immune system
NutraGammax	Proliant Inc., USA	Serum protein isolate	Dietary supplement target the sports nutrition world
Fibrimex	Harimex B.V., the Netherlands	Isolate of thrombin and fibrinogen precipitated from plasma	Natural binder for whole muscle processing
Harimix P	Sonac B.V., the Netherlands	Hemoglobin	Natural meat colorant
Plasma powder FG	Sonac B.V., the Netherlands	Plasma	A binder in meat products
Prietin	Lican Functional Protein Source, Chile	Whole blood	For making Morcilla (blood sausage)
Vepro globin	VEOS Group, Belgium	Globin	An emulsifier in meat products

of the foaming and leavening properties of blood plasma proteins [14,21]. Substituting spray-dried plasma for eggs, however, only produces cakes with desirable qualities, if the substitution is partial. Because egg products are among the more costly ingredients used in the bakery industry, the use of blood plasma would provide a low-cost substitute for some of the eggs in bakery products [21].

Food-based strategies remain the most sustainable means of combating iron deficiency anemia (IDA), which is the most widespread micro-nutrient deficiency disease globally, particularly in developing countries. Bovine blood, which has a high concentration of heme iron, has been found to be a suitable way to fortify commonly consumed food products in order to combat IDA. The heme iron content of bovine blood powder is estimated to be as high as 195.46 mg/100 g of bovine blood powder—more than 10 times that of bovine liver (17 mg/100 g of bovine liver) which is usually considered an iron rich food [22]. Studies by Walter and coworkers [23], where children were fed bovine hemoglobin fortified cookies through a nationwide school lunch program in Chile, revealed significant differences in hemoglobin concentrations and serum ferritin levels between the fortified and non-fortified groups. Thus, fortification of commonly consumed food items with blood products is a feasible and effective way to improve iron levels in iron-deficient populations. Other studies have shown that globin (hemoglobin without the heme group), which is deficient in isoleucine, serves as an excellent complement to widely consumed plant products such as corn and wheat that are low in lysine but rich in isoleucine, providing a nutritionally beneficial end product [24].

2.2.2 Need for Methods to Detect Blood and Blood Products in Food

Despite the nutritional, environmental, and economic benefits that can be derived from the use of animal blood, there is a concern about the widespread use of these products as a result of the advent of bovine spongiform encephalopathy (BSE), known colloquially as mad cow disease. There is strong evidence to suggest that blood from ruminant animals carries some level of infectivity for transmissible spongiform encephalopathies (TSEs) [25–29], a group of related fatal, progressive degenerative diseases, including BSE, which affect the central nervous system (CNS) in both humans and animals. In addition, certain individuals, for example Jews, Muslims, and vegetarians must avoid blood-tainted food products as a result of dietary restrictions imposed by their religion [30], or simply as a matter of preference [31]. Others restrict blood from their diets because of an allergy to blood

proteins such as serum albumin [32,33]. Yet other concerns regarding the use of animal blood relate to the labeling issue. Federal regulation requires that the percentage of meat or poultry in products identified as containing meat be declared on the label. Undeclared blood proteins in meat products would increase the nitrogen content of the product and hence, falsify the actual meat content, as meat content is usually estimated based on the nitrogen content of the product. Partial replacement of lean meat with blood plasma content thus offers great economic advantage to the manufacturer, as the addition of 2% of blood plasma to a meat product can boost yield by 4%–5% and substitute for up to 10% lean meat content [34]. Manufacturers may therefore be tempted to add more plasma to meat than permitted by law in order to boost profits. For all these reasons, it is necessary that effective methods be developed to detect blood in both raw and processed food as a product quality control measure to enforce labeling regulations and address consumer safety concerns. Accordingly, a great deal of effort has been devoted to developing effective ways to detect and quantify the amount of animal blood in food formulations. Some of the methods that have been developed for the purpose will be reviewed and their limitations discussed in the following section.

2.2.3 Detection Methods

Several methods, including spectrophotometric methods, ultra-thin layer isoelectric focusing, Kjeldahl, and immunological methods, have been used for detecting animal blood that is added to meat products. A spectrophotometric method developed by Maxstadt and Pollman [35] was designed to estimate the added blood in raw ground beef. This method is based on the detection of hemoglobin as a measure of the amount of added blood and involves the extraction of hemoglobin with water. Myoglobin is extracted alongside the hemoglobin from the meat sample, and these two heme pigments must then be separated by treating the extract with 85% $(NH_4)_2SO_4$ to precipitate the hemoglobin, leaving the myoglobin in solution. The hemoglobin is then converted to cyanomethemoglobin and quantified by its absorbance at 422 nm using a standard curve prepared using cyanomethemoglobin standards. The method has the advantage of being easy and fast to perform, with good repeatability. However, it is not species specific, and hence does not discriminate between blood of bovine origin and blood from other species, leading to BSE concerns, and the amount of hemoglobin extracted is the sum of that from both the added blood and the residual blood in the meat sample. Hence it is necessary to subtract a correction factor of 171 mg hemoglobin/100 g meat, which represents the normal residual hemoglobin content of ground beef, from the amount of hemoglobin measured to estimate the amount of hemoglobin (and hence blood) added. Since many factors may affect the quantity of residual blood in meat, this method cannot accurately determine the amount of added blood at low levels to enable effective regulatory decisions to be made.

The Kjeldahl method has been adopted as an alternative way to estimate the amount of hemoglobin in meat products [36]. This method uses hazardous chemicals to digest samples and measures the total nitrogen content to provide an estimate of the crude protein content. The crude protein content is determined by multiplying the nitrogen content by an appropriate conversion factor. Normally 6.25 is used as the average conversion factor, but the factors 5.94, 4.94, and 5.65 have been calculated based on biochemical data to estimate the amount of muscle protein, collagen, and hemoglobin, respectively, in the sample. This method is time consuming and at best gives only an approximation of the amount of blood present in the sample. Although it is the most commonly used method for crude protein determination in a wide range of samples, it does not distinguish between added blood and residual blood and is by no means either species- or tissue-specific.

Bauer and Stachelberger [37] employed ultra-thin layer isoelectric focusing to detect added blood in heat-treated meat, reporting that this method is not affected by heat-treatment, and has a detection limit of 0.2% of dry blood plasma in sausage. However, it is not able to discriminate between different species and requires a laborious gel chromatographic step to desalt the samples and then to concentrate the desalted samples by adsorption of the protein on hydroxyl apatite, followed by subsequent elution.

A liquid chromatography tandem mass spectrometric (LC-MS-MS) method has also been developed to detect the presence of a commercial bovine [38] and porcine [39] blood-based food binding agent in food products. The blood-based binding agent is sold under the brand name Fibrimex® and consists primarily of thrombin and fibrinogen. The method is based on the specific detection of fibrinopeptide A and B, which are cleaved by the blood protease thrombin from the N-terminus of the alpha and beta chains of fibrinogen, respectively. This method, however, suffers from matrix interference, as different matrices (fish, chicken, and meat) spiked with 5% (v/w) of the binding agent produced marked differences in the amount (peak size) of fibrinopeptides A and B detected. Although fibrinopeptides A and B could be detected when meat samples (beef, pork, and lamb) were spiked with these target peptides, neither was detected in 5% spiked cod fish samples. Detection in cod was only possible when spiking of the binding agent was above 10% level. Similarly the signal due to fibrinopeptide A in chicken samples spiked at 5% was considerably lower than that for comparable levels in meat samples. Although the authors asserted that this method was not hampered by the heat-treatment (80°C for 15 min) given to samples, it is questionable if the method will still be effective with samples that have undergone more severe or prolonged heat treatment. In addition, the fibrinopeptides could also be detected faintly in non-spiked control samples, indicating that the method may not adequately distinguish between added blood and residual blood.

Immunological methods have begun to emerge as important and effective methods for qualitative and quantitative analyses of numerous food ingredients due to the simplicity, specificity, and speed of the assays produced. These methods are all based on the specific binding between the antigen (the analyte) and its corresponding antibody and are available in many different formats, including immunodiffusion, immunoglutination, enzyme-linked immunosorbent assay (ELISA), immunoblot, and immunohistochromatography (lateral flow assay). The effectiveness of the assay mainly depends on the quality of the antibody used, which is either a polyclonal antibody (PAb) or a monoclonal antibody (MAb). Otto and Sinell [40] produced two antisera against extracts of dried bovine blood plasma and dried porcine plasma, for the detection of added dried bovine and porcine plasma in heat-treated meat mixtures, respectively. Plasma proteins were extracted with 7 M urea and tested against the antisera using either gel-diffusion or electro immune assays. Both methods proved useful for the detection of dried blood plasma, provided the tested samples had been properly diluted. The assay signal, however, was hampered by the heat treatment of the cooked samples. Thus, the concentration of plasma in a given sample can only be determined if the time–temperature regimen of the process experienced by the sample is known, and appropriate model samples tested for comparison. This method is therefore inadequate for most real life situations, where the details of the processing the sample has undergone are not available.

Blood and blood products used as the ingredients in food and feedstuffs could be produced from whole blood, plasma, serum, red blood cells, hemoglobin, and/or globin. Because of the easy availability of this wide range of blood products, the above-mentioned methods developed for detecting blood in food products that depend on detecting specific analytes may be useful for detecting only particular blood products. For example, the spectrophotometric method proposed by Maxstadt and Pollman [35], which is based on the specific detection of hemoglobin as a measure of the amount of blood that has been added to meat products, would not be useful for detecting the presence of non-hemoglobin-containing blood products such as plasma or serum in meat products. It is therefore necessary that the scope of these methods be better defined to streamline the application of these methods.

A competitive indirect enzyme-linked immunosorbent assay (ELISA) was recently developed for the detection of bovine blood in heat-processed meat [41]. This assay, which is based on a MAb, Bb1H9, that recognizes a 12 kDa thermal-stable antigenic protein in ruminant (bovine and ovine) red blood cells, is bovine and ovine blood specific and works effectively with severely heat-processed samples. It is also possible for the assay to distinguish between added blood and residual blood, with a detection limit of 0.5% bovine blood in beef. As mentioned earlier, decolorized blood, a useful blood product in food formulation, is produced through the removal of the heme group, which is responsible

for the negative sensory qualities of hemoglobin containing blood products. The 12 kDa antigenic protein recognized by the MAb Bb1H9 seems to be a monomer of the tetrameric hemoglobin molecule (Ofori and Hsieh, unpublished data). Consequently, this competitive ELISA can be used to detect any blood products containing hemoglobin or globin in a food sample, because MAb Bb1H9 has the ability to recognize hemoglobin both with and without the heme group. To the best of our knowledge, however, there is currently no single method available for the detection of globin in food products.

Ofori and Hsieh [42] have developed a sandwich ELISA for the detection of bovine blood in animal feed. This method is based on two MAbs, Bb6G12 and Bb3D6, that recognize an approximately 60 kDa thermal-stable antigenic protein in bovine blood. This assay also has the advantage of being bovine blood specific, tissue specific, and not hampered by any heat treatment the samples may have been subjected to. Further studies confirmed that the 60 kDa antigenic protein is present in the plasma fraction (Ofori and Hsieh, unpublished data) and that the assay has a detection limit of 0.3% bovine blood in autoclaved (121°C at 1.2 bar for 15 min) beef. These two methods [41,42] therefore complement each other and can be used together to detect the whole range of blood and blood products that may be used as ingredients both in food and feed. In addition, these two assays offer a specific and sensitive determination of added bovine blood in heat-processed products.

2.3 COLLAGEN

Collagen is an insoluble fibrous protein that constitutes the major component of connective tissues, skin, and bones. It is the raw material for the production of gelatin and is also used in the food industry as is, without processing into gelatin. It is the most abundant protein in vertebrates, accounting for about one-fourth of total proteins [43]. Unlike other mammalian proteins, collagen has high concentrations of 4-hydroxyproline, which is formed post-translationally from proline side chains [44]. Collagen has a triple helix structure composed of three almost identical polypeptide chains with a repeating triplet sequence (Gly-X-Y)*n*, where X and Y are usually proline or 4-hydroxyproline. The triple helical structure is stabilized by the presence of 4-hydroxyproline through the formation of hydrogen-bonded water bridges [45]. Over 90% of the different collagen types fall into three categories, namely Type I, Type II, and Type III, with Type I being the most abundant form, and the most widely distributed within the body. Each year, over 200,000 metric tons of collagen and gelatin are produced for use by the food, pharmaceutical, and cosmetic industry [46]. Most collagen is produced from bovine and porcine skin. A patented procedure of a collagen manufacturing process from calcified tissues such as animal skin, without a prior decalcification step is described in Figure 2.1.

2.3.1 Use of Collagen as a Food Ingredient

Collagen performs a wide range of functions in different food products. For example, collagen preparations are utilized in processed meat products to enhance the tenderness, texture, and yield of the final product [47]. Isinglass, a collagen fining agent obtained from the swim bladders of fish, has for years been used as a clarifying agent in the manufacture of both beer and wine. However, as a result of BSE-related fears and for religious reasons, raw materials from fish sources are now the consumer-preferred alternative for the production of collagen [48]. Collagen in the form of collagen hydrolyzate and collagen fiber preparation can also serve as a carrier of antioxidants when added to meat products in order to prevent meat lipid oxidation, which is responsible for the production of several chemical compounds that affect the nutritional and other quality attributes of meat products [49]. However, collagen is currently underutilized in the food industry because it suffers from low solubility, weak water-retention and emulsion-forming properties, and a poor amino acid profile, lacking the essential amino acid, tryptophan. In addition, collagen is poorly digested due to the low levels of collagenase in the human gastrointestinal tract [50]. Interestingly, individual fractions of collagen, which can be produced by various techniques, such as water-salt extraction or enzyme

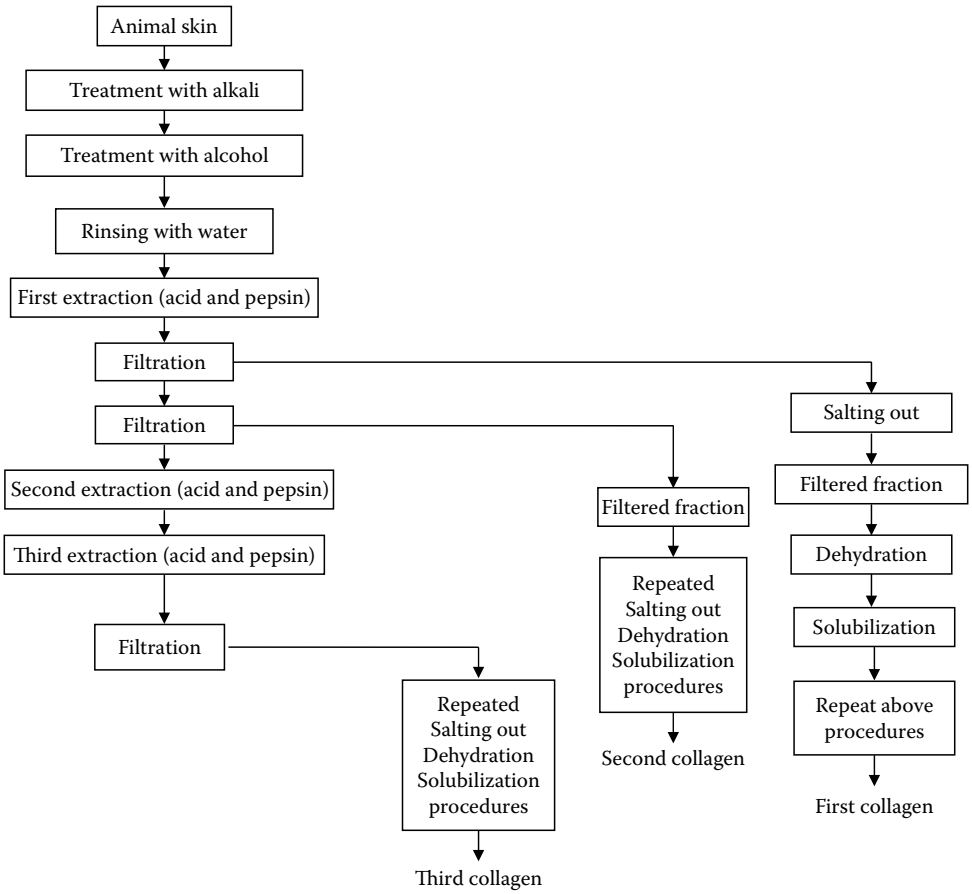

FIGURE 2.1 Manufacturing process of collagen from animal skin. (Adapted from Losso, J.N. et al., Extraction of collagen from calcified tissues, U.S. Patent 7109300, 2006. http://www.freepatentsonline.com/7109300.html. Accessed on November 28, 2010.)

preparation, exhibit a pronounced capacity for water retention and emulsion formation. It has therefore been suggested that these fractions be used as a replacement for gelatin, as research findings indicate that these collagen fractions have higher moisture-absorbing and gelling properties, and are also cheaper to produce than gelatin [51].

2.3.2 Need for Methods to Detect Collagen in Food

Methods have been developed to detect the presence of collagen in food products for a variety of reasons. For example, collagen serves as a measure of the quality of meat and meat products; poor quality meat-based products tend to be rich in connective tissues, which are high in collagen [52,53]. Accordingly, the amount of collagen gives an indication of the economic value of the raw material (meat) used in the manufacture of the meat-based product, as well as the nutritional value of the product [52,53]. The Food Safety and Inspection Service (FSIS) of the USDA thus considers estimation of the collagen content to be an easy and practical method for ascertaining the protein quality of meat products [54]. Many countries specify the maximum allowable level of collagen in comminuted meat products in order to prevent adulteration and partial substitution of economically high value meat portions with less expensive alternatives. Compared with other mammalian proteins, collagen has a high concentration of the imino acid 4-hydroxyproline; hence, estimating the content of 4-hydroxyproline provides an indirect measure of the collagen content.

2.3.3 Detection Methods

The most commonly used method for 4-hydroxyproline content determination as a measure of collagenous material in meat and meat products is the AOAC official method 990.26 [55]. The colorimetric method involves a time consuming (16 h) acid hydrolysis step. Hydroxyproline in the filtered and diluted hydrolysate is then oxidized to pyrrole by reacting with chloramines-T, followed by adding 4-dimethylaminobenzaldehyde to develop a red-purple color. The absorbance of the solution is measured spectrophotometrically and the amount of 4-hydroxyproline present in samples is calculated from a calibration curve obtained from absorbance readings of standard solutions of hydroxyproline. The amount of collagenous connective tissue present in the sample is computed as the hydroxyproline content multiplying a factor of 8, as the collagenous connective tissue contains 12.5% 4-hydroxyproline, if nitrogen to protein factor is 6.25. This calculation is different in some countries based on different nitrogen and hydroxhproline factors, and this method is not applicable to analysis of freeze-dried material. Recently, microwave hydrolysis of proteins combined with high performance anion exchange chromatography, and pulsed amperometric detection (HPAEC-PAD) analysis of 4-hydroxyproline, was reported as a new way to rapidly determine the collagen content in meat-based foods [52]. Briefly, an aliquot of sample corresponding to 25 mg of protein was subjected to hydrolysis in acid using a microwave digestion system. Filtered hydrolyzed samples evaporated to dryness and dissolved in 0.1 N HCl were then diluted with ultra-pure water, filtered and injected into the chromatographic system. Hydroxyproline quantification was then carried out alongside other amino acids using amino acid standards. The collagen content was also calculated by multiplying the 4-hydroxyproline content (g/100 g of sample) by 8. The use of microwave hydrolysis allowed the hydrolysis time to be reduced from 16 to 24 h typical of traditional hydrolysis to 20 min. The ratio of collagen to protein gives an indication of the quality of meat used, with higher ratios indicating the use of low quality meat and lower ratios indicating the use of high quality meat. However, there is an issue with the interpretation of the results for the above two methods. For example, a low collagen to protein ratio may not necessarily indicate the use of high quality meat but could be as a result of the use of non-meat protein in the preparation of the product.

Several instrumental methods have also been developed for the same purposes. The use of near infrared reflectance (NIR) spectroscopy has been reported for rapid estimation of components (fat, protein, collagen-free protein [CFP], moisture, and starch) in meat patties [56]. The instrument was first calibrated using results obtained from the analysis of each component from 50 meat patties using standard chemical reference methods. After calibration, the protein, water, fat, CFP, and starch contents of the meat patties were determined with the near infrared spectroscope and the results compared with the chemical data. The standard error between the two sets of data served as an estimate of the difference between the NIR and reference methods. After calibration, 43 additional meat patties were analyzed using both NIR and reference methods. The correlation coefficient (r), standard error of prediction (SEP), and coefficient of variation (CV) were then calculated to assess the accuracy of the NIR method. Correlation coefficients of 0.943 and 0.983 were recorded for CFP and crude protein, respectively. The collagen content of the sample was calculated as the difference between crude protein and CFP (crude protein minus CFP). The drawback of this method is that it suffers from a high prediction error due to the necessity to calculate SEP, and may thus be inaccurate. The researchers therefore recommended that samples be defatted and dehydrated to make the determination of CFP more accurate.

Autofluorescence spectroscopy is another instrumental method that has been proposed as a rapid way to quantify the hydroxyproline content of ground beef [53]. This method is based on the fact that connective tissue (such as collagen) is autofluorescent, giving off a bluish fluorescence [57]. Five excitation wavelengths, namely 300, 332, 365, 380, and 400 nm, were investigated (based on previous data) and autofluorescence spectra obtained using an optical system. The measurements were performed using an optical bench system and light from a 300 W Xenon light source. The light was focused onto samples (contained in specially designed cuvettes) at an angle of 45°.

Detection was via a detector coupled to the optical device. The emission intensities at 300, 365, and 380 nm increased as the concentration of collagen increased, while at 332 nm the samples exhibited emission spectra that provided information about both their fat and collagen contents. The emission spectra at 400 nm gave the least reliable results, as other chromophores influenced the spectra. This method was performed under ideal conditions and may therefore not be suitable under field conditions, given that autofluorescence is a very sensitive technique that may be influenced by several factors in the environment, including pH and color variations in the sample (i.e., dark or white meat) [53].

In addition to the above methods for quantifying the endogenous collagen in meat products as a measure of meat quality, methods are also developed to detect collagen that has been added to food products for the concern of the potential allergenicity of collagen proteins. For example, a semi-quantitative sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)/densitometer has been used to detect trace amounts of native collagen in beer [45]. As the method does not detect denatured collagen, beer samples were first heat treated to denature any collagen that might be present. Some samples were then spiked with known amounts of acid soluble collagen in amounts ranging from 0.03 to 0.05 ppm to determine the detection limit, while other samples were tested without spiking. Supernatants obtained from samples that had undergone settling, centrifugation, treatment with acetic acid, and rotary evaporation were subjected to SDS-PAGE on pre-cast 7.5% or 4%–15% gels, and the resolved protein bands stained with Coomassie Stain for visualization. Gels were then scanned on an imaging densitometer. This method was based on the extraction of acid soluble collagen as fibers under elevated ionic strength brought about by the addition of salt. SDS-PAGE revealed three bands: α_1I and α_3I, co-eluted, while α_2I remained separate. The first band, α_1I/α_3I was easily visualized and also better resolved from contaminating proteins, so this was used for relative quantitative comparisons using the densitometer. The detection limit of the assay was reported to be 0.03 ppm for some beers and 0.05 ppm for others. The researchers noted that the recoveries of this method at different spiking levels were not always proportional to the amount of collagen added. Another disadvantage of this method is that background in the gel has a tendency to mask increases in the loading of samples, so it is important to establish correct loadings in order to produce a linear response to quantity on the gel.

In summary, all the above-mentioned methods developed for the detection of both endogenous and added collagen in food products suffer from shortcomings that limit their application. These problems include prediction errors, interference from environmental factors, errors in interpretation of results, low recoveries, and/or interference from background. In addition to their individual shortcomings, none of these methods is species-specific. Detection of native collagen is therefore still an analytical challenge.

2.4 GELATIN

Gelatin is a mixture of peptides commonly obtained through the heat dissolution at acidic or alkaline pH of collagen from animal skins, tendons, and bones [58]. As discussed in the previous section, collagen is the principal protein constituent of animal skins, bones, tendons, and loose connective tissues. The amount of collagen present, as well as its nature, varies considerably from tissue to tissue and also from one species to another, so differences in the gelatin manufacturing technology are dictated by the raw material (collagen), although the principle of gelatin production remains the same. The raw material is pre-treated, either with an acid or an alkaline solution, then subjected to increasing temperatures to extract the gelatin. The final product is obtained through centrifugation, filtration, or settling. The quality in terms of clarity, transparency, and purity of the gelatin produced depends on the type of extraction method (acid or alkaline), source, and history of the raw material, the thermal conditions used, and the subsequent processing [59]. Five types of gelatin are produced industrially for various applications, namely bovine gelatin produced by alkali

(BA gelatin) or acid (hydrochloric acid) treatment (BHA), porcine gelatin produced by alkali (PA) or acid (PHA) treatment, and fish gelatin (F gelatin) [60].

In Europe, about 80% of edible gelatin is derived from pig skin. Of the remaining 20%, 15% is obtained from bovine hide splits and 5% is extracted from the bones of bovine, porcine, poultry, or fish species [61]. In North America, pig skin is currently the major raw material source for the production of edible gelatin, while cattle hide is the least used raw material. Pig skins are obtained from slaughterhouses and meat processing plants already trimmed of fat, flesh, and hair, and supplied as either fresh or frozen [58]. However, the preferred source of collagen for producing high-quality gelatin is cow bone. Gelatin extracted from fish by-products has poor functional properties and therefore requires chemical or enzymatic modifications to improve its functionality [62]. Gelatins are sometimes referred to as edible, photographic, technical, or pharmaceutical grade, but these terms refer to their uses rather than the method of manufacture [58]. In the United States, gelatin manufactured for food, drug, and cosmetic uses must be produced from the skin and bones of healthy animals that have been slaughtered in plants inspected by the USDA. Such materials are also sourced to a small extent from other countries with comparable veterinary health services inspection systems. In Canada, gelatin manufactured for food, drug, and cosmetic usage is made solely from the skin of healthy pigs slaughtered in plants inspected by the Canadian Food Inspection Agency (CFIA) or in plants inspected by the USDA. Accordingly, in the United States, gelatin produced locally or imported from companies in countries that are affiliated to the Gelatin Manufacturers Institute of America (GMIA) should comply fully with the current recommendations of the U.S. Food and Drug Administration (FDA) Guidance [63].

2.4.1 Use of Gelatin in the Food Industry

Gelatin has traditionally been used in the food industry as a stabilizer, clarifier, or protective coating material, and is commonly found as an ingredient in food products such as candies, baked products, desserts, meat products, ice-cream, and dairy products. The major use of gelatin in the food industry is as a clarifying agent and as a stabilizer, for example, in drinks and beverages that contain tannins, where the gelatin reacts with the tannins to form a gelatin-tannin complex that precipitates out as sediment. In the United States, about 50% of edible gelatin produced is used for such purposes. The foaming capacity of gelatin is utilized in the manufacture of marshmallow, a common confectionery in the American diet, to produce a stable foam system that imparts an airy and light texture to the product. In the bakery industry, gelatin is widely used as a setting agent, a stabilizer, and as a foaming agent in products such as cakes, breads, and pies, and also as a stabilizer in different kinds of icing and dairy products. The meat industry makes use of a considerable amount of gelatin in products such as meat loaves, sausages, and meat jellies, where the principal function of added gelatin is to absorb the juices that separate out during cooking, or as a coating material [59]. Gelatin has also been suggested as a coating on meat products to extend the shelf life because the gelatin matrix is believed to act as a barrier to water and oxygen, which would slow down the oxidation of myoglobin and lipids and decrease water loss [64]. In developed countries, about one tenth of the edible gelatin produced goes into the pharmaceutical industry, primarily for use as capsules and emulsions. Gelatin has been subjected to several chemical modifications with the aim of producing food-grade gelatin with improved functional properties [59].

2.4.2 Need for Methods to Detect Gelatin and Sources of Gelatin in Food

It has become necessary for methods to be developed to detect the presence of gelatin in food products and also to differentiate between the species of origin of gelatin products. In some individuals, foods containing gelatin may trigger allergic reactions [65,66]. Most of these gelatin-sensitive patients develop allergic reactions to porcine and bovine gelatin but do not react to fish gelatin [67,68], so some countries require that the source of gelatin used in food products be appropriately

labeled. For example, the Ministry of Ordinance in Japan has recommended bovine and porcine gelatin to be clearly labeled. Labeling precautions notwithstanding, there is still a high possibility of gelatin (from bovine or porcine sources) inadvertently becoming present in processed foods as undisclosed allergens as a result of cross-contamination during processing, particularly in establishments that produce different products with various ingredients on the same production line. This is a serious issue, as even small amounts of bovine or porcine gelatin have the potential to cause severe reactions [60]. Thus, detection methods for bovine and porcine gelatin contaminants in food products are highly necessary. They are also important to address consumer concerns as certain individuals, for example Muslims, Jews, and Hindus, do not accept gelatin produced from porcine and/or bovine sources for religious reasons [69,70]. It has also been suggested that foods contaminated with the BSE prion may be responsible for variant Creutzfeldt-Jakob disease (vCJD), raising concerns about the use of any bovine-derived food ingredient. According to both the FDA and the European Food Safety Authority (EFSA), the manufacturing guidelines established for edible gelatin production that gelatin manufacturers are required to adhere to sufficiently reduce the likelihood of BSE infectivity to protect human health. Edible gelatins produced according to these guidelines can thus be assumed to be safe. This notwithstanding, there is still the need for methods to differentiate bovine gelatin from porcine or fish gelatin for labeling enforcement purposes, for the benefit of those individuals who may be concerned about the safety of bovine gelatin as a result of the BSE scare. Accordingly, immunological and DNA-based methods, as well as methods based on principal component analysis, have been designed for the speciation of gelatin, as outlined below.

2.4.3 Detection Methods

A sandwich ELISA has been developed for the detection of bovine and porcine gelatin in processed foods using different pairs of polyclonal antibodies [60]. Three polyclonal antibodies, PAb1, PAb2, and PAb3 were used for this assay. The antibodies PAb1 and PAb2 were obtained by immunizing rabbits with bovine gelatin, and PAb3 was obtained by immunizing goats with bovine gelatin. Several antibody pairs were evaluated and two antibody-pair systems, PAb2–PAb1 (PAb2 as first antibody and PAb1 as second antibody) and PAb3–PAb3 (with PAb3 as both first and second antibody) were selected for the ELISA. This ELISA, however, cross-reacted with heat-treated meat species and also with some sea foods tested, either raw or cooked, and also with gelatin from non-bovine species, although the nature of this cross-reaction varied, depending on the pair of PAbs used. The ELISA system made up of the antibody pair PAb2–PAb1 cross-reacted strongly with porcine gelatin and boiled squid, and slightly with fish gelatin, while the ELISA system made up of the antibody pair PAb3–PAb3 reacted strongly with porcine gelatin. Both antibody pairs reacted with different kinds of heat-treated meat samples. The researchers therefore pointed out the need for further studies to develop methods that are better able to discriminate between gelatin used as a food ingredient and gelatinized heated meat.

Another competitive ELISA method based on PAbs obtained by immunizing rabbits with tyrosylated bovine and porcine gelatins was reported by Venien and Levieux [70] to differentiate between bovine and porcine gelatin. Gelatin was tyrosylated to increase the immunogenicity of gelatin, which is traditionally known to be a weak immunogen. However, the antibodies were not species specific, as antibodies raised against porcine gelatin reacted with some of the bovine gelatins tested and vice versa. To circumvent this cross-reactivity, the authors used peptides synthesized from species-specific sequences of the bovine alpha 1(I) collagen chain as the immunogen to produce bovine-gelatin specific antibodies, reporting that this process was effective in producing antibodies capable of distinguishing bovine from porcine gelatin. However, neither the cross-reactivity of the antibodies with other proteins commonly used as ingredients in food nor the ability of the assay to differentiate between added gelatin and gelatin resulting from heat-treated meat samples was examined or discussed in this study.

Conventional and real-time PCR-based methods have also been used for the molecular detection and quantification of bovine species material in edible gelatin [61]. The method developed by Tasara et al. [61] involved the isolation of DNA from gelatin of bovine, fish, and porcine origin, followed by confirmation that sufficient amount and PCR-detectable template had been isolated given that the gelatin manufacturing process has a tendency to severely degrade nucleic acids. Several published species-specific PCR systems designed for bovine, porcine, and fish species detection were evaluated as potential tools for determining the species origin of the raw material used in the gelatin manufacture. A PCR system specific for bovine material in gelatin was selected after this preliminary evaluation, as most of the PCR systems tested were either incapable of identifying species of origin of gelatin or cross-reacted with DNA of other species. This bovine species-specific PCR primer set, which targets the ATPase 8 subunit gene in bovine mitochondrial DNA, was then optimized using both conventional and real-time PCR methodology. The conventional PCR assay had a detection limit of 0.1% and 0.5% bovine gelatin in porcine gelatin and fish gelatin, respectively. The real-time system had a better detection limit of 0.001% bovine gelatin in both pork and fish gelatin. However, this method only reveals the presence of bovine species material in gelatin, which does not necessarily mean it is gelatin, as DNA-based methods are not tissue specific. In addition, this method offers only an approximate estimate of bovine DNA, as absolute quantification of DNA in severely processed products such as gelatin is not feasible.

Principal component analysis (PCA), a technique that reduces the dimensionality of a data set while retaining the most significant information, and widely used in many classification studies, has also been used to differentiate between bovine and porcine gelatins [69]. Gelatin samples were hydrolyzed using hydrochloric acid (12 M HCl) to release their amino acid residues, then separated and analyzed using reversed-phase high performance liquid chromatography (RP-HPLC). Twenty peaks were detected by the HPLC for both bovine (14 samples) and porcine (5 samples) gelatin samples of high purity. PCA was then employed with the MATLAB® program using peak height, area, area percentage, and width to differentiate between bovine and porcine gelatin. PCA processes peak parameters, and extracts the principal components or significant variables (in this case, peaks), which are then used as the basis for classifying bovine and porcine gelatin. Twelve samples comprising of 9 bovine and 3 porcine gelatins, were first processed by PCA and presented in a two-dimensional graph. The remaining 7 samples, consisting of 5 bovine and 2 porcine gelatins, were employed as the prediction set and added to the first 12 samples. All 19 samples were then analyzed by PCA by comparing the two dimensional presentation graphs. Bovine and porcine gelatin was distinguished by a line from the PCA plot of HPLC data or peak height or peak width for bovine and porcine gelatins. However, one limitation of PCA is that finding the principal component direction becomes a problem when large numbers of data points are involved. In addition, it is not clear how to properly handle incomplete data sets in which some of the points are missing.

2.5 WHEY

Whey is a cheap by-product obtained during the production of cheese or casein from milk. It is the watery part of milk that separates from the curd when milk is curdled in the production of cheese. For every kilogram of cheese manufactured, about 9 kg of whey is produced [71]. According to a 2004 report, cheese whey was manufactured by over 200 whey plants in the United States and their total output represented 25% (935,000 metric tons) of the global production of cheese whey [72]. The composition of whey varies depending on the type of cheese produced, with lactose being the main constituent at 70–80 g/100 g of dry matter [73]. Whey is broadly categorized as sweet whey or acid whey, depending on the raw material used for the coagulation of the milk, that is, whether rennet (enzymatic) coagulation or acid coagulation, respectively, is used. A typical composition of the two types of whey is shown in Table 2.3. The major proteins found in whey are β-lactoglobulin

TABLE 2.3
Composition and pH of Fresh Whey

Component	Sweet Whey	Acid Whey
Water	93%–94%	94%–95%
Dry matter	6%–6.5%	5%–6%
Lactose	4.5%–5%	3.8%–4.3%
Lactic acid	Traces	Up to 0.8%
Total protein	0.8%–1.0%	0.8%–1.0%
Whey protein	0.6%–0.65%	0.6%–0.65%
Citric acid	0.1%	0.1%
Minerals	0.5%–0.7%	0.5%–0.7%
pH	6.4–6.2	5.0–4.6

Source: http://www.dairyforall.com/whey.php. Accessed on November 28, 2010.

TABLE 2.4
Whey Protein Composition

Protein	Abundance
β-Lactoglobulin	50%–55%
α-Lactalbumin	20%–25%
Immunoglobulins	10%–15%
Bovine serum albumin	5%–10%
Lactoferrin	1%–2%
Lactoperoxidase	0.5%
Lysozyme	<0.1%
Glycomacropeptide	ND

Source: http://www.wheyoflife.org/facts.cfm#8. Accessed on November 28, 2010.
ND, not determined.

(BLG), α-lactalbumin (ALA), bovine serum albumin (BSA), and immunoglobulins (IgG), with BLG being the most abundant (~55%) of the whey proteins. The various proteins found in whey and their relative abundance are shown in Table 2.4.

2.5.1 Use of Whey and Whey Proteins as Food Ingredients

The use of whey proteins in formulated foods has increased in recent years, but in the past whey was considered merely as a waste product and dumped in water bodies, on agricultural lands, or in any convenient location. The trend toward processing whey into valuable end products instead of its disposal as waste is at least partly as a result of present day pollution standards designed to protect water bodies from run-offs, which makes casual disposal unattractive [74]. Currently, whey products such as whey powder, whey protein isolates, whey protein concentrates, hydrolyzed whey protein isolates and concentrates, deproteinized whey, and lactose are used as ingredients in a wide range of food and dietary supplements because of certain qualities they possess that are highlighted below. Whey proteins are nutritionally valuable because of their complete amino acid composition; they are not deficient in any amino acid and also have high contents of the essential amino acids

tryptophan and lysine. They are particularly rich in cysteine, which is considered conditionally essential for individuals who do not synthesize it in sufficient amounts [75]. In addition to their nutritional quality, whey proteins have excellent solubility, water-binding, gel formation, foaming, and emulsifying capacities. They can also protect against syneresis in a product such as yoghurt, which impairs the organoleptic quality of the product and often causes consumers to believe that the product has gone bad [76], and are used as fat replacers in low-fat products such as cheese, pasta, and yoghurt [77,78]. The unique functional and nutritional attributes of whey proteins make them desirable as a functional ingredient in processed foods such as beverages, sauces, meat products, and baked goods [79].

The widespread use of whey as an ingredient in food products due to its excellent functional and nutritional qualities, coupled with improvements in processing technologies such as ultra-filtration, osmosis, and ion-exchange, means that several different whey products are now available for use in food products to address particular needs. These mostly take the form of powder obtained from the drying of liquid whey. Examples of the wide range of whey products that are available commercially are shown in Table 2.5. However, despite the multiple benefits that are derived from processing whey into valuable end products, whey is seldom fully utilized because of the high cost inherent in its processing into value added products. Drying or concentrating liquid whey is very costly and this adds to production costs [80]. Only a fraction of the tons of the fluid whey generated annually is currently utilized in food and feed production [81]. A study of 11 dairies in Serbia indicated that 78.75% of whey was discharged into river systems, contributing significantly to the organic pollution of the environment [82]. Whey is usually dumped as a result of a lack of the technology needed to process it, mainly due to the inherent cost of concentrating or drying [80]. Accordingly, efforts are now being directed toward utilization of liquid whey, which is currently not used because of its high water content, to circumvent the high cost of processing and environmental concerns over the dumping of excess whey, and to facilitate its full utilization. For example, research by Yetim et al. [80] has indicated that fresh liquid whey can be added to frankfurter-type sausages without compromising product quality. Further research in this area is necessary and should be encouraged to ensure maximum utilization of this valuable product.

TABLE 2.5
Examples of Commercially Available Whey Products

Product	Description
Whey Protein Concentrate 80% (WPC 80%)	Product obtained by removing non-protein constituents from whey such that the final dry product contains not less than 80% protein
Whey Protein Isolate (WPI)	Product obtained by removing non-protein constituents from whey such that the final dry product contains not less than 90% protein
Whey permeate	Dairy solids obtained by the removal of protein, lactose and some minerals from whey
Reduced lactose whey or mineral concentrated whey	Product produced by the partial removal of lactose from whey
Demineralized whey	Product obtained by removing a portion of the minerals from whey
Hydrolyzed whey	Product obtained by hydrolyzing whey with specific enzymes
Sweet whey powder	Product obtained by drying fresh whey obtained as by-product in the production of cheeses such as Cheddar, Mozzarella, and Swiss
Acid whey powder	Product obtained by drying fresh whey obtained as by-product in the production of cheeses such as cream cheese, cottage cheese and ricotta

2.5.2 Need for Methods to Detect Whey Products in Food

Because of the multiple benefits of whey proteins, they are beginning to be used as ingredients in food products that hitherto would not have contained whey proteins. For example, whey proteins are now added to fruit juices for protein fortification. However, although whey ingredients are assuming an increasingly important role in the food industry, they are known to be potent allergens and as such, their widespread use by the food industry poses a serious health threat to consumers who are allergic to whey proteins. While the most abundant whey protein, β-lactoglobulin (BLG), represents the major allergen in whey, studies using large populations indicate that even those minor whey proteins present in trace amounts, such as bovine serum albumin (BSA), lactoferrin, and immunoglobulins (IgG), are potential allergens [83]. The Food Allergen Labeling and Consumer Protection Act of 2006 (FALCPA) currently requires packaged foods to clearly label ingredients derived from the eight major allergen foods, namely milk, eggs, soybean, wheat, peanuts, tree nuts, fish, and shellfish, all of which are considered Class I allergens. There is therefore an urgent need for methods to be developed to detect the presence of whey proteins in food products as a regulatory tool to protect consumers who are allergic to whey proteins. This is particularly important given that several fruit juices and soft drinks that may contain undeclared whey proteins have recently been recalled in Canada and the United States [84], especially since these products typically do not contain whey products. Analytical methods for whey detection are necessary not only to protect consumers who are allergic to whey, but also to deter unscrupulous manufacturers from adulterating expensive whole milk-based products with low-priced whey products. Because of the large quantity of inexpensive whey that is available, the use of whey to adulterate more costly dairy products such as liquid milk and milk powder is economically very attractive [85]. Not only does whey cost about four to five times less than milk, adding it to milk does not compromise the sensory qualities of the final product [86].

2.5.3 Detection Methods

Several methods have therefore been developed to detect whey proteins in food as part of the effort to enforce food allergen labeling laws as a consumer safety measure, as well as to protect the consumer against fraud. One approach that has been tried uses liquid chromatography with mass spectrometric detection to quantify whey allergen traces in mixed-fruit juices [84]. Here the whey proteins ALA, BLG, and alpha lactoglobulin (ALG) were simultaneously extracted using solid phase extraction (SPE), and injected into the LC-MS (liquid chromatographic system coupled to a quadrupole mass spectrometer) system. The LC–MS system allows the identification and quantification of whey proteins from the spectrum based on the retention time of individual protein peaks. This method has the capacity to unambiguously detect intact whey proteins at levels as low as 1 μg/mL in fruit juices. However, this approach is only suitable for intact proteins and may not be suitable in situations where processing has affected the structural integrity of these proteins.

For those individuals that are allergic to cow's milk, soybean dairy-like products are often used as an alternative. However, in some cases, whey proteins are added to the soybean-based products for enrichment. A perfusion reversed-phase high-performance liquid chromatographic (RP-HPLC) method has therefore been developed to simultaneously separate soybean and bovine whey proteins [87]. Extracts obtained from samples, together with a soybean protein isolate standard, were injected into the chromatographic system. When bovine proteins were present at very low levels, the sample extracts had to first undergo an acidic precipitation step to concentrate the proteins. A linear binary gradient water-acetonitrile-0.1% trifluoroacetic acid procedure is used to separate the soybean and whey proteins, which were then detected by UV absorption at 254 nm, a wavelength at which sensitivity for soybean proteins is higher than at the wavelengths commonly used to detect whey proteins. Whey proteins in the powdered soybean milk were then quantified from calibration curves of standard solutions containing known concentrations of ALA or BLG. This method allows

soybean proteins and whey proteins to be rapidly separated in about 5 min, although high errors have been reported in the estimation of the concentration of BLG, which the authors ascribed to the shape of the peaks and poor resolution between peaks in the chromatogram.

A competitive ELISA has been developed as an easy-to-use alternative screening method for the detection of bovine rennet whey powder in milk powder and buttermilk powder [88]. This method uses an anti-bovine-κ-casein MAb 4G10 that recognizes bovine caseinomacropeptide CMP, a compound specific to whey. Because MAb 4G10 also binds to the κ-casein ordinarily present in milk, samples had to be treated with optimal concentrations of trichloroacetic acid (TCA) to selectively precipitate interfering casein and whey proteins without compromising the CMP recovery. The assay has a detection limit of 0.1% (w/w) whey powder in skimmed milk powder. The main drawback of this method is that it requires this optimal TCA concentration to be determined based on several trials using spiked samples to ensure that interfering proteins such as κ-casein are absent in the samples, and also to maximize the recovery of CMP. Thus, in the case of unknown samples it would be difficult to determine the optimum TCA concentration to use in each case, which ultimately affects the results obtained.

Other instrumental methods including capillary electrophoresis [89,90] and HPLC [91,92] have been reported for the detection of whey proteins in food, based on the detection of caseinomacropeptide (CMP). CMP (residues 106–109) is the smaller of two peptides and remains in the whey when the milk protein κ-casein is hydrolyzed by the enzyme chymosin in the making of cheese. The larger peptide (residues 1–105), known as para-κ-casein, remains with the curd [93]. However, in samples such as ultra high temperature (UHT) milk, the presence of CMP may not necessarily be an indicator of adulteration with whey proteins. This is because it has been found that certain proteases from psychotropic bacteria have the capacity to split CMP from κ-casein during storage of long-life products such as UHT milk [89]. Thus, the presence of CMP in UHT milk is an exception to the rule that it can be used to indicate that a product has been adulterated with whey solids.

There are ELISA kits available commercially for the detection of milk or milk-derived products such as whey, in food products. One such product is RIDASCREEN®β-lactoglobulin, a competitive ELISA kit manufactured by R-Biopharm AG, Germany, for the quantitative analysis of native and processed BLG in such products as hydrolyzed milk products, or foods containing whey, milk, or milk powder (http://www.r-biopharm.com/product_site.php?language=english&product_id= 273). Although the manufacturers report that this kit is suitable for both raw and processed foods, studies supporting this claim have not been reported in the literature. This ELISA kit shows trace cross-reaction with α-, β-, and κ-casein and hence cannot be said to be whey specific. The assay also shows cross-reaction with ALA and BSA. However, since these two proteins are present in the whey fraction of milk, cross-reaction with these two proteins is an advantage as far as detection of whey in food is concerned. Other commercial ELISA kits based on the detection of BLG have been developed by companies such as ELISA Systems Australia (http://www.elisas.com.au/allergens/allergen_2/index.htm) and Tepnel, U.K. (BioKits BLG) (http://www.tepnel.com/elisa-blg-assay-kit.asp). Both the kits from ELISA Systems and Tepnel are claimed to work as well with raw samples as with processed samples. The Tepnel kit also shows trace cross-reactivity with caseins. The cross reaction with non-whey proteins of these methods, so far as detection of whey products are concerned, is understandable, as they were primarily developed for the detection of milk, not just whey protein in food products. The above-mentioned assays have as yet not been validated by any research reports and hence their effectiveness in whey protein detection in different food products is not confirmed.

There is also a need for further work on developing better hydrolyzed whey protein detection methods. Hydrolyzed whey proteins are one of the fastest growing commercial products in the food industry. These hydrolyzed whey formulas are referred to as hypoallergenic formulas (HFs) and are often used in infant formula and medicinal dietary supplements. Their allergenic potential has been lowered through enzymatic hydrolysis, and they are considered a suitable alternative for infants or individuals that suffer from an allergy to cow's milk. HFs are classified based on the degree of hydrolysis as extensive (EHWFs) or partial (PHWF) protein hydrolysates. However, it

is important to note that hypoallergenic formulas may still contain residual allergenicity due to inadequate hydrolysis or filtration, which results in peptides that are large enough to induce allergic reactions. Even after extensive hydrolysis, peptides that are large enough to be antigenic may remain intact [94]. In addition, smaller peptides resulting from hydrolysis may aggregate into larger peptides with allergenic potential, which may explain the presence of higher molecular weight particles in HFs [95]. Hence, despite the use of HFs in child nutrition, cases of allergic reactions have been reported for EHWFs and, more commonly, for PHWFs [96]. Moreover, it has been reported that PHWFs may induce allergic reactions even in infants not previously sensitized to cow's milk [97]. Unfortunately, there are as yet no methods available for the detection of hydrolyzed whey proteins, and current methods based on intact protein molecules cannot be appropriated for the detection of hydrolyzed whey proteins. It is therefore imperative that new methods be developed for the detection of the presence of these modified whey proteins in a variety of food products.

2.6 CONCLUSIONS

In conclusion, the utilization of animal by-products as described in this chapter offers great economic, nutritional, and environmental benefits. As such, there is the need for more effort to be directed toward full utilization of these valuable by-products to ensure that maximum benefits are derived. However, there is also a need for methods to be developed to detect these by-products in both feed and food materials, to enforce labeling laws. This is necessary to protect the health of individuals who may be allergic to some of these by-products, or who avoid these by-products for cultural, religious, or personal reasons, and also to protect consumers from being cheated by rogue manufacturers who may be tempted to adulterate high priced food commodities with these lower priced by-products. Analytical methods are not only needed to detect animal by-products that are deliberately added to the food items, but also those that are present naturally in the food as a measure of the quality or economic value of the food. Despite the extensive work that has been done in the area of method development, there is still room for improvement; most of the methods developed so far suffer from one or more limitations that affect their suitability for the intended application. In some cases, as with hydrolyzed whey proteins, where processing has significantly altered the protein structure, as yet no methods are available that are capable of reliably detecting altered proteins that still possess allergenic potential. The availability of methods that can easily and quickly detect the whole range of by-products would enable the nutritional, environmental, and economic benefits of these products to be fully realized, while at the same time protecting the consumer from fraud or allergic reactions that may result from inadvertent exposure to these by-products.

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3 Analysis of Rendered Fats

Stephen B. Smith and Dana R. Smith

CONTENTS

3.1 INTRODUCTION

Between 45% and 50% of the entire live weight of livestock species does not enter directly into the human food chain [1]. These by-products of animal slaughter and fabrication include muscle trim and a large quantity of the carcass fat (especially subcutaneous and internal fat depots) that are not used in processed meats. In addition, many animal internal organs are included in total by-products. Altogether these by-products contain approximately 20% extractable fat. With rendering some of this extractable fat can be recovered and converted into more useful and profitable materials such as lard or tallow allowing part of this fat to reenter the human food chain [1,2].

In addition to tissue from slaughterhouses, material for rendering also comes from restaurant grease, butcher shop trimmings, and expired grocery store meat. The quality of the rendered materials determines the fate of the finished product. This chapter will focus on the rendering of fat, its chemical composition, and its uses in the industry.

3.2 RENDERING PROCESS

Early rendering involved the addition of water to the by-products in an open kettle or the injection of steam into a sealed autoclave. This type of process is referred to as "tanking" and could be used for both edible and inedible products [3,4]. The fat produced by this method was relatively light in color, but the increased presence of added water resulted in an elevation in the free fatty acid content reducing the quality and making the product more susceptible to oxidation. Therefore, for economic reasons dry rendering is most commonly used today.

In the past, each slaughter facility had its own steam-jacketed kettle and rendered its own fat. Although the kettle was jacketed in steam, the by-products were cooked in their own juices with no added water; therefore, this was considered a dry rendering method. Currently in the United States, there has been a general shift to centralized rendering operations that collect animal by-products and ship them to a central rendering facility. This is evident by the decline in rendering plants from 823 in 1921 to 273 in 2006 [4].

Rendering fat involves applying heat, extracting moisture and separating the fat. To accomplish this, by-products are first ground into a consistent particle size and then cooked at 115°C–145°C for up to 90 min. With the heating process, microorganisms such as bacteria and viruses are inactivated (an advantage over other waste product disposal methods). While the by-products are cooked, the jacket steam pressure must be controlled, the mixture agitated and the temperature monitored for the desired end-point. Over the years there have been advances in the uses of the rendered products, as well in the rendering methods. Today various more energy efficient, continuous processes are used to allow the re-use of process vapors to preheat or dry the materials. Filtering and bleaching systems, as well as refining equipment to remove free fatty acids, may be part of the rendering process.

During rendering, the melted fat floats to the top of the unit by virtue of its lesser density, whereas protein and bone solids settle to the bottom of the rendering kettle. Traditionally, the rendered fat then was skimmed from the top of the rendering kettle. This has been replaced with a screw press that draws off the melted fat, and the melted fat is stored and/or transported in tanks.

3.3 FATE OF RENDERED FATS

For the 2009 calendar year, the U.S. rendering industry produced an estimated 0.9 billion kg edible tallow, 1.5 billion kg inedible tallow, 0.6 billion kg yellow grease, 0.1 billion kg lard, and 0.6 billion kg poultry fat [5]. Tallow is primarily derived from rendered beef fat. Choice white cooking grease is derived from pork fat. Yellow grease (not to be confused with off-colored white cooking grease) is restaurant-quality grease or cooking oil and may be from blended sources. Rendered fat provides concentrated sources of energy for use in feed for poultry, aquaculture, and pets. Other uses of rendered fat include soap, candles, and biodiesel.

3.4 CHEMISTRY OF RENDERED FATS

Fatty acids in meat are located primarily in adipose tissues, and the majority of the fatty acids are stored as highly nonpolar triacylglycerols. Triacylglycerols coalesce into the large lipid vacuoles that are the central features of adipocytes, and these adipocytes comprise the various fat depots in animals [6]. The triacylglycerol structure consists of a glycerol (i.e., three-carbon alcohol) backbone containing three fatty acids in ester linkages. The glycerol primarily is derived from glycerol-3-phosphate, which in turn is derived from the metabolism of glucose or, in liver, from the phosphorylation of free glycerol by glycerokinase. In adipose tissue and intestinal mucosal cells, 2-monoacylglycerol (derived from partial hydrolysis of triacylglycerols) provides a portion of the carbon backbone for triacylglycerol synthesis.

Rendered fats consist primarily of the triacylglycerols. The characteristics of the rendered fat are determined by the composition of fatty acids attached to the glycerol backbone. The most abundant fatty acids of animal fat triacylglycerols are palmitic (16:0), stearic (18:0), and oleic acid (18:1n-9), typically comprising 20%–25%, 10%–30%, and 30%–55% of the total fatty acids in muscle and adipose tissue [7]. Smaller but significant quantities of myristic (14:0), palmitoleic (16:1n-7), linoleic (18:2n-6), and α-linoleic acid (18:3n-3) are contained in triacylglycerols, especially in rendered poultry fat. Lipids from ruminant tissues also contain measurable amounts of odd-chained fatty acids, branched-chain fatty acids, *trans*-fatty acids, and conjugated fatty acids, due to the absorption and metabolism of the products of ruminal fermentation. Virtually all Δ9 desaturase products of saturated fatty acids can be detected in triacylglycerols from animal tissues. However, with the exception of oleic acid, the concentrations of other monounsaturated fatty acids are low [8]. Lard and tallow also contains free fatty acids (primarily as oleic acid), but these must be no more than 0.5% in edible lard and 0.75% in edible tallow [2].

When the rendered fat contains more saturated fatty acids, the fat is referred to a hard fat. It is more solid at room temperature and has a higher melting point. When the rendered fat contains more unsaturated fatty acids, the fat is referred to as a soft fat. It is not as solid at room temperature and has a lower melting point.

3.5 ANALYSIS OF LIPIDS SPECIES IN RENDERED FAT

The composition of rendered lipids can be analyzed for total fatty acid composition; the triacylglycerol classes can be separated by silver-ion chromatography; the positional distribution of fatty acids can be quantified by specific lipase digestion; or some combination of these methods can be used to provide essentially complete information about the composition of the triacylglycerols [9–11]. However, so much detailed information about the composition of rendered fat is rarely needed, and more rapid analyses such as iodine number or detailed analysis by gas/liquid chromatography are typically performed.

3.5.1 Iodine Number

Iodine number provides a rapid means of estimating the amount of unsaturated fatty acids and is a measurement of hardness/softness of the fat. Iodine number is defined as the grams of iodine that will react with 100 g of lipid. There are two primary methods for measuring iodine number, the Hanus method and the Wijs method [12], which differ primarily in their choice of iodine source. In both procedures, an extracted lipid sample is dissolved in a small amount of organic solvent, and titrated with sodium thiosulfate ($Na_2S_2O_3$) until the yellow/brown color of the iodine solution becomes colorless. A starch indicator is used at the final steps to ensure complete titration. Titrated samples are compared to blanks titrated concurrently, and the difference is the amount of iodine that was taken up by the double bonds of the fatty acids.

Because iodine reacts with the carbons at the double bonds in the fatty acid chains, a more unsaturated fat will have a higher iodine number. Inversely, a more saturated fat will have a lower iodine number. Poultry fat, being highly polyunsaturated, will have a high iodine number (77–80). Pork fat also will have a relatively high number (63–65). With beef and lamb being more saturated, fat from these species will have lower values. The iodine number for beef is 43–45, and for lamb, it is 42–43.

3.5.2 Measurement of Fatty Acid Composition by Gas/Liquid Chromatography

Gas/liquid chromatography of fatty acid methyl ester derivatives of tissue fatty acids is the method of choice for quantifying fatty acids, based on its high reproducibility and relatively low cost. This is the most commonly used procedure for the analysis of the fatty acid composition of animal tissues. Identification of the various 18-carbon monounsaturated fatty acids, in addition to less abundant *trans*-fatty acids, has been made possible by the development of the capillary columns of up to 100 m in length (e.g., 10).

Methylation of the triacylglycerol fatty acids is prerequisite to their quantification by gas/liquid chromatography. After extraction of lipids in chloroform/methanol [13], the extracted lipids are *trans*-esterified in boron trifluoride under alkaline conditions at 70°C [14], which methylates only those fatty acids in ester linkage. Alternatively, *trans*-methylation can be conducted under acidic conditions, resulting in methylation of both glyceride-fatty acids and nonesterified fatty acids [15].

3.5.3 Melting Point

The melting point of fat is determined by chain length and the saturation of the fatty acids. Although there are several methods to measure melting point, one of the simplest is slip point [10]. After extraction of lipids and complete removal of organic solvents, the lipids are heated gently and drawn into capillary tubes. After chilling, the tubes are immersed in water and the temperature is raised gradually. The slip point (melting point) is that temperature at which the lipids begin to slip up the capillary tube.

Longer-chain fatty acids have higher melting points than shorter-chain fatty acids, and more saturated fatty acids have higher melting points than unsaturated fatty acids. Because of the abundance of stearic and palmitic acids, animal fats are typically solids at room temperature. At slightly

higher temperatures (90°F–100°F), rendered pork fat will separate with a liquid upper layer and solid material in the lower layer. The solid portion is known as stearin (from the Greek for animal fat) and is composed primarily of stearic acid. The liquid portion is composed of the glycerol backbone in the form of esters with the less saturated fatty acids, primarily oleic acid, and is known as lard oil or olein (from the Latin for oil).

Due to differences in fatty acid composition, the melting points of fat from different species vary. Lamb fat typically has the highest melting point, followed by beef fat and then pork fat. With a high polyunsaturated fat content, poultry fat has the lowest melting point of the four species (80°F–110°F). Related to the higher internal temperature, the internal fats have a higher saturated fatty acid content and a higher melting point. The fat in the outer layers of the animal body is less saturated with a lower melting point. This corresponds to the lower temperature near the body surface and the need to maintain a more liquid state. For example beef kidney fat has a melting point of 104°F–122°F, while the beef external fat has a melting point of only 89°F–110°F. Beef brisket subcutaneous fat has an unusually low melting point (77°F), due to its high concentration of oleic acid [8]. Pork leaf fat has a melting point of 110°F–118°F; pork back fat has a melting point of 86°F–104°F.

Tallow and lard melting points are standardized as titers, which is the minimum temperature at which fat congeals [2]. For edible tallow, the minimum titer is 105°F, whereas for edible lard, the minimum titer is 100°F.

3.5.4 Oxidative Rancidity

Rendered fat high in *polyunsaturated* fatty acids will react chemically with oxygen resulting in off-flavors and odors known as rancidity. Oxidative rancidity is catalyzed by the presence of heat, light, salt and iron, as well as other elements. Because rancidity is not chemically defined or quantifiable, measurements of oxidation products are often used to indicate quality of the product. One such test is the peroxide value test that measures the milliequivalents (mEq) of peroxide per kilogram fat. A low peroxide value (<10 mEq) indicates a non-rancid fat. Lard and leaf lard (from pig abdominal fat) should have a peroxide value no higher than 5 to be considered high quality [2].

To increase the value of rendered fats reentering the human food supply, antioxidants are often added. Even with the pet food industry, antioxidants are recommended due to the loss of fat-soluble vitamins A, D, and E that occurs with oxidative rancidity. Approved antioxidants are odorless, tasteless, and non-toxic to humans. Approved antioxidants include butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), glycine, propyl gallate, resin guaiac, tertiary butylhydroquinone (TBHQ), and tocopherols. Approved combinations of these antioxidants have been shown to be effective in not only protecting the rendered fat from oxidative rancidity, but to also protect products made from these rendered fats.

3.6 CONCLUSION

Rendered fats have a great deal of functionality, depending on the source of the fat and the separation techniques. Rendering allows an outlet for animal by-products that would otherwise be unusable and accumulate, creating significant economic and environmental problems. Differential rendering of fat trims that vary in melting points improves the functionality and usefulness of the rendered fat.

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4 Analysis of Cholesterol in Edible Animal By-Products

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CONTENTS

4.1 INTRODUCTION

Cholesterol (5-cholesten-3β-ol) is an essential structural component of cell membranes and lipoproteins and serves as the precursor for steroid hormones, bile acids, and vitamin D.[1] However, numerous studies have shown that an increase in the intake of dietary cholesterol can increase the plasma low density lipoprotein (LDL) cholesterol.[2–8] There is an association between blood levels of cholesterol and the risk of coronary heart diseases[9] (CHD) and premature development of atherosclerosis in humans.[10] On the other hand, the increasing amount of the dietary cholesterol on LDL has been relatively small in comparison to the well-established LDL-raising effects of dietary saturated fat[3] and it is highly variable between individuals;[2,5,6] however, several studies do not link dietary cholesterol to the risk of CHD.[11–13] The main dietary modification needed to reduce plasma LDL-cholesterol concentration involves decreased intakes of dietary cholesterol and total fat.[14] One of the implications of the dietary guidelines for public health policy is the need for food labels showing the total fat, saturated fat, and cholesterol content.

Dietary cholesterol is strictly linked with foods of animal origin since cholesterol is a constituent of animal cells. However, little information is available on the consumption and mainly the estimate of cholesterol content in edible animal by-products used as human foods. Earlier methods for the determination of cholesterol were neither sufficiently precise nor standardized and the data published in different studies were not always comparable.

4.2 CHOLESTEROL: A STEROL

The cholesterol is a small molecule highly recognized since thirteen Nobel Prizes awarded between 1910 and 1985 were associated with work on sterols. In 1974, cholesterol was isolated from biliar calculus and continues to be the focus of research activities in the field of many chemists, biochemists, as well as clinical workers due to its relation to atherosclerosis. The other sterols that have great impact on science research are phytosterol esters because of their lowering effect on blood cholesterol and antipolymerization effect on frying fats.

Cholesterol has a C27 carbon skeleton being a sterol characteristic of higher animals and present in very small amounts in plants and marine algae, while phytosterols contain 28 and 29 carbon atoms and are present in minor amounts in vegetable oils, nuts seeds, cereals, and beans.

4.3 CHOLESTEROL METABOLISM IN HUMANS

The critical question about the metabolism of cholesterol in humans is: does a lower intake of dietary cholesterol contribute significantly to lowering of plasma cholesterol reducing risk of coronary disease? The lipid hypothesis of coronary heart diseases has received wide acceptance; however, it has many limitations and deficiencies, which have been discussed in detail.[11–13]

The works that show the dietary influences on plasma cholesterol commonly use as model species rabbits, which are very sensitive to dietary cholesterol, developing severe hypercholesterolemia in response to modest dietary supplementation.[15] On the other hand, humans and other model species such as rats are less responsive since the intestinal absorption is far from complete. The absorption of dietary cholesterol averages 60% in the human population with individual values ranging from 20% to 80% at a daily intake of 200–300 mg of cholesterol.[16] The rate of absorption depends on a number of factors including the total dietary cholesterol load; when the quantity was increased to 800 mg/day the fractional absorption was only 55%, demonstrating that an increase in dietary cholesterol supply had no substantial effect on the plasma lipids. In addition, it is now accepted that for most people the influence of dietary cholesterol on plasma cholesterol is really quite small. Harman et al.[17] showed that increasing dietary cholesterol by consuming two eggs a day produces no increase in plasma LDL when accompanied by energy restriction and moderate weight loss.

Endogenous cholesterol synthesis ranges between 11 and 13 mg/day per kg body weight, so a man with 70 kg will produce around 770–910 mg/day. If the body synthesizes 840 mg cholesterol per day and absorbs a dietary cholesterol input of 270 mg/day (60% of 450 mg/day, which is an average dietary cholesterol intake of an American), the total amount of cholesterol the body must handle would be equal to 1110 mg/day: 76% from synthesis and 24% from diet. Nearly 20%–30% of this cholesterol will synthesize bile acid, and small amounts will be used for steroid hormone production and tissue repair. The body has no mechanisms to degrade steroids, so most of this daily input must be excreted as unabsorbed bile acids and biliary cholesterol.[16]

In addition, the synthesis of cholesterol by the human organism is under feedback control; increased dietary cholesterol supply decreases the rate of endogenous cholesterol synthesis by 20%. Normally, the population responds to increased cholesterol intake through lower intestinal absorption, suppression of endogenous synthesis, increased sterol excretion, and/or increased deposition in the tissues. In general, the compensation is such that the influence of dietary cholesterol is minimal. According to McNamara,[16] only 31% of the test subjects showed an increased serum cholesterol level after a higher dietary cholesterol intake, while in the other people it remained unchanged and

the cholesterol synthesis in the mononuclear leukocytes decreased by 26%. It demonstrated that most of the individuals have a precise feedback control mechanism maintaining the level of cholesterol serum relatively constant when the cholesterol supply changes moderately. Nevertheless, there are individuals who exhibit raised cholesterol by dietary cholesterol although in any population it is relatively small.

4.4 METHODS OF CHOLESTEROL DETERMINATION

The determination of the sterol components, particularly cholesterol, among the unsaponifiable lipids of food is of great importance to the food industry. The qualitative and quantitative evaluation of cholesterol is of interest to the consumer because of an apparent relationship between increased incidence of coronary heart disease and elevation of serum cholesterol.[14]

Methods for cholesterol analysis have been developed for decades, beginning in the late nineteenth century when Salkowski described a color reaction for this analyte, which was isolated from gallstones about a century ago.[18] A great number of the methods, gravimetric, colorimetric, or chromatographic, for the determination of cholesterol in foods have been developed over the years. Prior to the qualitative and quantitative steps, the cholesterol must be separated from the apolar fractions such as triglycerides. Methods for isolation and identification of cholesterol have been much improved since the early method of precipitation with digitonin or tomatin[19] to high performance liquid chromatography (HPLC) with detector of mass spectrometry (MS). Nowadays, to determine cholesterol in foods, gas chromatography (GC) and HPLC are chosen over the colorimetric and enzymatic methods since the former are more accurate due to their greater specificity. This is especially true when samples contain both cholesterol and phytosterols.

4.4.1 Saponification and Extraction

Saponification is a vital step, which is conducted routinely for two reasons. The first is to convert cholesterol esters to free cholesterol in the determination of total cholesterol; if this step was not carried out the results must be expressed in free cholesterol or the results of total cholesterol are subestimated. The second reason is to remove mainly triglyceride and free fatty acid.

Two saponification procedures, saponification of the lipids and saponification of the food normally designed to direct saponification, are often employed. Direct saponification has been the method of choice in meat products[20–26] because saponification of the lipids are time-consuming procedures, laborious, and costly, and they use highly toxic and flammable chemicals in large quantities. In addition, many reports have shown that direct saponification produces results that are slightly higher than or comparable to saponification of the lipids because there are fewer steps.[20–22,25,27–29]

After saponification, addition of water is necessary to increase the polarity and improve the partitioning of nonpolar compounds into the organic phase to extract the unsaponifiable material. The organic solvent normally used are *n*-hexane,[30–36] benzene,[37] cyclohexane,[22,28,38] toluene,[21] ether/hexane,[29] diethyl ether, and petroleum ether.[39] Al-Hasani et al.[23] compared toluene and *n*-hexane and as the results were similar, recommended the use of *n*-hexane. King et al.[40] compared the solubility of cholesterol in six solvents (hexane, diethyl ether, cyclohexane + 1% isopropanol, toluene, and methylene chloride) and observed good recovery of cholesterol with toluene or hexane; but toluene has the disadvantage of being toxic. Tsai and Hudson[41] suggested the use of diethyl ether, and Thompson and Merola[38] cyclohexane since water has an extremely low solubility in this solvent.

4.4.2 HPLC Analysis

HPLC and GC are the most frequently used chromatographic methods for analysis of cholesterol in meat. HPLC has some advantages as it is often carried out under ambient temperatures: provides an

ideal means, for sample, recovery and purification, simplifies the quantification procedure, shortens the analysis time, and introduces fewer artifacts.

The triglycerides of the fatty acids comprise up to 99% of the total lipids in most foods and interfere with cholesterol detection and determination since its content is relatively small in comparison to the triglycerides. Consequently, the HPLC methods for determination of cholesterol in food have been developed using direct saponification[26,42,43] or extraction of the lipid and subsequent saponification.[30,31,44,45] Reversed-phase systems with mobile phase from nonpolar (2-propanol:hexane)[46] to extremely polar (methanol)[47] and ultraviolet (UV) detection at 205, 210, 230 and 254 nm have been used. The most common HPLC conditions used for the analysis of cholesterol in meat consist of a C18 column (150 mm, 4.6 mm, 5 μm) with a mixture of acetonitrile and 2-propanol as mobile phases and UV detection set at 210 nm. Comparison of the retention times of the samples with those of the cholesterol standard, co-chromatography, and spectra taken at 190–300 nm when using a photodiode array detector have been used for the identification of the cholesterol. Since HPLC is a nondestructive technique, it is possible to recover the cholesterol and confirm its identity by GC-MS or other techniques.[25] For the quantification of cholesterol, most of the publications report the use of a calibration curve obtained by relating the cholesterol concentration and the corresponding area. When possible, an internal standard like 6-cetocholesterol,[42] stigmasterol,[48] or pregnolone[29] is used.

A procedure for quantification of cholesterol by HPLC in muscle and organs was optimized as show a Figure 4.1. Using this method, the mean of recovery was 95 ± 1% and the coefficient of variation was 1.4% when cholesterol was added in meat.[42] This method was validated also using meat standard reference material (SRM 1546, NIST) and the results obtained (75.0 ± 0.5 mg/100 g) were similar to those declared on the certificate (75 ± 7 mg/100 g).

4.4.3 GC Analysis

Among the chromatographic methods to determine cholesterol in meat, the most commonly used is the GC. There are numerous GC methods available which are generally cumbersome and time-consuming.[37,49,50] Besides, there is little uniformity among these methods regarding the respective extractions of total fat and the unsaponifiable from total fat. Most of the methods for cholesterol determination by GC consist of direct saponification, extraction of nonsaponifiable materials with an organic solvent, and derivatization prior to analysis. Derivatization of sterols for GC is still a common practice in the methodologies of the analysis of cholesterol,[24,38,40,51] although Kanada et al.[52] showed that derivatization was non essential since this procedure is not only time-consuming but also contributes to increase the noise and to decrease linearity due to silicon deposits on the flame ionization detector (FID) originated from the trimethylsilyl (TMS) derivatives.[53] However, King et al.[40] obtained higher recovery in turkey meat using derivatized samples than underivatized samples.

To determine cholesterol by GC the most common system of detection is by flame ionization detector (FID) with separation being performed with nonpolar columns (5% diphenyl and 95% dimethylpolysiloxane) or medium and low polarity columns. Most works use slightly polar column as HP-5, DB-5, Ultra2, or HP-1 (5% phenyl-methyl silicone or methyl silicone) fused silica bonded phase capillary column with a length of 25 m. The conditions used for cholesterol identification are the same as for HPLC, i.e., comparison of the retention times of the samples with those of the cholesterol standard, co-chromatography and confirmed by MS.[22,40] The majority of the works quantify cholesterol by internal standardization using 5α-cholestane.[23,28,29,40,52] However, Thompson and Merola[38] recommended 5α-cholestanol instead of 5α-cholestane since it is an alkane, and so does not have the same chemical and physical proprieties of cholesterol or one of the plant sterols when the samples do not already contain such sterol. In this way, the choices of the internal standard depend to the greatest extent on the type of samples to be analyzed.[38]

Piironen et al.[51] determined cholesterol in meat and organs by GC using direct saponification, with two internal standards (epicholesterol mixed with the sample and dihydrocholesterol during

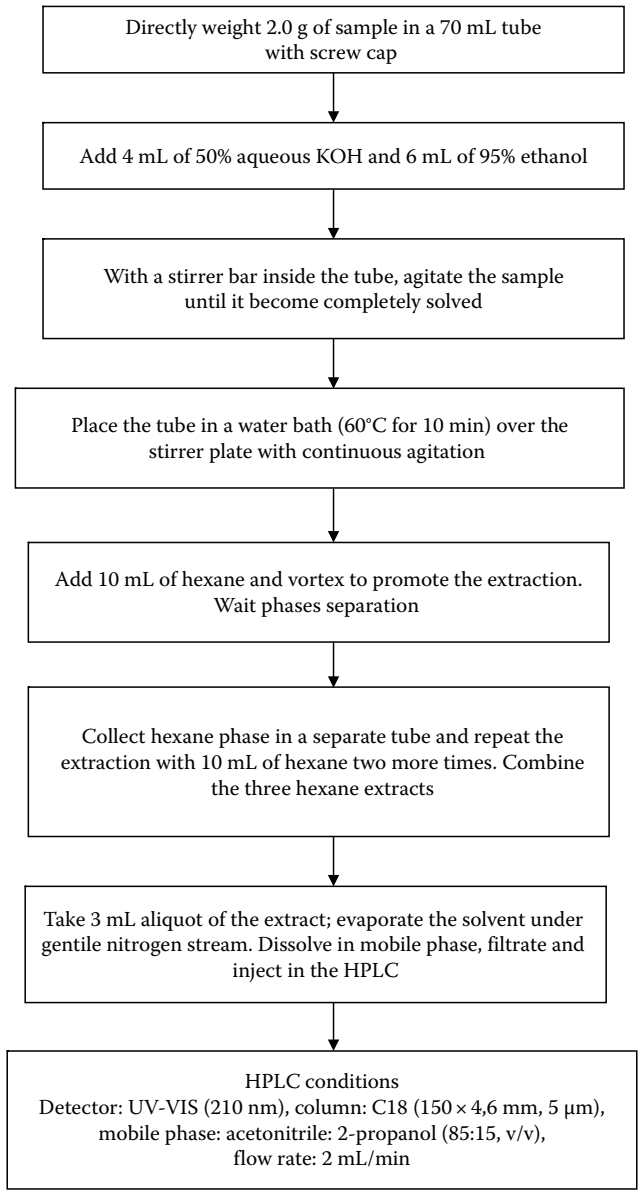

FIGURE 4.1 Cholesterol assay fluxogram.

saponification) and saturated aqueous KOH in absolute ethanol at 59°C–85°C for 30 min. The unsaponifiable matter was extracted with 20 mL of cyclohexane from the hydrolyzate diluted with 12 mL of water. The sterols were derivatized to trimethylsilyl (TMS) ethers and analyzed with capillary GC. Anhydrous milk fat (274.7 ± 9.0 mg/100 g; BCR CRM 164; European Commission, Geel, Belgium) was used as a certified reference material to confirm the analytical values. The cholesterol levels obtained were 246 ± 8 mg for triplicates and 266 ± 6 mg for six analysis, when epicholesterol and dihidrocholesterol were used as the internal standards, respectively, in agreement with the certified values. Comparing the date obtained by two internal standards, the values with dihidrocholesterol were 3.3 ± 2.3 mg higher than those obtained with epicholesterol in meat and meat products. The percentage of recovery of cholesterol for meat and meat products was 95% ± 4%.

4.4.4 Colorimetric and Enzymatic Analysis

The tendency to overestimate the cholesterol content in foods, together with the presence of other chromogens present in the samples, limit the use of colorimetric methods. However, it has been largely used for measuring the cholesterol extracted from meat.[54–62] Enzymatic methods as in colorimetric methods also are not specific for cholesterol, since all sterols with a 3 β-OH group react with this enzyme, normally cholesterol oxidase, leading to an overestimation of the cholesterol content in foods, especially those containing mixtures of animal and vegetable origin. This method has also been used to determine cholesterol in meat and meat products,[22,42,63–65] but in lower extension than the colorimetric methods.

The overestimation depends mainly on the sample analyzed and the method used. When the samples have higher amount of cholesterol than the other sterols such as meat, egg, and cheese, the results of the cholesterol obtained by colorimetric methods and by GC[36] and HPLC[65] are similar. Similarly, the enzymatic method showed no significant difference in bovine meat, milk, and egg cholesterol values when compared to the results obtained by HPLC.[42,66] In the same way, when *biceps femoris* was analyzed, no differences were observed between the enzymatic method and HPLC methods; but higher cholesterol levels by the enzymatic method than by HPLC were obtained in *semi membranous*, chicken drumsticks and chicken thighs.[26] In order to obtain accurate results using enzymatic and colorimetric methods, it is necessary to control the analytical conditions, mainly temperature and time of reaction.

4.5 CHOLESTEROL CONTENTS IN EDIBLE ANIMAL BY-PRODUCTS

Edible offal is defined as the edible part of the carcass of any animal ordinarily consumed by man, other than the meat flesh of that carcass, and includes liver, kidneys, heart, tongue, brains, pancreas, thymus, and spleen. Since cholesterol is the major component of cell membranes and of nerves and is an active metabolite within the cells of organs and glandular meats, heart, kidney, liver, and sweetbreads contain higher concentrations of cholesterol than regular cuts of meat, with or without fat, the concentration being highest in brains. However, little information is available in the literature about cholesterol levels in these foods. As the methods for the determination of cholesterol in the past were neither sufficiently accurate nor standardized, Tables 4.1 through 4.10 show the results obtained mainly with GC and HPLC methods. However, some results obtained with colorimetric and enzymatic method were also added; but only the ones obtained after 1976, since by that time data about cholesterol in food including offal were reviewed by Sweeney and Weihrauch[19] for the period 1972–1976 and Feeley et al.[67] on data prior to 1972.

4.5.1 Cholesterol Contents in Brain

Although the results shown in Table 4.1 vary greatly, they indicate that the cholesterol content of brain is high in all animals analyzed compared with the other offal. The lowest values in raw samples were obtained in lamb brain and the highest in beef brain. The results of raw lamb brain showed little variation (939 mg/100 g[74] to 1352 mg/100 g[62]) while raw beef brain showed great variation (1456 mg/100 g[68] to 3010 mg/100 g[71]). The results obtained in raw mutton brain by colorimetric method are similar to the cholesterol values in raw lamb brain obtained by enzymatic method, and these results are lower comparing with the other results in brains of other animals. However, the sample preparation used in these methods was carried out by extraction of lipids followed by the determination of cholesterol without saponification, which can explain the low results because these results must be expressed in free cholesterol and not total cholesterol. Similarly, in raw brain beef, lower results were obtained by colorimetric methods than by chromatographic methods, showing that the cholesterol levels depend on the sample preparation.

TABLE 4.1
Cholesterol Contents in Brain

Animals and References	Methods			Cholesterol Level (mg/100 g, Wet Basis)
	Lipid Extraction	Saponification	Measurement	
Beef				
Raw[68]	Ether	No	Colorimetric	1456 ± 55
Raw[69]	C/M	Saponification	Colorimetric	2154 ± 300
Raw[70]	ND	ND	ND	2335 (2000–2670)
Raw[71]	No	Direct saponification	GC-DIC, TMS, IS	3010
Cooked[68]	Ether	No	Colorimetric	1395 ± 81
Pan-fried[71]				1995
Simmered[71]				3100
Calf				
Raw[70]	ND	ND	ND	2000
Lamb				
Raw[71]	No	Direct saponification	GC-DIC, TMS, IS	1352
Raw[72]	C/M	Saponification	CG, TMS, IS	1352
Breed, raw				
Altamurana[73]	No	Direct saponification	Enzymatic	1067 ± 169
Comisana[73]				939 ± 184
Gentile di Puglia[73]				987 ± 166
Males[73]				997 ± 104
Females[73]				999 ± 212
Singles[73]				1002 ± 159
Twins[73]				994 ± 189
Braised[71]	No	Direct saponification	GC-DIC, TMS, IS	2043
Pan-fried[71]				2504
Cooked[72]	C/M	Saponification	CG, TMS, IS	1886
Boiled[74]	ND	ND	GC	2200
Mutton				
Raw[68]	Ether	No	Colorimetric	968 ± 74
Raw[70]	ND	ND	ND	2200
Cooked[68]				1408 ± 109
Pork				
Raw[70]	ND	ND	ND	2550 (2000–3100)
Raw[71]	No	Direct saponification	GC-DIC, TMCS, IS	2195
Braised[71]				2552
Veal				
Raw[71]	No		GC-DIC, TMCS, IS	1590
Braised[71]				3100
Pan-fried[71]				2120

Note: C/M, extraction with chloroform and methanol; TMS, with derivatization; IS, internal standard (5α-cholestane); ND, not described.

TABLE 4.2
Cholesterol Contents in Gizzard

Animals and References	Methods			Cholesterol Level (mg/100 g, Wet Basis)
	Lipid Extraction	Saponification	Measurement	
Chicken				
Raw[69]	C/M	Saponification	Colorimetric	260 ± 16
Raw[71]	No	Direct saponification	GC-DIC, TMS, IS	240
Simmered[71]				370
Cooked in water[75]	No	Direct saponification	GC-FID, IS	73 ± 16
Turkey				
Raw[71]	No	Direct saponification	GC-DIC, TMS, IS	197
Simmered[71]				203

Note: C/M, extraction with chloroform and methanol; TMS, with derivatization; IS, 5α-cholestane.

4.5.2 Cholesterol Contents in Gizzard

As shown in Table 4.2, the results of cholesterol found by Labrador and Sangronis[69] in raw chicken gizzard (260 ± 16 mg/100 g) using colorimetric method were similar to the ones described in USDA[71] (240 mg/100 g) using GC. On the other hand, the results obtained after thermal treatment were very different considering that both samples were cooked in the same way. Both methods were carried out by GC using direct saponification, a small difference being that one used derivatization, while the other did not. The results obtained by Pereira et al.[75] were very lower than those described in USDA[71]; however the samples were not the same since the animals were different from each other and the cholesterol in chicken can be varied with diet, breed, species, and hen's age. Comparing the results of cholesterol levels in gizzard between chicken and turkey, it can be observed that turkey showed lower levels than chicken sieving of the results by Pereira et al.[75] in cooked chicken gizzard.

4.5.3 Cholesterol Contents in Heart

There are several results of cholesterol reported for heart in beef, chicken, lamb, pork, turkey, and veal, which varied between the limits from 64 mg/100 g in raw mutton to 238 mg/100 g in raw chicken and in cooked samples from 92 mg/100 g in mutton to 291 mg/100 g in lamb (Table 4.3). The lower results obtained in raw and cooked samples were in mutton,[68] being similar to the results obtained in raw and cooked heart beef[68] and raw heart pork. The other results were higher than these and in general the samples after thermal treatment showed higher levels of cholesterol than the raw samples. However, these results were expected since all results are expressed in wet weight because during heating there is a loss of water and consequently the other compounds were concentrated. However, the results showed great variation between different and equal animals, and it is interesting to note that the results obtained in heart veal from American and Australian samples were similar.

4.5.4 Cholesterol Contents in Kidney

Although the results obtained vary greatly between the same and different animals, they indicate that the cholesterol content in kidney is high but lower than in brain (Table 4.4). The largest variation can be observed in raw (100–517 mg/100 g) and cooked (200–716 mg/100 g) beef kidney. Comparing the results obtained in beef kidney by colorimetric methods, it is possible to see the great difference between colorimetric methods where one is very low and the other very high. Both analyses were carried out with extraction of the lipids and no saponification step, so the results are in relation to

TABLE 4.3
Cholesterol Contents in Heart

Animals and References	Methods			Cholesterol Level (mg/100 g, Wet Basis)
	Lipid Extraction	Saponification	Measurement	
Beef				
Raw[68]	Ether	No	Colorimetric	72 ± 6
Raw[70]	ND	ND	ND	138 (125–150)
Raw[71]	No	Direct saponification	GC-DIC, TMS, IS	124
Raw[72]	C/M	Saponification	CG, TMS, IS	103
Raw[74]	ND	ND	GC	140
Raw[76]	ND	ND	ND	91
Barbecued[68]	Ether	No	Colorimetric	93 ± 5
Simmered[71]	No	Direct saponification	GC-DIC, TMS, IS	212
Cooked[72]	C/M	Saponification	CG, TMS, IS	179
Stewed[74]	ND	ND	GC	230
Cooked[76]	ND	ND	ND	128
Stewed with pork fat and margarine[76]				102
Calf				
Raw[70]	ND	ND	ND	2000
Chicken				
Raw[69]	C/M	Saponification	Colorimetric	238 ± 40
Raw[70]	ND	ND	ND	170
Raw[71]	No	Direct saponification	GC-DIC, TMS, IS	136
Raw[77]	No	Direct saponification	HPLC	161 ± 24
Raw[78]	ND	ND	ND	170
Simmered[71]	No	Direct saponification	GC-DIC, TMS, IS	242
Roosted[75]	No	Direct saponification	GC-FID, IS	213 ± 19
Fried[77]	No	Direct saponification	HPLC	292 ± 66
Lamb				
Raw[71]	No	Direct saponification	GC-DIC, TMS, IS	135
Raw[72]	C/M	Saponification	CG, TMS, IS	129
Raw[74]	ND	ND	GC	140
Braised[71]	No	Direct saponification	GC-DIC, TMS, IS	249
Cooked[72]	C/M	Saponification	CG, TMS, IS	199
Roasted[79]	ND	ND	GC	260
Mutton				
Raw[68]	Ether	No	Colorimetric	64 ± 3
Raw[70]	ND	ND	ND	130
Barbecued[68]	Ether	No	Colorimetric	90 ± 5
Pork				
Raw[70]	ND	ND	ND	154 (150–158)
Raw[71]	No	Direct saponification	GC-DIC, TMS, IS	131
Raw[74]	ND	ND	GC	79
Raw[76]	ND	ND	ND	92
Raw[78]	ND	ND	ND	150
Braised[71]				221
Stewed with pork fat and margarine[76]	ND	ND	ND	112

(*continued*)

TABLE 4.3 (continued)
Cholesterol Contents in Heart

Animals and References	Methods			Cholesterol Level (mg/100 g, Wet Basis)
	Lipid Extraction	Saponification	Measurement	
Turkey				
Raw[71]	No	Direct saponification	GC-DIC, TMS, IS	147
Simmered[71]				184
Veal				
Raw[71]	No	Direct saponification	GC-DIC, TMS, IS	104
Raw[72]	C/M	Saponification	CG, TMS, IS	104
Braised[71]	No	Direct saponification	GC-DIC, TMS, IS	176
Cooked[72]	C/M	Saponification	CG, TMS, IS	172

Note: C/M, extraction with chloroform and methanol; TMS, with derivatization; IS, 5α-cholestane; ND, not described.

free cholesterol and not total cholesterol, which can explain the lower results. Moreover, depending upon the conditions used in colorimetric method, the results can be overestimated, which might explain the high results. Using colorimetric methods, Mustafa[68] obtained lower levels of cholesterol in beef and mutton although both the results were similar. However, the results obtained with raw and cooked lamb were very similar independent of the method of the analysis. In addition, kidneys from different breeds of lamb have same cholesterol levels with the exception of the Altamurana breed that showed higher levels than the other breeds.

4.5.5 Cholesterol Contents in Liver

Because the liver is a common food, there is more information about cholesterol as can be seen in Table 4.5, which shows the cholesterol contents in beef, calf, chicken, duck, goose, lamb, mutton, pork, turkey, and veal. The results cover a wide range from 105 mg/100 g[81] to 712 mg/100 g[69] in raw liver chicken and from 192 mg/100 g[75] in liver chicken to 585 mg/100 g in liver lamb[72] when cooked. The highest result found in liver chicken by Labrador and Sangronis[69] was obtained by colorimetric method that can explain this result. On the other hand, the results in cooked liver chicken showed great variability from 192 mg/100 g[81] mg/100 g[75] to 563 mg/100 g[71] although both methods used chromatography determination demonstrating that the variation of the results could be attributed to the samples.

Cholesterol levels mostly increased on cooking, due to the effects of concentration and subsequent the moisture loss; however, the results obtained in cooked beef, chicken, and lamb were lower than raw.[73]

The literature is limited regarding changes in liver cholesterol due to the effect of cholesterol-reducing agents, especially in beef, pork, lamb, and chicken. When feeding chickens (male Ross × Ross) with garlic powder, linear reductions of cholesterol in liver were observed as garlic supplementation increased.[81] In addition, a decrease of about 28% in liver cholesterol was found in birds fed garlic, copper, and combination of garlic and copper in comparison to the control.

The free cholesterol and its esters, as well as total cholesterol content were determined in beef, chicken and pork liver raw (Table 4.6). The ester cholesterol is higher than free cholesterol varying from 69% in chicken liver to 80% in beef liver. Perona and Ruiz-Gutierrez[83] found 71% and 18% of the esterified cholesterol in raw muscle and abdominal adipose tissue of Iberian pig, respectively. According to Bhattacharyya and Strong,[84] cholesterol is present in atherosclerotic lesions primarily

TABLE 4.4
Cholesterol Contents in Kidney

Animals and References	Methods			Cholesterol Level (mg/100 g, Wet Basis)
	Lipid Extraction	Saponification	Measurement	
Beef				
Raw[68]	Ether	No	Colorimetric	100 ± 12
Raw[69]	C/M	Saponification	Colorimetric	517 ± 18
Raw[70]	ND	ND	ND	358 (340–375)
Raw[71]	No	Direct saponification	GC-DIC, TMS, IS	411
Raw[72]	C/M	Saponification	CG, TMS, IS	313
Raw[74]	ND	ND	GC	265
Raw[76]	ND	ND	ND	213
Simmered[71]	No	Direct saponification	GC-DIC, TMS, IS	716
Cooked[72]	C/M	Saponification	CG, TMS, IS	549
Barbecued[68]	Ether	No	Colorimetric	200 ± 13
Stewed[79]	ND	ND	GC	460
Calf				
Raw[70]	ND	ND	ND	380
Lamb				
Raw[71]	No	Direct saponification	GC-DIC, TMCS, IS	337
Raw[72]	C/M Folch	Saponification	CG, TMS, IS	338
Raw[74]	ND	ND	GC	315
Breed, raw				
Altamurana[74]	No	Direct saponification	Enzymatic	374 ± 90
Comisana[74]				320 ± 61
Gentile di Puglia[74]				298 ± 59
Males[74]				329 ± 92
Females[74]				332 ± 59
Singles[74]				321 ± 72
Twins[74]				339 ± 73
Braised[71]	No	Direct saponification	GC-DIC, TMCS, IS	565
Cooked[72]	C/M Folch	Saponification	CG, TMS, IS	550
Fried[79]	ND	ND	GC	610
Mutton				
Raw[68]	Ether	—	Colorimetric	102 ± 6
Raw[70]	ND	ND	ND	365 (354–375)
Raw[76]	ND	ND	ND	276
Barbecued[68]	Ether	—	Colorimetric	214 ± 14
Fried with margarine[76]	ND	ND	ND	401
Pork				
Raw[70]	ND	ND	ND	385 (365–405)
Raw[71]	No	Direct saponification	GC-DIC, TMCS, IS	319
Raw[74]	ND	ND	GC	410
Raw[76]	ND	ND	ND	395
Braised[71]	No	Direct saponification	GC-DIC, TMCS, IS	480
Stewed[79]	ND	ND	GC	700
Fried with margarine[76]	ND	ND	ND	483
Veal				
Raw[71]	No	Direct saponification	GC-DIC, TMCS, IS	364
Raw[72]	C/M	Saponification	CG, TMS, IS	272
Braised[71]	No	Direct saponification	GC-DIC, TMCS, IS	791
Cooked[72]	C/M	Saponification	CG, TMS, IS	434

Note: C/M, extraction with chloroform and methanol; TMCS = with derivatization; IS: 5α-cholestane; ND, not described.

TABLE 4.5
Cholesterol Contents in Liver

Animals and References	Methods			Cholesterol Level (mg/100 g, Wet Basis)
	Lipid Extraction	Saponification	Measurement	
Beef				
Raw[51]	No	Direct saponification	GC-FID, TMS	228
Raw[68]	Ether	No	Colorimetric	192 ± 10
Raw[69]	C/M	Saponification	Colorimetric	334 ± 21
Raw[70]	ND	ND	ND	261 (257–265)
Raw[71]	No	Direct saponification	GC-DIC, TMCS, IS	275
Raw[72]	C/M	Saponification	CG, TMS, IS	271
Raw[74]	ND	ND	GC	270
Raw[76]	ND	ND	ND	283
Raw[77]	No	Direct saponification	HPLC	338 ± 31
Raw[80]	C/M	Saponification	HPLC	230 ± 16
Barbecued[68]				385 ± 31
Braised[71]	No	Direct saponification	GC-DIC, TMCS, IS	396
Immered[71]				381
Cooked[72]	C/M	Saponification	CG, TMS, IS	409
Fried[76]				297
Fried[77]	No	Direct saponification	HPLC	460 ± 23
Stewed[79]	ND	ND	GC	240
Fried[82]	No	Direct saponification	GC-FID	265 ± 35
Raw[70]	ND	ND	ND	360
Raw[74]	ND	ND	GC	370
Raw[76]	ND	ND	ND	286
Fried with pork fat and margarine[76]				284
Fried[76]				310
Grilled[76]				387
Fried[79]				330
Chicken				
Raw[69]	C/M	Saponification	Colorimetric	712 ± 40
Raw[70]	ND	ND	ND	492 (429–555)
Raw[71]	No	Direct saponification	GC-FID, TMCS, IS	345
Raw[74]	No	Direct saponification	GC-FID, TMCS, IS	380
Raw[80]	C/M	Saponification	HPLC	228 ± 12
Raw, diet 1[81]	C/M	Cholesterol esterase	Enzymatic	
Control[81]				105 ± 10
1.5% Garlic[81]				91 ± 15
3% Garlic[81]				73 ± 15
4.5% Garlic[81]				54 ± 9
Raw, diet 2[81]				
Control[81]				208 ± 25
3% Garlic + 0 mg Cu/kg[81]				150 ± 10
0% Garlic + 180 mg Cu/kg[81]				152 ± 12
3% Garlic + 180 mg Cu/kg[81]				176 ± 14

TABLE 4.5 (continued)
Cholesterol Contents in Liver

Animals and References	Methods			Cholesterol Level (mg/100 g, Wet Basis)
	Lipid Extraction	Saponification	Measurement	
Pan-fried[71]	No	Direct saponification	GC-FID, TMCS, IS	564
Simmered[71]				563
Fried[79]	ND	ND	GC	350
Fried[75]	No	Direct saponification	GC-FID, IS	192 ± 31
Cooked[80]	C/M	Saponification	HPLC	410 ± 18
Duck				
Raw[71]	No	Direct saponification	GC-DIC, TMCS, IS	515
Goose				
Raw[71]	No	Direct saponification	GC-DIC, TMCS, IS	515
Lamb				
Raw[71]	No	Direct saponification	GC-DIC, TMCS, IS	371
Raw[72]	C/M	Saponification	CG, TMS, IS	433
Raw[74]	ND	ND	GC	430
Raw, breed[73]	No	Direct saponification	Enzymatic	
Altamurana[73]				456 ± 127
Comisana[73]				298 ± 49
Gentile di Puglia[73]				362 ± 120
Males[73]				350 ± 134
Females[73]				394 ± 112
Singles[73]				354 ± 90
Twins[73]				390 ± 130
Braised[71]	No	Direct saponification	GC-DIC, TMCS, IS	501
Pan-fried[71]				493
Cooked[72]	C/M	Saponification	CG, TMS, IS	585
Fried[79]	ND	ND	GC	400
Mutton				
Raw[68]	Ether	No	Colorimetric	279 ± 35
Raw[70]	ND	ND	ND	312 (300–323)
Barbecued[68]	Ether	No	Colorimetric	356 ± 47
Pork				
Raw[51]	No	Direct saponification	GC-FID, TMS	225
Raw[70]	ND	ND	ND	354 (340–368)
Raw[71]	No	Direct saponification	GC-DIC, TMCS, IS	301
Raw[74]	ND	ND	GC	260
Raw[76]	ND	ND	ND	237
Raw[80]	C/M	Saponification	HPLC	214 ± 8
Braised[71]	No	Direct saponification	GC-DIC, TMCS, IS	355
Fried with pork fat and margarine[76]	ND	ND	ND	233
Fried[76]				256
Grilled[76]				267
Stewed[79]	ND	ND	GC	290
Cooked[80]	C/M	Saponification	HPLC	289 ± 32

(*continued*)

TABLE 4.5 (continued)
Cholesterol Contents in Liver

Animals and References	Methods			Cholesterol Level (mg/100 g, Wet Basis)
	Lipid Extraction	Saponification	Measurement	
Turkey				
Raw[71]	No	Direct saponification	GC-DIC, TMCS, IS	331
Simmered[71]				388
Veal				
Raw[71]	No	Direct saponification	GC-DIC, TMCS, IS	334
Raw[72]	C/M	Saponification	CG, TMS, IS	206
Braised[71]	No	Direct saponification	GC-DIC, TMCS, IS	511
Pain-fried[71]				485
Cooked[72]	C/M	Saponification	CG, TMS, IS	244

Note: C/M, extraction with chloroform and methanol, TMCS = with derivatization; IS, 5α-cholestane; ND, not described.

TABLE 4.6
Total, Free, and Esterified Cholesterol Contents in Raw Liver

Animals	Total Cholesterol	Free Cholesterol		Esterified Cholesterol	
	mg/100 g	mg/100 g	%	mg/100 g	%
Beef	230 ± 16	47 ± 2	20	183 ± 16	80
Chicken	228 ± 12	70 ± 3	31	158 ± 13	69
Pork	214 ± 8	51 ± 4	24	163 ± 8	76

Source: Santos, C.C. and Bragagnolo, N., Unpublished data, 2009.

in the form of cholesterol esters, and such accumulation of cholesterol esters within the arterial wall is the hallmark of atherosclerotic lesions.

4.5.6 Cholesterol Contents in Lungs

The values reported for pork lungs, raw or braised, were higher than that reported for beef, lamb, and veal lungs. Cholesterol levels found in pork lungs was 320 and 387 mg/100 g in raw and braised, respectively, while in the beef, lamb and veal lungs were near to 229 and 263 mg/100 g for raw and braised, respectively (Table 4.7).

4.5.7 Cholesterol Contents in Pancreas

The results are given for raw and braised beef, pork, lamb, and veal being the values reported higher in raw and braised lamb pancreas than in raw and braised beef, pork, and veal pancreas which in these animal the results is similar. All results reported in Table 4.8 are by USDA Nutrient Database[71] using GC methods. The data for beef, pork, and veal pancreas are also generally similar to those obtained for beef, lamb, and veal lungs, while the cholesterol results for pork lungs agree with lamb pancreas.

TABLE 4.7
Cholesterol Contents in Lungs

Animals and References	Methods			Cholesterol Level (mg/100 g, Wet Basis)
	Lipid Extraction	Saponification	Measurement	
Beef				
Raw[70]	ND	ND	ND	234
Raw[71]	No	Direct saponification	GC-DIC, TMCS, IS	242
Braised[71]				277
Lamb				
Braised[71]	No	Direct saponification	GC-DIC, TMCS, IS	284
Mutton				
Raw[70]	ND	ND	ND	215
Pork				
Raw[70]	ND	ND	ND	314
Raw[71]	No	Direct saponification	GC-DIC, TMCS, IS	320
Braised[71]				387
Veal				
Raw[71]	No	Direct saponification	GC-DIC, TMCS, IS	229
Braised[71]				263

Note: TMCS, with derivatization; IS, 5α-cholestane; ND, not described.

TABLE 4.8
Cholesterol Contents in Pancreas

Animals and References	Methods			Cholesterol Level (mg/100 g, Wet Basis)
	Lipid Extraction	Saponification	Measurement	
Beef				
Raw[71]	No	Direct saponification	GC-DIC, TMCS, IS	205
Braised[71]				262
Lamb				
Raw[71]	No	Direct saponification	GC-DIC, TMCS, IS	260
Braised[71]				400
Pork				
Raw[71]	No	Direct saponification	GC-DIC, TMCS, IS	193
Braised[71]				315
Veal				
Raw[71]	No	Direct saponification	GC-DIC, TMCS, IS	173

Note: TMCS, with derivatization; IS, 5α-cholestane; ND, not described.

4.5.8 Cholesterol Contents in Tongue

The cholesterol levels of the tongue are shown in Table 4.9. The raw tongue cholesterol was lowest in veal (62 mg/100 g), beef was in the range 78–171 mg/100 g, pork was 101 mg/100 g, lamb was in the range 132–189 mg/100 g, while mutton had 197 mg/100 g. When the samples were cooked, the cholesterol values ranged from 104 to 270 mg/100 g in beef tongue,

TABLE 4.9
Cholesterol Contents in Tongue

Animals and References	Methods			Cholesterol Level (mg/100 g, Wet Basis)
	Lipid Extraction	Saponification	Measurement	
Beef				
Raw[68]	Ether	No	Colorimetric	120 ± 10
Raw[69]	C/M	Saponification	Colorimetric	171 ± 21
Raw[70]	ND	ND	ND	105 (102–108)
Raw[71]	No	Direct saponification	GC-DIC, TMCS, IS	87
Raw[72]	C/M	Saponification	CG, TMS, IS	103
Raw[74]	ND	ND	GC	78
Raw[76]	ND	ND	ND	89
Cooked[68]	Ether	No	Colorimetric	211 ± 32
Simmered[71]	No	Direct saponification	GC-DIC, TMCS, IS	132
Cooked[72]	C/M	Saponification	CG, TMS, IS	104
Steewed[76]	ND	ND	ND	95
Lamb				
Raw[71]	No	Direct saponification	GC-DIC, TMCS, IS	156
Raw[72]	C/M	Saponification	CG, TMS, IS	132
Raw[74]	ND	ND	GC	180
Braised[71]	No	Direct saponification	GC-DIC, TMCS, IS	189
Cooked[72]	C/M	Saponification	CG, TMS, IS	146
Mutton				
Raw[68]	Ether	No	Colorimetric	197 ± 14
Raw[70]	ND	ND	ND	147
Cooked[68]	Ether	No	Colorimetric	192 ± 14
Stewed[79]	ND	ND	GC	270
Pork				
Raw[70]	ND	ND	ND	116
Raw[71]	No	Direct saponification	GC-DIC, TMCS, IS	101
Raw[78]	ND	ND	ND	87
Braised[71]	No	Direct saponification	GC-DIC, TMCS, IS	146
Veal				
Raw[71]	No	Direct saponification	GC-DIC, TMCS, IS	62
Simmered[71]				238

Note: C/M, extraction with chloroform and methanol; TMCS, with derivatization; IS, 5α-cholestane; ND, not described.

146–189 mg/100 g in lamb tongue, and 146–270 mg/100 g in pork tongue; it was 197 mg/100 g in mutton and 238 mg/100 g in veal.

4.5.9 Cholesterol Contents in Other Offal

Table 4.10 shows the results of cholesterol in beef blood and intestine, pork chitterlings, chicken and pork feet, pork jowl, beef and pork stomach, beef tail, tripe as well as beef and veal thymus. The value reported in beef intestine is much higher than the other organs but similar to the values reported in raw beef brain[68] and raw veal brains[71]; raw pork jowl and beef tail had lower cholesterol (60 and 61 mg/100 g, respectively); raw chicken and pork feet and beef tripe showed similar levels

TABLE 4.10
Cholesterol Contents in Other Offal

Animals and References	Methods			Cholesterol Level (mg/100 g, Wet Basis)
	Lipid Extraction	Saponification	Measurement	
Blood, beef, raw[70]	ND	ND	ND	190
Blood, pork, raw[70]				40
Intestine, beef, raw[69]	C/M	Saponification	Colorimetric	350 ± 13
Chitterlings, pork, raw[71]	No	Direct saponification	GC-DIC, TMCS, IS	154
Chitterlings, pork, simmered[71]				277
Feet, chicken, boiled[71]	No	Direct saponification	GC-DIC, TMCS, IS	84
Feet, pork, raw[71]				88
Feet, pork, cured-pickled[71]				83
Feet, pork, simmered[71]				107
Jowl, pork, raw[78]	ND	ND	ND	60
Stomach, beef, raw[69]	C/M	Saponification	Colorimetric	155 ± 21
Stomach, beef, raw[77]	No	Direct saponification	HPLC	168 ± 7
Stomach, beef, cooked[77]				255 ± 24
Stomach, pork, raw[71]	No	Direct saponification	GC-DIC, TMCS, IS	223
Stomach, pork, raw[78]	ND	ND	ND	141
Stomach, pork, simmered[71]	No	Direct saponification	GC-DIC, TMCS, IS	316
Tail, beef, raw[72]	C/M	Saponification	CG, TMS, IS	61
Tail, beef, cooked[72]				59
Tripe, beef, raw[72]	C/M	Saponification	CG, TMS, IS	82
Tripe, beef, cooked[72]				112
Thymus, beef, raw[71]	No	Direct saponification	GC-DIC, TMCS, IS	223
Thymus, beef, braised[71]				294
Thymus, veal, raw[71]				250
Thymus, veal, braised[71]				350

Note: C/M, extraction with chloroform and methanol; TMCS, with derivatization; IS, 5α-cholestane; ND, not described.

among 82–88 mg/100 g; raw pork chitterlings and beef stomach had cholesterol values ranging from 154 to 168 mg/100 g; raw beef blood had 190 mg/100 g and raw pork stomach, beef, and veal thymus reported values from 223 to 250 mg/100 g.

4.6 EFFECT OF BREED IN CHOLESTEROL CONTENTS IN OFFAL

There are limited data available on the cholesterol content in offal from different breeds. Sevi et al.[74] observed significant differences in cholesterol content of the kidney from different breeds of lamb, which was markedly higher in Altamurana (374 mg/100 g) than in Gentile di Puglia lambs (298 mg/100 g), and in liver it was higher in Altamurana (456 mg/100 g) than in Comisana lamb (298 mg/100 g). However, in these works, all results of cholesterol in Altamurana lambs were higher than the other breeds of lamb, and the authors suggested this fact to be ascribed either to a larger output and depot of endogenous cholesterol in Altamurana lambs or to a different composition of the dams' milk.

4.7 CONCLUSIONS

The cholesterol content of the organs is higher than in all meats because much of the cholesterol is synthesized in organs such as kidney and liver and in the structural part of brain. The highest cholesterol levels are found in the brain, kidney, and liver and the lowest in tripe, tail, jowl, and feet, which, at times, are very similar to the meat.

There is little information in the literature about the cholesterol levels in these foods and the great variation in the cholesterol levels among different offal derived mainly from the differences in the type of muscle, i.e., red or white, as pig stomach is rich in smooth muscle fibers and connective tissue. Moreover, cholesterol values in the same organ often differ among investigators by more than 100%, and this fact can be attributed to the inherent differences among samples and the analytical methodologies.

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5 Oxidation

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CONTENTS

5.1 INTRODUCTION: OXIDATION IN EDIBLE ANIMAL BY-PRODUCTS

The oxidative degradation of biomolecules involves several molecular mechanisms that lead to the generation of oxygen-rich precursors of reactive, chain-propagating free radicals. Subsequent reactions lead to the degradation of the reactive-oxygen species (ROS) formed in early stages to yield a large variety of final oxidation products.[1] Oxidative reactions occur in living animal tissues as a result of an imbalance between the production of ROS and endogenous antioxidant mechanisms. Upon slaughter, the occurrence of oxidative reactions is accelerated as the defense mechanisms partially collapse and the tissues are exposed to oxygen and other pro-oxidant factors. Subsequent manipulation of animal tissues during handling, processing, and/or storage greatly enhances the onset of oxidative reactions.[2] The study of oxidation phenomena in animal tissues has been traditionally focused on lipids, specifically polyunsaturated fatty acids and cholesterol, whereas the oxidation of some other major components such as proteins is an issue of recent interest.

The subject of this chapter, namely, edible animal by-products, comprises a heterogeneous group of edible tissues and internal organs obtained from animal dressing. Most of these tissues are highly susceptible to suffer oxidative reactions owing to their high fat content (i.e., lard, tallow, brains), the high unsaturation index of their fat (i.e., liver, heart, bone marrow fat), and the presence of high amount of transition metals such as iron and other oxidation promoters (i.e., heart, liver, kidneys). Whereas the consumption rate of these by-products as such can be highly variable among customs and countries, these tissues are frequently used as ingredients for the manufacture of a large variety of processed-food products worldwide. In this sense, these tissues are very commonly subjected to numerous processing operations that enhances oxidation by facilitating the reaction between molecular oxygen and unsaturated lipids (size reduction, mixing) or by accelerating the oxidative reactions through high temperatures (cooking, pasteurization, sterilization).

Overall, the oxidation of animal-based foods is considered a major threat to their quality. The loss of essential fatty acids, amino acids, and vitamins as a result of their oxidative degradation

largely diminishes the nutritional value of the food. Relevant sensory traits such as color, flavor, and texture are also affected by the onset of lipid and protein oxidation.[2,3] As a result of the oxidative reactions, porcine lard or beef tallow might become rancid,[4] whereas oxidized liver develops a very unpleasant "fishy" and "rancid" off-flavors.[5] The analysis of the oxidative stability of an edible animal by-product might be necessary whenever this is used as an ingredient for the manufacture of a processed foodstuff. The initial oxidative status of the tissue predicts its suitability for being subjected to a technological process and an eventual storage. Raw materials with high oxidation rates lead to a final product with undesirable sensory properties and might not be appropriate for processing.[4] The analysis of the final product for lipid and protein oxidation is also of great interest as the extent of the oxidation reflects the suitability of the processing and the nutritional, sensory, and technological quality of the resulting product. In addition to the oxidation-induced loss of quality, the intake of highly oxidizable foods is associated with the development of serious diseases such as colorectal cancer.[6] Certain lipid oxidation products such as malondialdehyde (MDA) have been recently highlighted as compounds with mutagenic and toxic potential.[7]

The enormous impact of oxidation on food quality challenges food chemists to assess the extent of the oxidative reactions in food systems by means of sensitive and reliable methodology. However, the complexity of the chemistry involved in the oxidative reactions affecting lipids and proteins hinders the possibility of one, general, analytical test for unambiguous evaluation of the oxidative deterioration of edible animal by-products. The deep understanding of lipid oxidation mechanisms and the development of improved techniques for the isolation, identification, and quantification of lipid oxidation products have enabled the development of methodology to obtain accurate results. Most methods used for assessing lipid oxidation in edible animal by-products are commonly employed in other animal-based products such as muscle foods with the detection of thiobarbituric acid substances (TBA-RS) and hexanal being the most popular procedures. Whereas some routine methods employed for measuring lipid oxidation has been used for several decades, the methodology currently used for assessing protein oxidation is considerably novel and very recently some innovative methods have been developed. In most cases, the analytical methods used in biomedical sciences are being successfully extrapolated to muscle food systems. The dinitrophenylhydrazine (DNPH) method is now commonly used as a routine procedure for quantifying protein carbonyls in most animal-based products, whereas some advanced methodologies employ liquid chromatography coupled to electrospray ionization and mass spectrometry (LC-ESI-MS) for the accurate detection of specific protein oxidation products.[8]

This chapter reviews routine and advanced methodologies for assessing lipid and protein oxidation in raw and processed edible animal by-products.

5.2 LIPID OXIDATION

Lipid oxidative reactions leads to the generation of toxic compounds and are responsible for the loss of sensory quality in animal-based foods including aroma, color, and texture deterioration.[2] The use of edible animal by-products such as liver, rendered fat, or natural casings for the manufacture of these foods would likely affect the oxidative stability and the quality of the final product. The onset of lipid oxidation in self-stable animal products is generally recognized as the main cause of food spoilage and a major concern in the food industry. Great efforts have been made to shed light on the mechanisms involved in the oxidation of animal lipids, to characterize the lipid-derived oxidation products and to assess their effects on food quality and human health. The deep knowledge of the chemical insight into mechanisms involved in the oxidative degradation of unsaturated lipids provides a solid basis upon which specific, reliable, and highly sensitive techniques have been developed. A brief overview of the mechanisms and factors affecting these complex reactions would assist the reader in the comprehension of the methodologies subsequently reported.

5.2.1 Mechanisms and Factors

Lipid oxidation is a radical reaction described as a combination of a various chain reactions, consisting of three phases[9]: initiation, propagation, and termination.

During the initial phase, in the presence of initiators or the reactive oxygen species, unsaturated lipids lose hydrogen radical to form a lipid free radical.[9] The direct reaction between the fatty acid and molecular oxygen is highly improbable since lipid molecule has a singlet electronic state and the oxygen molecule has a triplet ground state. This spin barrier between lipids and oxygen can be overcome with the presence of initiators that can produce radicals by different mechanisms: (1) thermal dissociation, (2) decomposition of hydroperoxides catalyzed by redox metals, (3) exposure to light in the presence of a sensitizer such as ketone.[9] Unsaturated lipids are easily oxidized by the ROS, which include oxygen radicals and non-radical derivatives of oxygen.[10] The hydroxy radical is highlighted as mainly responsible for the initiation of lipid oxidation in animal tissues.[10]

During the propagation stage, the alkyl radical of an unsaturated lipid containing a labile hydrogen reacts very rapidly with molecular oxygen (O_2) to form peroxide radicals. This reaction is always much faster than the following hydrogen transfer reaction with unsaturated lipids to form hydroperoxides (ROOH), which are considered the main primary products of lipid oxidation.[9] The newly formed hydroperoxy radical can abstract hydrogen from an adjacent unsaturated fatty acid since the reaction sequences goes through 8–14 propagation cycles before termination.[9,10] Hydroperoxides are considered the most important initial reaction products from lipid oxidation. At the last stages of oxidation, the radical species react with each other and self-destruct to form non-radical products by different mechanisms. Alkoxyl radicals can react with unsaturated lipids to form stable and innocuous alcohols or undergo transformation into unsaturated aldehydes, such as MDA, and other unstable compounds.

The susceptibility of animal tissues to undergo lipid oxidation depends upon a variety of factors including the fat content, fatty acid composition, and the presence of transition metals such as iron and copper and endogenous antioxidant compounds such as tocopherols.[2] The extent of the oxidative reactions in animal tissues during processing and storage is largely affected by the conditions and characteristics of the technologies applied with the temperature and exposure to light and oxygen being the most influential factors.[2]

5.2.2 Assessment of Lipid Oxidation

There are many analytical methods for measuring oxidative status of animal edible by-products, ranging from simple sensorial evaluations to more complex chemical methods. Chemical methods may be performed by a variety of procedures, measuring either primary oxidative changes or secondary changes that originate from primary oxidation products decomposition. Selecting an optimum test for lipid oxidation in animal by-products is difficult due to the complexity of the chemical processes involved and the diversity of the food matrices. Thus, the suitability of each method depends on the type of animal by-product and the way it has been processed or stored and, in many cases, no single method can suffice for analyzing lipid oxidation.

5.2.2.1 Primary Oxidation Changes

Numerous analytical methods have been developed for assessing primary oxidative changes in raw and processed edible animal by-products (Table 5.1). Among them, hydroperoxide determinations by titrimetric or colorimetric methods and lipid stability tests have been the most widely used at industry and academic research. Alternatively, other advanced techniques such as enzymatic, chromatographic (GC and HPLC) or spectroscopic (ESR and NMR) methods have been proposed (reviewed by Dobarganes and Velasco[11] and Prior and Loliger[12]). However, these methods are not easily adapted to routine screening of large numbers of samples and have been more frequently used in biological studies.

TABLE 5.1
Summary of Techniques Used for Evaluating Primary Lipid Oxidation Changes in Edible Animal By-Products

Sample	Technique	References
Mechanically deboned meat	Peroxide value by the iodometric assay	Hassan and Fan[21] and Ozkececi et al.[22]
	Peroxide value by the iron oxidation assay	Olsen et al.[30] and Raghavan and Richards[31]
	Conjugated diene determination	Nissen et al.[34]
Liver	Peroxide value by the iodometric assay	Farag et al.[23]
	Conjugated diene determination	Bernacchi et al.[35]
Lard	Peroxide value by the iodometric assay	Liang and Schwarzer[24] and Milos et al.[25]
	Peroxide value by the iron oxidation assay	Kang and Dasgupta[20] and Osawa et al.[28]
	Conjugated diene determination	Rigby et al.[36]
	Active oxygen method	Liang and Schwarzer[24] and DeMan and DeMan[38]
	Rancimat and Oil Stability Instrument	Liang and Schwarzer,[24] Aruoma et al.,[39] and De Leonardis et al.[40]
	Oxygen absorption method	Kang and Dasgupta,[20] Liang and Schwarzer,[24] and Koga and Terao[45]
Tallow	Peroxide value by the iodometric assay	Liang and Schwarzer[24]
	Active oxygen method	Liang and Schwarzer[24]
	Rancimat and Oil Stability Instrument	Liang and Schwarzer[24]
	Oxygen absorption method	Liang and Schwarzer[24]
Pork and beef back fat	Peroxide value by the iodometric assay	Hertzman et al.[26] and Lasta et al.[27]
	Active oxygen method	Hertzman et al.[26]
	Rancimat and Oil Stability Instrument	Flachowsky et al.[41] and Gebert et al.[42]
Poultry fat	Peroxide value by the iodometric assay	Kang and Dasgupta[20]
	Peroxide value by the iron oxidation assay	Kang and Dasgupta[20] and Shantha and Decker[29]
Shortenings	Active oxygen method	DeMan and DeMan[38]
	Rancimat and oil stability instrument	DeMan and DeMan[38] and Anwar et al.[43]

5.2.2.1.1 Primary Oxidation Products: Hydroperoxides

Since the primary products of lipid oxidation are hydroperoxides, it is reasonable to determine their concentration as a measure of oxidation. Hydroperoxides are formed during the autoxidation of unsaturated lipids and have little or no direct impact on the odor and flavor of the food product. Nevertheless, hydroperoxides are easily decomposed to a high variety of nonvolatile and volatile secondary products, of which some have very low sensory thresholds.[9] Hydroperoxide determination in animal by-products may be limited by the inherent chemical instability of these compounds. Consequently, when hydroperoxides breakdown is as fast as or faster than hydroperoxides formation, lipid hydroperoxides are not good indicators of oxidation.[9] This fact can occur in animal by-products, especially in those with high susceptibility to undergo oxidative phenomena. The analysis of lipid hydroperoxides in animal by-products other than pure fat (i.e., tallow, lard) namely, mechanically deboned meat or viscera, requires a previous lipid extraction with solvents. These solvents, mainly chloroform or chloroform-methanol mixtures,[13,14] must be eventually removed to avoid the decomposition of hydroperoxides or loss during solvent evaporation.[15]

One of the most widely used tests for lipid hydroperoxide determination is the peroxide value. Peroxide value is a measure of the concentration of hydroperoxides formed in the initial stages of lipid oxidation and is commonly expressed as the milliequivalents (mEq) of peroxides per kilogram

of sample. As an indicative value, samples with a peroxide value greater than 15–20 mEq kg^{-1} can be considered as rancid.[15] Peroxide value can be measured based on their ability to liberate iodine from potassium iodide (iodometric assay), or to oxidize iron ions from the ferrous to the ferric state:

5.2.2.1.1.1 Determination of Peroxide Value by the Iodometric Assay The iodometric method is a volumetric analysis based on the titration of iodine released from potassium iodide by hydroperoxides. The standard method, described by American Oil Chemists' Society (AOCS) (Official Method Cd 8–53)[16] and by Association of Official Analytical Chemists (AOAC) (Official Method 965.33),[17] involves the reaction of the sample dissolved in acetic acid/chloroform (3:2 v/v) or isooctane, with aqueous KI for 1 min (Equation 5.1). The amount of released iodine is assessed by titration against a standardized solution of sodium thiosulfate ($Na_2S_2O_3$) using a starch indicator (Equation 5.2):

$$ROOH + 2H^+ + 2KI \rightarrow I_2 + ROH + H_2O + 2K^+ \quad (5.1)$$

$$I_2 + 2Na_2S_2O_3 \rightarrow Na_2S_4O_6 + 2NaI \quad (5.2)$$

Iodometric methods are easy to perform and produce consistent results and the equipment and glassware required are readily available in most laboratories although any modification in the procedure may cause large variations in the results.[18] Potential drawbacks of this method are the absorption of iodine by fatty acid double bonds and liberation of iodine from potassium iodide by oxygen present in the solution during titration.[19] Samples with low peroxide value cannot be adequately measured by the standard iodometric method because of difficulties encountered in determination of the titration end point. However, this drawback can be resolved by determining the titration end point by electrochemical techniques.[20] Despite these disadvantages, the iodometric assay of peroxide value is one of the commonest methods to determine oxidative stability of lipids and has been frequently used in different animal by-products, such as mechanically deboned meat,[21,22] liver,[23] tallow,[24] lard,[24,25] pork and beef backfat,[26,27] and poultry fat.[28]

5.2.2.1.1.2 Determination of Peroxide Value by the Iron Oxidation Assay Methods based on the formation of iron complexes have been also proposed to improve the sensitivity of the classical iodometric procedures. These methods are based on the ability of peroxides to oxidize iron (II) to iron (III).[29] Ammonium thiocyanate reacts with ferric ions, resulting in a colored complex that can be measured spectrophotometrically at 500 nm. Peroxide values as low as 0.1 mEq kg^{-1} sample can be determined with this method, providing a clear advantage over the iodometric titration procedure.[18] Alternatively, this determination can be carried out by the ferrous oxidation-xylenol orange (FOX) method.[29] In this case, the resulting ferric ion is bound to the xylenol orange dye producing a blue-purple complex that can be measured spectrophotometrically with a maximum of absorbance between 550 and 600 nm. These methods have been reported to provide a sensitivity comparable or even better than that of the spectrophotometric iodometric assay. It has been successfully applied to the analysis of edible animal by-products, such as mechanically deboned meat,[30,31] lard[20,28] and chicken fat.[20,29]

5.2.2.1.2 Primary Oxidation Products: Conjugated Dienes

During the formation of hydroperoxides from polyunsaturated fatty acids, conjugated dienes are the typical yield. When the conjugated diene moiety is present in fatty acids it shows absorption at 233 nm that can be determined spectrophotometrically. Comprehensible and developed methods for conjugated diene analysis can be found in IUPAC Standard Method 2.505[32] and in the review reported by Pegg.[18] The conjugated diene determination offers several advantages over the peroxide value method as it is faster, requires no chemical reagents or does not depend upon a chemical reaction or color development. However, the magnitude of the value obtained depends mainly upon

the fatty acid composition of the analyzed sample, and therefore, conjugated diene value cannot be compared amongst different food matrices, especially when large differences in fatty acid composition occurs.[33] Conjugated diene determination has been used in different animal by-product, such as mechanically deboned meat,[34] liver,[35] and lard.[36]

5.2.2.1.3 Predictive Methods

Stability of lipids during storage can be anticipated on the basis of special tests called predictive or stability tests. These oxidative stability tests are designed to accelerate the oxidation process by manipulating pro-oxidant conditions, such as temperature, metal catalysts or oxygen pressure.[18] Although predictive methods were primarily developed for oil samples, it is also possible to use these methods in animal by-products containing high amount or fat (i.e., lard, tallow, backfat). For other animal by-products, lipids should first be extracted with solvents.[13,14] These methods have been widely used in industry and academic research and may be useful to determine the quality of raw ingredients, to measure the effectiveness of preservatives, or to estimate the shelf life of processed animal by-products (Reviewed by Liang and Schwarzer[24] and Wan[37]). However, many of these methods have been also criticized because they run at elevated temperature, usually far above the ambient or storage temperature, at which the mechanism of lipid oxidation changes.[9] Some of the most commonly used accelerated stability methods are described briefly below:

Active Oxygen Method (*AOM*): This method predicts the stability of a lipid sample by bubbling air through a solution of the fat. According to the AOCS official method (AOCS Cd 12–57),[16] the fat sample is heated to 98.7°C and bubbled with a stream of dry air (140 mL min^{-1} flow rate). AOM value is determined by the time at which the fat sample reaches a fixed peroxide value (usually 20 or 100 mEq kg^{-1}, depending on the type of fat).[18,19] The method is very time-consuming since a stable fat may require 48 h or more before reaching the endpoint and periodic titrations to determine peroxide value is also required. The AOM has been used extensively in lipid analysis over the years, including animal by-product as lard,[24,38] pork backfat,[26] tallow,[24] or shortenings,[38] but this method has inherent deficiencies and difficulties and is being supplanted by faster automated techniques.

The Rancimat and Oil Stability Instrument: The Rancimat and the Oil Stability Instrument are two modified and automated versions of AOM. Both methods measure the conductivity in deionized water as it increases due to the absorption of volatile acids and other products formed from the oxidative decomposition of lipids. Increasing conductivity is an indication of hydroperoxide breakdown that occurs at the same time as peroxide value increases. Conductivity of the water is monitored continuously and the endpoint (Oil Stability Index) is defined as the hours required for the rate of conductivity change to reach a predetermined value.[18] This Oil Stability Index is an official method (Cd 12b-92) of AOCS.[16] These methods rely on either of two commercially available instruments; the Oil Stability Instrument is produced commercially by Omnion Inc. (Rockland, MA) and is capable of running up to 24 samples at the same time. The Rancimat method employs a commercial equipment marketed by Metrohm Ltd. (Herisau, Switzerland) and is capable of running only eight samples simultaneously. These methods have been profusely used in animal by-product analysis, particularly in those containing high amounts of fat, such as lard,[24,39,40] pork backfat,[41,42] tallow,[24] and animal shortenings.[38,43]

Oxygen Absorption Method: The consumption of oxygen during formation of hydroperoxides from lipids can be measured during initial stages of lipid autoxidation. In the classical method, a sample of fat is weighted into petri dishes and stored in an oven at a fixed temperature.[19] Oxygen uptake is followed by weighing samples at intervals during storage. Other versions of this method confined a sample in a reactor and consumption of oxygen is monitored by an oxygen manometer or by means of a gas-phase flow injection analysis.[44] An advantage of this technique is its ability to measure stability of the complete product without prior extraction of the fat. These methods have been applied to predict lipid stability in lard[20,24,45] and tallow.[24]

5.2.2.2 Detection of Secondary Changes

As indicators of lipid oxidation, the secondary oxidation products are more reliable compared to primary products as they reflect the deterioration of the quality of edible animal by-products as a consequence of oxidative reactions. Secondary products are generally odor-active and stable compounds, whereas primary products are colorless, flavorless, and commonly labile compounds.[19] Amongst these compounds, aldehydes are considered the most important breakdown products because they possess low threshold values and are the major contributors to the development of rancid off-flavors.[46] The most commonly used techniques for assessing secondary lipid oxidation changes are summarized in Table 5.2.

5.2.2.2.1 Secondary Oxidation Products: Malonaldehyde

Malonaldehyde (1,3-propanedial), is a three-carbon dialdehyde with carbonyl groups at the C-1 and C-3 positions. The generation of MDA is promoted by the oxidation of polyunsaturated fatty acids (PUFA) leading to a highly reactive compound that remains bound to other food ingredients. An acid/heat treatment of the food presumably releases the bound MDA.[47] Besides the deterioration of the sensory quality caused by the presence of MDA, its implication on health should be taking into consideration as MDA acts as a catalyst in the formation of *N*-nitrosamines and also as mutagenic promoter.[7,48] Different methods have been developed to evaluate the lipid oxidation extent by MDA detection and quantification, most of which were firstly applied in muscle foods and subsequently in different edible animal by-products.

5.2.2.2.1.1 Thiobarbituric Acid (TBA) Test The 2-thiobarbituric acid (TBA) test is the most widely used method for the assessment of the oxidative status of a great variety of edible animal by-products. The technique is based on the formation of a color red pigment (MDA-TBA adduct) as consequence of the attack of the monoenolic form of MDA by the active methylene groups of TBA.[47] The complex shows a wavelength of maximum absorbance at 532–535 nm and a secondary one at 245–305 nm.[49] MDA is the major TBA reactive substance although other oxidation products, such as α, β-unsaturated aldehydes (for instance 4-hydroxyalkenals) and certain unidentified nonvolatile precursors of these substances may also react with TBA, which are generally referred to as TBA-reactive substances (TBARS).[49]

TABLE 5.2
Summary of Methodologies Used to Analyze Secondary Lipid Oxidation Changes in Edible Animal By-Products

Sample	Technique	References
Spleen and liver (pork)	TBA test[a]	Gebert et al.[42]
Liver and heart (chicken)	TBA test[b]	Tang et al.[56]
Mechanically deboned chicken meat	TBA test[a]	Gomesa et al.[63]
Mechanically deboned turkey meat	TBA test[c]	Pettersen et al.[55]
Lard	TBA test[a]	Houben et al.[58]
Back fat	TBA test[a]	Verma et al.[59]
Adipose tissue	FTIR	Guillén et al.[71]
Cured lard	Indol agent	Paleari et al.[70]
Oxidized liver	GC	Im et al.[5]
Cured lard	GC	Paleari et al.[70]
Mechanically deboned turkey meat	GC	Pettersen et al.[55]

[a] Distillation method.
[b] Direct method.
[c] Extraction method.

Different methods have been proposed to perform the TBA test: (1) by directly heating the sample with TBA in acidic conditions followed by separation of the red pigment by centrifugation,[50] (2) by distillation of the sample followed by the reaction of the distillate with the TBA,[51] (3) by extraction of MDA using aqueous trichloroacetic or perchloric acid and subsequent reaction with TBA,[52] (4) and by extraction of the lipid fraction of the sample with organic solvents and reaction of the extract with the TBA.[53] The selection of one of these methods is usually made according to the characteristics of the matrix of the product. Sørensen and Jørgensen[54] reported a higher reproducibility (5–10 times) of the direct acid extraction method compared to an optimized distillation procedure, which highlights the use of the direct extraction method in food matrices with less than 14%–18% of fat and with no artificial colorants. Pettersen et al.[55] and Tang et al.[56] employed the direct acid extraction method for measuring MDA in mechanically deboned turkey meat and in chicken liver and heart, respectively. Nevertheless, the distillation procedures are considered as more sensitive and also more suitable for high fat samples (>10%) where turbidity may occur in the extracted samples.[49] Several authors have assessed TBARS numbers using the distillation method in a great variety of edible by-products such as backfat, liver, and porcine spleen,[42] edible broiler skin fat,[57] frozen pork backfat,[58] and additive-free lard.[59]

Amongst animal edible by-products, special attention has been paid to the development of lipid oxidation in mechanically deboned meat since oxidative reactions are enhanced by the addition of unsaturated fatty acids from bone marrow[60] and during the deboning process due to high temperatures and the incorporation of air and heme pigments.[61] The distillation procedure with modifications (incorporation of butylated hydroxytoluene before the blending step, to prevent auto-oxidation) is the most widely used TBA method to evaluate the oxidative status of mechanically deboned meat, followed by the direct extraction method.[21,62–64] Moreover, the direct extraction TBA method has been also applied to sausages including mechanically deboned meat as well as in different meat products elaborated using edible by-products as raw material.[65]

Overestimation of TBARS counts may occur as acidic and heating conditions enhance the formation of MDA and other TBARS.[48] Moreover, the main drawback reported for TBA test is the development of interfering reactions. Yellow chromogens (max. absorbance at 450–460 nm) derived from the reaction of sugar-derived compounds and TBA may be formed and hence, overlap the spectrophotometric measurement of the MDA-TBA adducts.[66] Numerous organic (proteins, pigments, aldehydes) and inorganic (transition metals) compounds could act as interfering compounds in animal edible by-products leading to the formation of colored compounds. The inclusion of sulphanilamide during performance of the TBA test in cured lard is highly recommendable as this compound prevents the nitrozation of MDA by residual nitrite and thus, reduces an eventual underestimation of TBARS counts.[67] Some strategies have been proposed in order to reduce these interfering reactions such as decreasing the reaction temperature (from boiling to room temperature),[68] removing the interfering compounds by solid-phase extraction,[52] or adjusting the reaction conditions (pH, TBA concentration and temperature of incubation).[69]

Some other procedures have been employed to detect and quantify MDA in animal edible by-products. Paleari et al.[70] analyzed free MDA in cured lard by using 1-methyl-2-phenylindol as a reactive agent. This compound reacts with MDA and other lipid derived aldehydes such as 4-hydroxy-2-nonenal to yield a stable chromophore with intense maximal absorbance at 586 nm.

The Fourier transform infrared spectroscopy (FTIR) has been successfully applied to different edible oils including porcine adipose tissue to assess lipid oxidation through the modifications in the Fourier transform infrared spectra of the melted lipids under oxidative conditions.[71]

5.2.2.2.1.2 Determination of MDA by GC and HPLC GC and HPLC have been extensively used for MDA determination in a great variety of foods.[72] Hydrazine-based reagents, such as 2,4-dinitrophenylhydrazine and *N*-methylhydrazine have been preferred as derivatization agents for

GC-MS analysis since these substances are able to form stable pyrazole derivatives with MDA and 4-hydroxynonenal.[72] Most reported GC methods provide a measurement of the total, free and bound forms of MDA because the assay conditions are sufficient to hydrolyze or decompose bound MDA during sample preparation. Kakuda et al.[73] originally used HPLC to quantify MDA in distilled water and found a linear correlation between TBARS numbers and HPLC results. Subsequently, different applications of HPLC to MDA detection have been reported in meat[74] and smoked meat products.[75] Therefore, it may be interesting to apply the reported chromatographic techniques to evaluate the extent of lipid oxidation also in edible animal by-products.

5.2.2.2.2 Secondary Oxidation Products: Lipid Oxidation-Derived Volatiles

Lipid oxidation of unsaturated fatty acids results in a wide range of volatile aldehydes, such as hexanal, propanal, or 4-hydroxy-2 nonenal, which have been commonly used as indicators of rancidity.[76] The most widely used techniques developed for the extraction and isolation of lipid-derived volatiles are the solvent extraction, the simultaneous distillation extraction procedure, the dynamic headspace or purge and trap, and the solid-phase microextraction (SPME) (reviewed by Ross and Smith[76]). The extracted volatiles are separated, identified, and quantified using GC or HPLC usually coupled to MS. Amongst the techniques for the analysis of lipid-derived volatiles, the use of SPME is growing in popularity due to its sensitivity and ease of use.[76]

Amongst the volatile compounds derived from lipid oxidation, hexanal can be considered the dominant in most food matrices and several authors have pointed out that this compound is the best indicator of lipid oxidation, particularly in meat and meat-derived products.[77] Accordingly, Paleari et al.[70] found that hexanal, nonanal, 2-nonenal, and 2,4-decadienal were the predominant aldehydes in cured lard analyzed by simultaneous distillation extraction. Im et al.[5] extracted the volatile compounds from the headspace of oxidized pork liver using SPME, with hexanal, 1-octen-3-one, 4-heptenal, 2-octenal, and 2,4-decadienal being the most abundant lipid-derived volatiles. Mielnik et al.[65] found 30-lipid derived volatiles in the headspace of comminuted sausages formulated with mechanically deboned poultry meat. These authors reported a good correlation between these compounds, particularly hexanal and TBA results. Pettersen et al.[55] used the hexanal content analyzed by dynamic headspace as index of lipid oxidation development in frozen mechanically deboned turkey meat.

5.3 PROTEIN OXIDATION

The oxidation of lipids captured the attention of the food industry throughout the last decades, while it was greatly ignored that some other major components of animal foods such as proteins are highly sensitive to oxidative deterioration.[78,79] Currently, protein oxidation is a hot topic of increasing interest among food technologists. Recent studies have shown the complex mechanisms implicated in protein oxidation, the effects of oxidation in protein functionality, and the large variety of oxidation products derived from oxidized proteins.[79–81] Certain edible animal by-products are significant sources of protein with interesting technological properties. In fact, the usage of blood plasma, animal gelatins, or pork rind powder in particular formulations and recipes is fully justified by the benefits contributed by their proteins to the final product in terms of consistency, stability, and flavor.[82] Recent studies have reported that specific technological properties (i.e., gelling, emulsifying) of myofibrillar and other animal proteins are seriously affected by oxidative reactions. The oxidation of muscle and liver proteins during refrigerated storage of liver sausages and frankfurters results in modifications of the color and texture of these products.[3]

It becomes, hence, essential to develop accurate procedures to assess the oxidative reactions affecting proteins during handling, processing, and storage of animal by-products and the foods elaborated with these. In order to comprehend the methodological approach of the analytical procedures, it is necessary to present a brief overview of the chemical aspects regarding the oxidative modification of proteins.

5.3.1 Mechanisms and Factors

Oxidation of proteins is manifested as a free radical chain reaction similar to that of lipid oxidation including initiation, propagation, and termination stages. Unlike the oxidative degradation of unsaturated fatty acids, the oxidation of each specific amino acid follows particular pathways and yields specific oxidation products. Lipid and protein oxidation take place concurrently in food systems and actually, timely interactions are known to happen between lipids and proteins during oxidative reactions. In fact, primary and secondary lipid oxidation products, mainly hydroperoxides and aldehydes are known to promote protein oxidation.[83,84]

Besides oxidizing lipids, numerous chemicals can initiate the oxidation of food proteins, including transition metals (i.e., iron, copper), myoglobin, hydrogen peroxide, reducing sugars, and ROS formed as a result of the interaction of the aforementioned compounds.[8,84–87] It is generally accepted that the nature of the protein oxidation products formed is highly dependent on how oxidation is initiated. In general, the more reactive the formed radicals are, the less selective reactions are initiated.[88] It is plausible that heme and non-heme iron are main promoters of protein oxidation in animal tissues acting (1) directly on amino acid side chains for the formation of carbonyl derivatives[8] and/or (2) indirectly through the formation of ROS and hypervalent reactive species.[89,90] As a direct consequence of the oxidative damage of food proteins, several chemical modifications can be observed such as the formation of cross-links between proteins, peptide scissions and the oxidative modification of single amino acids.[78,91] These modifications include the loss of sulfydryl groups, the generation of oxidized derivatives (i.e., sulfoxides from methionine), and the conversion of an amino acid residue to a different one.[78] Additionally, the oxidation of side chains of certain amino acids (arginine, lysine, proline, and threonine) leads to the generation of carbonyl residues through deamination reactions. Other relevant consequences derived from the oxidation of animal proteins are the formation of aggregates through covalent and noncovalent linkages. The formation of non-covalent aggregates are enhanced by the generation of hydrogen bonds and the complexes formed between proteins and oxidized lipids.[92] In animal tissues and animal-based products, the formation of covalent cross-links is usually ascribed to the reaction between two sulfur containing amino acids or two tyrosines to yield disulfide bonds or dityrosines, respectively.[86,93]

The oxidation of proteins and amino acids is affected by numerous environmental factors including pH, temperature, water activity, and the presence of catalysts or inhibitors such as transition metals and phenolic compounds.[8,89,91] Additionally, the three-dimensional structure of the protein as well as their amino acid composition influences the susceptibility of proteins to undergo oxidative reactions.[8,79,83] Heterocyclic amino acids and those containing reactive side chains (sulfhydryl, thioether, aminogroup imidazole, and indole rings) are particularly sensitive to undergo oxidative degradation due to the presence of OH-, S- or N- containing groups.[91] In a recent study, Estévez et al.[8] reported that animal proteins such as bovine serum albumin (BSA), whey proteins and myofibrillar proteins are more susceptible to ROS than soy proteins.

5.3.2 Assessment of Protein Oxidation

Most of the analytical procedures used for assessing protein oxidation in food systems have been adapted from methods originally developed for analyzing biological samples. These techniques are aimed to either (1) prove the oxidative modification of proteins and/or amino acids or (2) to detect protein oxidation products. Table 5.3 summarizes the methods employed for assessing protein oxidation products in animal tissues. Taking into account the extremely fast progress in this innovative field, some of the most novel and advanced techniques reported in the present chapter have not been applied yet in edible by-products. Nevertheless, their successful application in other animal-based tissues and certain biological samples predicts their suitability for being applied in all kinds of animal tissues and justifies their inclusion in this review.

TABLE 5.3
Summary of Methodologies Used to Detect Protein Oxidation Products in Animal Tissues and By-Products

Protein Source	Protein Oxidation Product	Technique	References
Skeletal muscle	Total protein carbonyls	DNPH	Lund et al.[80] and Ventanas et al.[104]
	AAS and GGS	LC-ESI-MS	Estévez et al.[8]
	Disulfide bond	Spectrophotometry	Liu et al.[98]
	CL-MHC	SDS-PAGE electrophoresis	Lund et al.[80]
	Dityrosines	Spectrofluorometry	Xiong et al.[118]
		HPLC	Decker et al.[117]
Liver	Total protein carbonyls	DNPH	Ibrahim et al.[99] and Bahramikia et al.[100]
	AAS and GGS	GC-MS	Requena et al.[112]
Heart	Total protein carbonyls	DNPH	Srinivasan et al.[102]
	CL-MHC	SDS-PAGE electrophoresis	Parkington et al.[119]
Plasma	AAS and GGS	LC-ESI-MS	Estévez et al.[8]
Whey	AAS and GGS	LC-ESI-MS	Estévez et al.[8]

5.3.2.1 Assessment of the Oxidative Modification of Proteins

5.3.2.1.1 Tryptophan Depletion

Tryptophan, one of the most sensitive amino acids to undergo oxidative decomposition,[78] emits natural fluorescence at around 350 nm when excited at around 280 nm. Physicochemical modifications of its original structure such as those caused by ROS, involves the loss of its natural fluorescence.[94] Hence, the oxidation of tryptophan can be easily assessed by measuring the loss of its natural fluorescence using fluorescence spectroscopy. For this purpose, the proteins are usually diluted at appropriate concentrations in organic solutions, distilled water, or specific buffers.[84,95,96] In food systems, the depletion of tryptophan through spectrofluorometric measurements has been used as an indicator of oxidative damage of whey proteins,[95,96] plasma proteins,[84,95] and myofibrillar proteins.[79,84] A recent study evaluated the oxidative stability of a processed meat product made of animal by-products (low-quality meat and adipose tissue) by means of tryptophan depletion using fluorescence spectroscopy.[97] The present procedure is simple and fast and could be used as an interesting alternative for time-consuming techniques. However, the method might require previous optimization when applied to a particular food sample for the first time as the nature of the protein and environment influence the sensitivity of the measurement, the quantum yield, and wavelength of maximum fluorescence emission of tryptophan.[94]

5.3.2.2 Assessment of Protein Oxidation Products

5.3.2.2.1 Quantification of Total Protein Carbonyls by the DNPH Method

The formation of carbonyl compounds from oxidatively modified amino acid side chains is one of the most remarkable changes occurring during protein oxidation.[78] The 2,4 dinitrophenylhydrazine (DNPH) method enables a simple estimation of the total amount of protein carbonyls in biological and food samples. This procedure is currently used as a routine method for the assessment of protein oxidation in animal tissues such as skeletal muscle[90,98] and liver,[99,100] particularly animal edible by-products such as beef heart surimi,[101,102] other muscle and liver foods,[3,103] and most processed animal-derived products.[104,105] The method is based on the reaction between the DNPH with protein carbonyl compounds to form a 2,4-dinitrophenyl (DNP) hydrazone product that displays a maximum absorbance peak at around 370 nm (Figure 5.1). The procedure involves a simultaneous

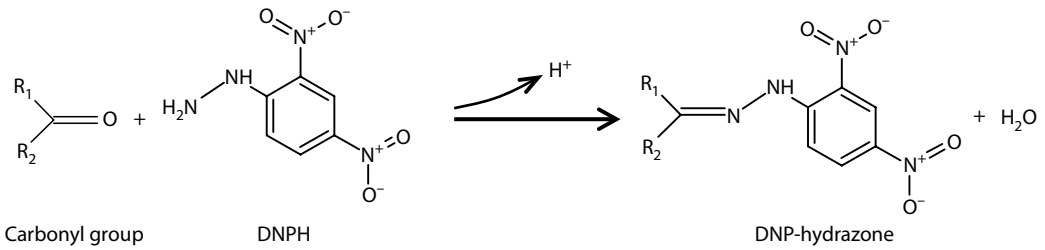

FIGURE 5.1 Derivatization of protein carbonyls using DNPH.

determination of carbonyl derivatives and protein content of the sample.[106] The concentration of DNP hydrazones is calculated by measuring reacted DNPH spectrophotometrically on the basis of an absorption of 22,000 M^{-1} cm^{-1} at 370 nm. Concentration of protein is determined in a control sample (without added DNPH) at 280 nm using BSA as standard. Obtained results are usually expressed as nmols DNP hydrazones per mg of protein. The original method[106] was developed for analyzing oxidative stress in biological samples and has been subsequently employed with minor modifications by food scientists. These modifications include treating the samples with a hydrochloric acid-acetone solution in order to remove potentially interfering chromophore substances (e.g., hemoglobin, myoglobin, retinoids)[107] and using high ionic strength buffers to facilitate the suspension of particular animal proteins such as myofibrillar proteins.[8,80]

Whereas the simplicity and convenience of this assay make it a widespread procedure for assessing protein oxidation, several drawbacks have been reported on this method. The total amount of carbonyl compounds in animal tissues and complex food systems might not consistently reflect the extent of protein oxidation. For example, certain oxidative modifications in proteins (i.e., oxidation of aromatic amino acids) might not lead to the generation of carbonyl compounds and carbonyl moieties can be present in proteins according to mechanisms that do not involve the oxidation of amino acid residues.[108] Whereas the method is simple and requires ordinary laboratory resources, it is relatively lengthy and numerous solutions and solvents are eventually employed. An optimized DNPH-ELISA analysis recently proposed by Jongberg et al.[109] enables a higher sample throughput for the detection and quantification of protein carbonyls in meat. According to another recent study, the DNPH method might overestimate the amount of protein carbonyls by accounting lipid-derived carbonyls from the samples.[110] This overestimation would be particularly problematical in animal tissues with high fat content and comminuted meat products. However, performing additional washing steps with organic solvents might remove oxidized lipids and improve the consistency of the results. The complexity of having an initial homogeneous protein suspension should also be considered a major drawback of this technique. An erroneous initial sampling from the protein suspension would lead to eventual unexpected, unreliable results. Whenever more specific, reliable, and sensitive methods are required for measuring protein carbonyls in food samples, advanced methodologies such as HPLC-MS should be employed.

5.3.2.2.2 Analysis of Specific Protein Semialdehydes by LC-ESI-MS

This advanced methodology has been recently adapted from medical research for the detection of specific protein oxidation products, namely, α-amino adipic semialdehyde and γ-glutamic semialdehyde (AAS and GGS, respectively) in food proteins using LC-ESI-MS.[8] AAS is an oxidative deamination product of lysine, whereas GGS originates from arginine and proline residues (Figure 5.2). Both compounds have been reported as major carbonyl products of metal-catalyzed oxidation of plasma and liver proteins.[111,112] In medical research, these semialdehydes have been used as biomarkers of oxidative stress and indicators of serious age-related disorders such as Alzheimer's disease.[113] Several procedures have been reported for the analysis of AAS and GGS in biological samples. They involve a preparative derivatization step using either $NaBH_4$ or fluoresceinamine

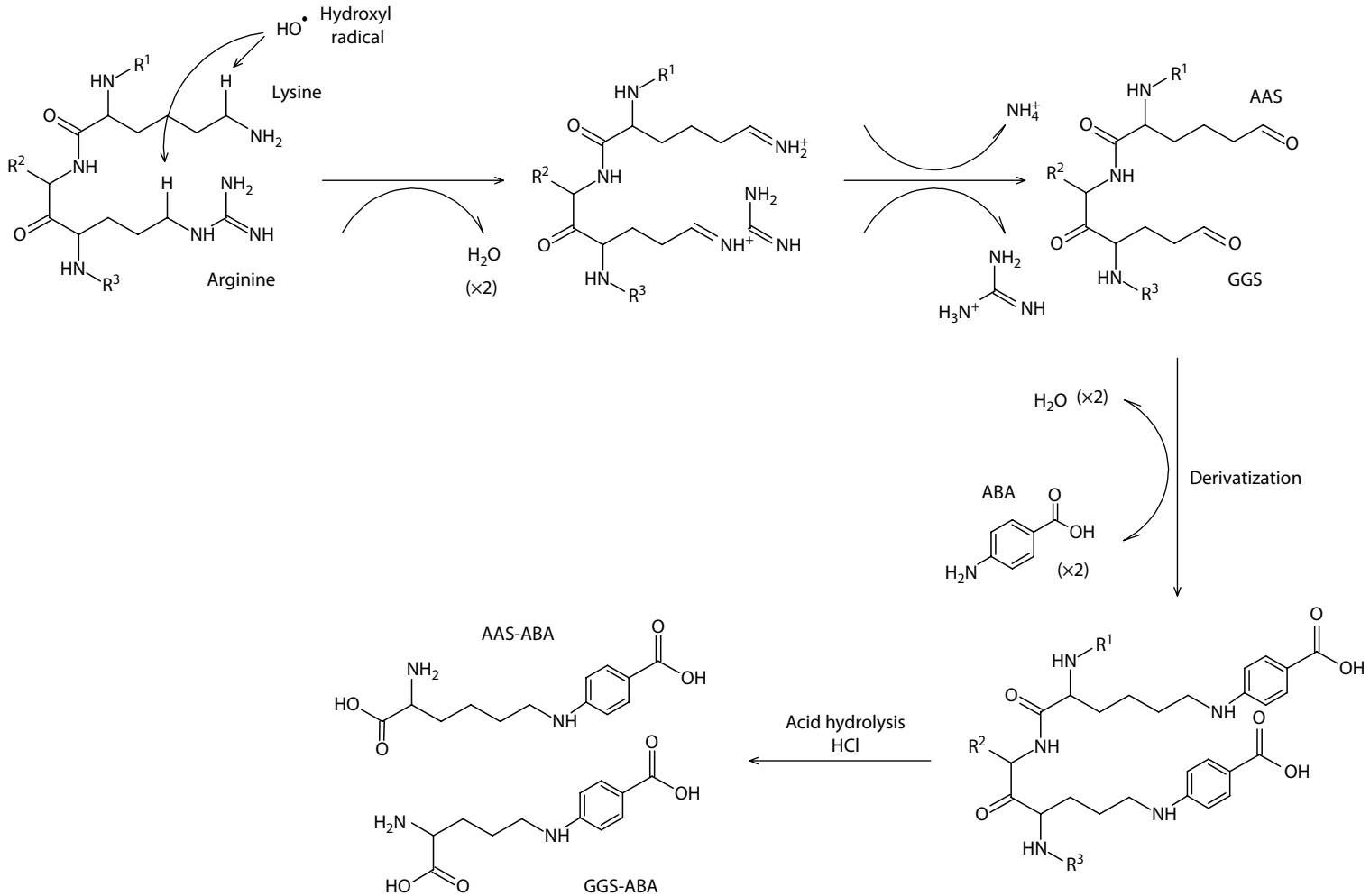

FIGURE 5.2 Formation of AAS and GGS from protein-bound lysine and arginine and subsequent derivatization for LC-MS analysis.

($FINH_2$) followed by gas chromatography-mass spectrometry (GC-MS) or high-performance liquid chromatography-diode-array detector (HPLC-DAD) analysis, respectively.[111,112] More recently, Akagawa et al.[114] developed a novel derivatization procedure using *p*-aminobenzoic acid (ABA) coupled to HPLC coupled to fluorescence detection (FLD). This derivatization procedure (Figure 5.2) provides some advantages as ABA-derivatized semialdehydes show great stability against acidic hydrolysis and cold storage.[87] Three years later, Estévez et al.[8] first detected both AAS and GGS semialdehydes in food proteins, namely myofibrillar, α-lactalbumin, soy proteins, and BSA by using the ABA derivatization procedure coupled to HPLC-ESI-MS. In the aforementioned study, AAS and GGS were positively identified by comparing their fragmentation patterns at MS^3 with those displayed by the standard compounds. The results obtained by Estévez et al.[8] highlight the suitability of using these semialdehydes as specific oxidation indicators in animal edible by-products containing myofibrillar proteins (e.g., mechanically deboned meat, heart, stomach, or intestines), α-lactalbumin (whey), and BSA (plasma). The study carried out by Armenteros et al.[110] shows the suitability of using these semialdehydes as protein oxidation markers in a large variety of muscle foods including those produced using edible by-products such as cooked sausages and liver pâté.

Whereas the procedure enables an accurate detection of specific protein oxidation products, complex and expensive equipments as well as highly qualified personnel are required. In addition, the whole procedure involves such a long procedure that it usually takes between 24 and 36 h to obtain definitive MS results. Unsuitable to be used as a routine method, this technique should be employed whenever the chemical insight into protein oxidation mechanisms is to be thoroughly studied.

5.3.2.2.3 Detection of Protein Cross-Links

There are three methods of interest for the evaluation of protein cross-links in animal tissues and related foods: (1) determination of disulfide bonds, (2) estimation of dityrosine formation, and (3) assessment of the formation of cross-linked myosin heavy chains.

The procedure for the determination of disulfide bonds was originally described by Thannhauser et al.[115] and subsequently improved by Damodaran.[116] The method is based on the quantification of 2-nitro-5-thiobenzoate (NTB) formed from the reaction of 2-nitro-5-thiosulfobenzoate (NTSB) with disulfides in the presence of excess sodium sulfite. The amount of the chromophoric derivative is determined through the measurement of absorbance at 412 nm and using a molar extinction coefficient of 13,600 M^{-1} cm^{-1}. Following this method, Liu et al.[98] have detected disulfide bonds in myofibril proteins from chicken muscles.

Identification and quantification of dityrosine in muscle protein hydrolysates is usually carried out by HPLC.[117] Some other approaches to the detection of dityrosines in meat samples involves using spectrofluorometry, with 320 and 420 nm being the excitation and emission wavelengths, respectively.[118]

Finally, the formation of protein cross-links can be assessed by the detection of cross-linked myosin heavy chain through electrophoresis techniques. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) of oxidized proteins show the appearance of a band corresponding to molecular weight higher than 500 kDa that have been identified as cross-linked myosin heavy chain (CL-MHC) by mass spectrometry[80] and usually attributed to the generation of disulfide bonds.[86] This technique has been applied for detecting protein cross-links in animal by-products such as heart beef surimi.[119]

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Part II

Technological Quality

6 Color Measurements on Edible Animal By-Products and Muscle-Based Foods

José Angel Pérez-Alvarez, María Estrella Sayas-Barberá, Esther Sendra Nadal, and Juana Fernández-López

CONTENTS

6.1 GENERAL ASPECTS OF COLOR

The first impression that a consumer receives concerning a raw material or food product is established visually, and among the properties observed are color, form, and surface characteristics. The power of color for food is not in doubt. Color appearance, contrast, and differences of edible animal by-products (EABP) and muscle-based foods (MBFs) can have a deep effect on an individual's moods and feelings, and food technologists can exploit these effects.

6.1.1 Color Attributes

Color is the main aspect that defines a food's quality and a product may be rejected simply because of its color, even before other properties, such as aroma, texture, and taste, can be evaluated [1]. All edible animal by-products and MBFs have a number of visually perceived attributes that contribute to their color and appearance as well as to their overall quality. According to Lozano [2], the appearance can be divided in three different categories: color, cesia, and spatial properties or spatiality.

Color is related to optical power spectral properties of the stimulus detected by observers [3]. Cesia includes transparency, translucence, gloss, luster, haze, lightness, opacity, and matt and is related to the properties of reflecting, transmitting, or diffusing light by foods evaluated by human observation. Spatial properties are divided into two main groups: (1) modes of appearance in which color is modified depending on the angle of observation related to the light incidence angle, such as metallic, pearlescent, or iridescent materials, and (2) modes of appearance related to optical properties of surfaces or objects in which effects of ordered patterns (textures) or finishing characteristics of food (as roughness, polish, etc.).

6.1.1.1 Appearance

The total appearance of an object consists of visual structure, surface texture, and distributions of color, gloss, and translucency. It comprises the visual images within the observer. These images are controlled by viewer-dependent variables and scene-dependent variables. The first consists of the viewer's individual visual characteristics, upbringing and preferences, and immediate environment. The second consists of the physics of the constituent materials and their temporal properties combined with the way these are put together and the scene illumination providing light and shade to define the volume and texture of the scene. The model considers the buildup of the appearance image [3]. As regards the specific characteristics that contribute to the physical appearance of EABP and MBFs, color is the quality that most influences consumer choice [4]. This is why the appearance (optical properties, physical form, and presentation) of MBF at the sales point is of such importance for the meat industry [5].

6.1.1.2 Color as Quality Parameter

Some of the edible animal by-products are constituted by lean and fatty tissues. Thus, most of the considerations related to muscle color can applied to the lean tissues of EABP and MBF.

The relation between meat color and quality has been the subject of study since the 1950s, any deviation (nonuniform or anomalous coloring) is unacceptable [6]. The color of fresh meat and associated adipose tissue is, then, of great importance for its commercial acceptability, especially in the case of beef and lamb [7] and in certain countries like the United States and Canada, and there have been many studies to identify the factors controlling its stability. In poultry, the consumers of many countries also associate the skin color with the way in which the animal was raised (intensive or extensive) and fed (cereals, animal feed, etc.) [8]. There is little information related to MBF (cooked, salted, or dry-cured) elaborated with EABP [1].

Color of foods greatly influences consumers' preferences [9]. Color as quality factor on EABP can be appreciated in different ways in different countries. Thus, bovine and chicken fat quality affects consumers grading and the buyer's price. For example, in the United States and Mexico, chicken skin color plays a significant role in the acceptance of chicken [10,11]. In Spain, pinkness fat in whole dry-cured meat products such as dry-cured ham affects consumer's perception on this type of products. Also yellowness in fat of dry-cured meat products is associated with extended processing time [12]. Sensorial quality, especially color and appearance, of EABP or MBF can be affected by internal and external factors. As an example, ostriches fed with carotene-rich diets yield foie-grass-like color [13].

Food technologists have a special interest in the color of food for several reasons: first, because of the need to maintain a uniform color throughout processing; second, to prevent any external or internal agent from acting on the product during its processing, storage, and display; third, to improve or optimize a product's color and appearance; and, lastly, to bring the product's color into line with what the consumer expects [12].

In simple words, the color of EABP and MBFs is determined by the pigments present in them. These can be classified into four types: biological (carotenes and hemopigments), which are accumulated or synthesized in the organism *ante mortem* [5]; pigments produced as a result of damage during manipulation or inadequate processing conditions; pigments produced postmortem (through

enzymatic or nonenzymatic reactions) [14]; and, finally, those resulting from the addition of natural (paprika, saffron, etc.) or artificial colorants [15].

The color also provides information about raw materials [16,17], processing technologies [18], storage conditions, and shelf life [19] and defects [20]. As a quality parameter, color has been widely studied at slaughter [21], in fresh meat [22–24], fat [25], liver [13], and cooked products [26,27], while dry-cured meat products have received less attention [28,29] because in this type of product color formation takes place during the different processing stages [18,29]. Recently, the interest on the effect of functional ingredients in meat products has increased [30,31].

Color plays a fundamental role in the animal production sector, especially in meat production (beef, lamb, and poultry, basically) [32,33]. Thus, in poultry the skin and meat color (to slaughter house to retail points) in the market place have been well established. Ponsano and coworkers [32] described that *Rhodocyclus gelatinosus* supplementation resulted in more yellow breast skin and increased darkening and color purity of breast and thigh skins. Akiba and coworkers [34] reported that poultry meat and poultry meat products can be modified by adding cell-wall-fractured yeast *Phaffia rhodozyma* containing high concentrations of Astaxanthin (Ax). This natural colorant was detected in edible meats and liver in the concentration range from 0.1 to 1.1 µg/g tissue, and the concentration was mostly dependent on the dietary Ax concentration. These results show that the cell-wall-fractured Phaffia yeast containing high concentrations of Ax can be a useful source of Ax for the modification of meat color, thus meeting consumer preferences in relation to the qualities of poultry meat. Bianchi and coworkers [35] found correlation between the skin and breast meat yellowness. Lin and Hsu [36] reported that caponization can modify chicken skin color; thus, the breast skin color parameters (L^* and b^*) are higher than in the intact birds. Related to redness (a^*) capons showed the lowest values. Also, consumers can evaluate beef fat as undesirableness if it has yellow or dark color, excessive glossy, or lustrous [37].

In many countries, the color of bovine subcutaneous adipose tissue is associated with carcass and meat quality. It can, as well, authenticate the dietary history [35]. Poultry sector is also affected by color characteristics, according to Zhang and coworkers [38]. Skin color is used as indicator for chicken sex.

Color can also be used as an important tool by the slaughter plant food inspectors. Trampel and coworkers [39] determined that livers from full-fed birds were lighter in color than normal, and consequently a significant number of chicken carcasses can be condemned for human consumption because they are associated to higher hepatic lipid concentrations. Also, color can be used to characterize postmortem changes in rabbit liver (Figure 6.1). However, few studies are available on the color of EABP cuts and its MBFs.

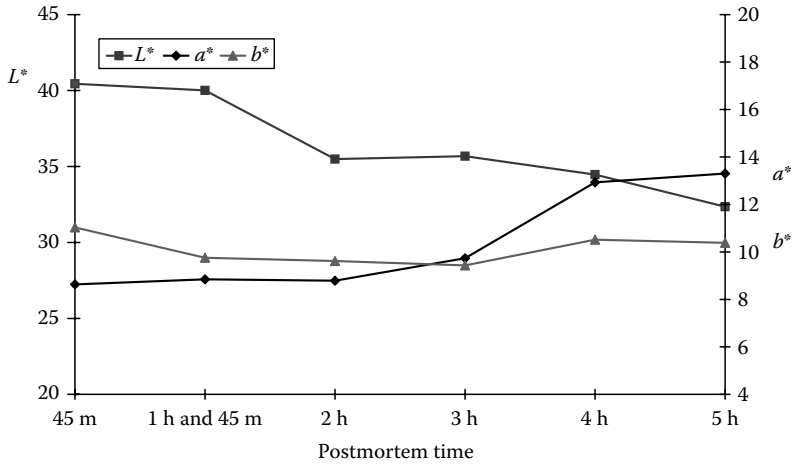

FIGURE 6.1 CIELAB color coordinates evolution during rabbit liver postmortem.

6.2 COLOR MEASUREMENT

6.2.1 Objective Methods

The color of foods can be defined as the interaction of a light, an object, an observer, and the surroundings of the food [40]. Recently, the International Commission on Illumination describes that background can influence the appreciation of color.

Optical methods have the advantage of being nondestructive, fast, and inexpensive and are considered suitable for online measurement. The color of foods can be studied in two main ways: chemically by analyzing the pigments present or physically by measuring the interaction of light. Color necessarily requires a light source that illuminates an object, which, in turn, modifies the light and reflects (or transmits) it to an observer. The observer senses the reflected light, and the combined factors provide the stimulus that the brain converts into our perception of color, a property which has three quantitatively definable dimensions: hue, chroma, and lightness [40].

Several methods are available for objectively measuring the color of foods, some of which depend on the extraction of pigments from food products followed by spectrophotometric determination of pigment concentration [41,42]. However, since such pigment extraction methods are time-consuming and tedious, some researchers have sought simpler methods of color measurement. For example, several methods measure the light reflected from the surface of foods. There are also tabulated coefficients of various objective values, which are correlated with panel scores [43]. These objective values consist of numerous combinations of percentage reflectance values and tristimulus values such as Hunter Lab, CIE XYZ, Munsell hue, chroma, and value or CIELAB [12]. The use of this color space was adopted as an internal standard. Thus in many countries the use of this color space is related to quality control, using as illuminant D_{65} and 10° as standard observer [43]. L^* is a measure of lightness: 0 equals black and 100 equals white. High, positive values of a^* indicate redness and large, negative values indicate greenness, b^* values indicate yellowness to blueness. In Table 6.1, CIELAB color coordinates of different EABP used for dry-salting process are showed.

Lindahl and coworkers [44] in a recent study reported that a^* values explained more than 90% of the variation in pigment content and form of pork muscles. Other study demonstrate that L^* value was the instrumental value most highly correlated with subjectively assessed color when this was based on the Japanese Color Standards [45]. Fat quality can be also related with optical characteristics [37].

Muscles are highly anisotropic optical materials owing to their structural organization and their composition: from muscle to sarcomere filaments, the components are elongated and roughly parallel, forming bundles of conjunctive tissue and myofibrils with widely different intrinsic optical properties [46]. This confers anisotropic optical properties. After slaughter, during rigor mortis and aging, structural damage appears that changes optical anisotropy [47].

Light propagation within meat and meat products is strongly modulated by the corresponding optical absorption and scattering properties of the product. Optical absorption depend on MBF type: (1) meat, (2) offals, (3) muscle-based meat products with various chemical components such as heme pigments (myoglobin and hemoglobin and its derivatives) [48], carotenes and added ingredients to meat products (1) salt, (2) phosphates, (3) curing agents, (4) starch, and (5) dietary fibers among others. Conversely, optical scattering properties of muscle are subjected to several effects: structural properties such as sarcomere length and collagen content [49,50] influence optical properties. Thus, in dry-cured meat products optical properties change during processing as several changes take place on the sarcomere [51].

6.2.1.1 Reflectance Measurements

The measured diffuse reflectance reflects photons that have survived absorption and been scattered diffusely in meat and eventually escaped from the meat surface. Hence, the conventional

TABLE 6.1
CIELAB Color Coordinates Evolution of Different Edible Animal By-Products during Dry-Salting Processing

Edible Meat Cut	Time (Weeks)	Lightness (L^*)	Redness (a^*)	Yellowness (b^*)
Lung	1	48.06	25.93	11.36
	2	49.45	11.65	11.89
	3	51.50	8.57	12.61
Heart	1	37.40	10.52	4.54
	2	33.63	7.35	2.63
	3	30.64	3.69	−1.63
Liver	1	37.77	7.34	2.35
	2	37.65	4.99	4.59
	3	43.42	4.80	5.67
Tongue	1	37.44	15.58	5.56
	2	36.85	6.61	2.24
	3	37.69	3.97	3.76
Backfat (muscular tissue)	1	43.64	7.43	5.76
	2	45.57	1.46	0.06
	3	43.26	0.55	1.73
Backfat (fatty tissue)	1	73.64	2.43	2.76
	2	76.14	1.36	4.78
	3	75.60	0.46	6.15
Backfat skin	1	65.41	9.97	6.53
	2	56.73	8.98	2.79
	3	65.58	3.37	4.7
Blood	1	37.44	15.58	5.56

absorbance is the combined result of the absorbing and scattering effects and is different from the derived absorption coefficient, which is independent of scattering. Absorbance cannot provide an accurate absorption spectrum because the scattering effect is not excluded. Similarly, the scattering coefficients are independent of sample chemical compositions and are solely determined by sample ultrastructure properties [52]. Dry-cured meat products color characteristics, has measured by reflectance values, which consist of indices and/or differences of reflectance at different wavelengths [53].

Myoglobin present in EABP absorbs light in the ultraviolet region and through practically the complete visible region of light [54]. Metmyoglobin (MMb), oxymyoglobin (OMb), nitrosomyoglobin (NOMb), and deoxymyoglobin (DMb) have maximum absorbances (>400 nm) at about 410, 418, 419, and 434 nm, respectively [55]. The absorbance band (AB) is typically much weaker at higher wavelengths (500–600 nm). Above 500 nm, OMb and NOMb have absorption maxima both at around 545 and 585 nm [55]. The NOMb complex maintains myoglobin in ferrous state, but is somewhat unstable and can be displaced and oxidized if stored with excess oxygen and light [56].

In adipose tissues, reflectance shows a minimum originated from the residual hemoglobin associated with the presence of capillaries or with hemorrhage. Irie [37] reported that internal fat is whiter, with less hemoglobin and with harder fat than subcutaneous fat. The hemoglobin derivatives showed different ABs, and, thus, ABs for methemyoglobin (MetHb) were 406, 500, and 630 nm, for oxyhemoglobin (OHb) were 418, 540–542, 516–578, 950 nm, and for deoxyhemoglobin (DHb) were 430, 555, 760, and 910 nm.

Objective color measurements may refer to include several properties or various ratios or color difference indices [40]. By summarizing all the reflected colors (wavelengths) and expressing them as one color [57]. The color as consumer sees can generally be described in one or two words, which

indicate the main color and its shade. However, color measurements whether descriptive or specific, must be made as carefully as other measurements [40].

Correlation between visual assessment and instrumental color measurement of MBFs are not very high, generally due to both technique and measurement conditions.

Rapid screening techniques to determine quality characteristics of meat are of great interest for both industry and consumers. Reflectance measurements closely relates to what the eye and brain see. This is a good method for examining the amount and chemical state of myoglobin in meat in situ. This method is also able to provide a procedure for estimating the percentages of myoglobin forms on the surface of meat. With this method, repeated measurements over time can be made on the same sample. In addition, the procedure is rapid and relatively easy. Reflectance measurements are affected by muscle structure, surface moisture, fat content, additives, and pigment concentrations [58]. Also, tissue structures are associated with the light scattering properties of meat. Several fat type colors can be described by its reflectance spectra. Thus, slight reddish white adipose tissue had a similar percentage of reflectance around 700 nm as the white adipose tissue but had a lower percentage of reflectance at the other visible wavelengths. For the slight dark white color, the reflectance minimum of DHb at 560 nm was characteristic. Yellow fats have lower reflectance than white fat and differed in the shape of the spectra. The reflectance spectra of white fat were low for the difference in the height between around 500 and 700 nm, but the spectra of colored fat were high for that difference. By visual observation, the dark yellow fat seemed to have more yellowness than the pink-yellow fat [59].

The increase in reflectance values of meat would be related with some factors as water-holding capacity (WHC). Its diminution caused by the falling pH might cause the meat structure to close up, driving out the intracellular water, hindering light penetration into the myofibrils, and increasing light scattering. Feldhusen [60] reported that this effect upon reflectance values could be related to the sarcoplasmic proteins denaturation and precipitation on the myofibrils, which result in increased light scattering and less light penetration [61].

Reflectance spectra also were used to evaluate growth rates [62], detection of poultry feces and ingesta [63], fecal contamination of chicken carcass [64], determine the use of nitrite in cooked meat products as Bologna type, etc. Swatland [48] reported that the use of nitrite was associated with lower reflectance at 400 and 410 nm, and with higher reflectance at 430 and 440 nm and from 600 to 700 nm.

Reflectance values of different myoglobin states (deoxymyoglobin, metmyoglobin, and oxymyoglobin) can be equal at several wavelengths (isobestic points). Thus by this method, myoglobin forms can be quantified. Swatland [48] found also isobestic at 580 nm in samples with/without use of nitrites. According with Snyder [65] in beef, several isobestic points are found at 474, 525, 572, and 610 nm. All myoglobins states can be stated at 525 nm in beef. This behavior was also found in pork by Fernández-López and coworkers [66] and Navarro [67]. This author also found more isobestic wavelengths for chicken meat and mechanically deboned poultry recovered (430, 440, 450, 460, 510, 560, 570, 610, 690 nm) meat than beef and pork. In Figure 6.2 reflectance spectra of the different myoglobin states of tuna "sangacho" (by-product from canned or dry-salted tuna processing) can be observed. In sangacho tuna, dark muscle there are not differences in several wavelengths between OMb and DMb myoglobin states reflectance spectra.

From a practical point of view, reflecto-spectrophotometry can be used as a method for the assessment of meat discoloration and MMb accumulation on meat surfaces. Reflectance measures may be excellent for describing appearance of meat color, but they offer very little information about Mb redox chemistry at subsurface levels. Although qualitative and quantitative reflectance data have been very useful in meat color chemistry and for providing insight into meat color problems, these data are not particularly useful for predicting or relating changes in Mb redox status, muscle oxygen status, and muscle color stability.

Liu and coworkers [68] found that intensities of two visible bands at 445 and 560 nm increase with the storage temperature and these wavelengths, possibly indicating a color change due to frozen

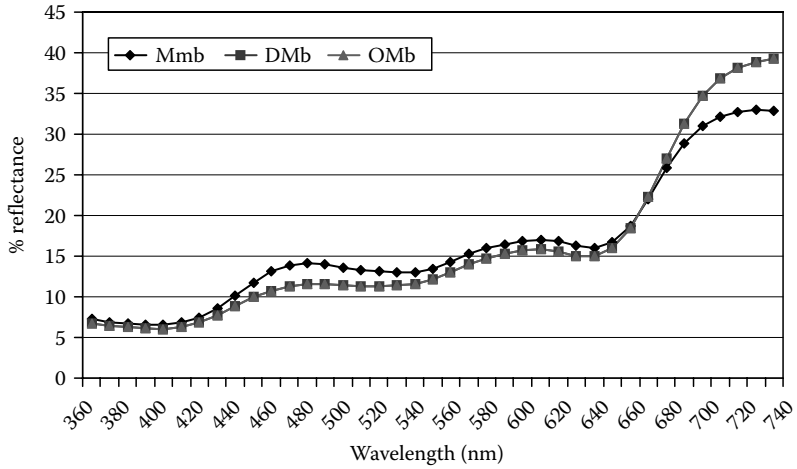

FIGURE 6.2 Reflectance spectra (360–740 nm) of different myoglobin states (OMb: oxymyoglobin; DMb: Deoxymyoglobin; MMb: Metmyoglobin) of tuna "sangacho" (dark muscle (*Thynnus Thynnus*)).

storage. The reduction of spectral intensities, probably indicate water loss and compositional alterations during the freeze–thaw process as well as the tenderization development in muscle storage. Chao and coworkers [69] determined that reflectance spectra, in the range of 400–867 nm, can be used for veterinarians to select wholesome and unwholesome carcass.

Perez-Alvarez and Fernandez-Lopez [1] reported that most additives and ingredients added to MBF do not modify the spectral form, but only modify its height.

Irie [37] reported that the main factors affecting bovine fat appearance are carotene concentration and hemoglobin concentration affecting on the yellowness and redness, respectively. Also the chemical state of hemoglobin and the translucency of fat affected the color and % reflectance or darkness of beef fat.

6.2.1.2 Near Infrared NIR Analysis

Near infrared (NIR) technology provides complete information about the molecular bonds and chemical constituents in a sample scanned. Optical devices coupled to computers offer potentially very fast data acquisition that may permit decision making on meat eating quality albeit from a selected small surface area only [70].

Near-infrared reflectance (NIR) spectroscopy can be useful for quality control. This technique can predict chemical composition of poultry meat and possibly some dietary treatments applied to the chicken [71]. Recent studies have therefore concentrated on looking for the application of this technique [72]. There is substantial interest to use NIR online to predict chemical parameters in the meat industry and to augment existing on video image scanning and analysis technology (VISA) systems [73]. New VISA systems [74] provide a noninvasive method operating at normal abattoir chain speeds and enable automatic acquisition of data on carcasses from the side and back view. However, these systems cannot classify on the basis of meat quality and need therefore to be augmented with other suitable systems to measure meat eating quality traits. Post rigor drip loss and WHC during storage can be predicted adequately by NIRS, also determine WHC specifically, rather than the occurrence of PSE, using techniques such as NIR spectroscopy [75].

The USDA Agricultural Research Service has developed a method and a hyperspectral imaging system to detect feces (from duodenum, ceca, and colon) and ingesta on poultry carcasses. The method first involves the use of multivariate data analysis on visible/near-infrared (Vis/NIR) reflectance spectra of fecal and uncontaminated skin samples for classification of contaminants [63].

Myoglobin contents vary with species and with fiber-type composition within specific specie. However, some authors [76] have found evidence for the possibility of estimating myoglobin in meat

samples using the visible region (400–800 nm) of the spectra. This would be feasible due to the different forms of myoglobin in the meat samples (oxymyoglobin, deoxymyoglobin, and metmyoglobin), thus giving rise to different colors (bright red, purple, and brown, respectively).

6.2.1.2.1 Near-Infrared Tissue Oximetry Analysis

Currently available meat color measurement techniques such as reflectance colorimetry and spectrophotometric methods are limited to surface measurements of meat color because of the inability of visible light to penetrate the meat surface.

NIR tissue oximetry has a potential as a noninvasive, rapid method for the assessment of meat color traits and may help to improve the meat color chemistry understanding. Thus, interactions between light and muscle pigments in meat allow the use of frequency-domain multidistance (FDMD) NIR tissue oximetry. FDMD NIR tissue oximeter penetrates several centimeters below the meat surface, and its ability to determine the oxygen-dependent absorption of Mb and Hb. [77,78]. Wavelengths of the NIR lights are differentially absorbed by the oxygenated and deoxygenated forms of Hb [43,79]. Hb and Mb have similar NIR absorption and given that because there are minimal amounts of Hb in well-bled animal tissues, it is possible to distinguish DMb, OMb, and MMb. Mohan et al. [79] reported that fiber orientation and Mb redox form affected NIR tissue oximetry (FDMD) data in postrigor muscle, allowing the direct measure of absolute concentrations of hemoglobin (Hb) and Mb oxygen saturation in skeletal muscle and brain tissue. Also, NIR tissue oximeter is based on the relative tissue transparency in the NIR region and on the O_2^- dependent absorption changes of Mb. The FDMD spectrophotometer permits the calculation of absolute concentrations of OMb and DMb (μM) using dynamic calculation of the reduced scattering coefficient. The disadvantage is that it is not possible to distinguish between Hb and Mb (both have nearly identical spectral characteristics). The absorption changes at the discrete wavelengths were converted into concentrations of OMb and DMb.

6.2.1.3 Digital Image Analysis

As mentioned above, visual properties are key factors for consumer acceptance. Visual inspection is often used, but it is highly subjective and is not appropriate for industrial settings. Different optical and imaging techniques have recently been developed [80].

The digital image analysis has several advantages: (1) nonstop working, (2) works at high speed, (3) amplifies the images, and (4) avoids human inspector failures by distraction or fatigue. The artificial intelligence does not see in the same way as the human beings. Cameras are different from the optics of the human eye: whereas the man sees as a whole, the computer has to see the pixels of the image one by one, and then built up the information [81].

Macro and microscopic research in EABP or MBFs needs simple technologies that allow characterizing the performance of ingredients or additives and changes during processing and shelf life. Therefore, digital image analysis technology can give good results to determine food appearance. The analysis of this property cannot be determined with colorimeters. These equipments cannot analyze the high variability and complexity of color distribution in many MBFs [82].

The development of computer-assisted vision grading systems, the changes in skin and meat color during and after processing have become important, based on calibrations and assessment values based on color. Thus color values for both skin and meat were studied. Both changed dramatically within the first 6 h postmortem, after which the changes were less pronounced. Also scalding process can be evaluated by VA; thus, the skin from semiscalded birds showed fewer changes than the skin from subscalded birds [83].

6.2.1.4 Photon Migration Techniques

Photon migration techniques (PMT) using visible to near infrared wavelengths have found widespread applications in the analysis of different material structures [84]. When light propagates in a turbid media, it is absorbed and randomly scattered. Eventually, some light escapes from the sample

surface and is measured as the optical reflectance. Those scattered photons carry vital information about optical properties of the media, which can be derived from reflectance measurements based on light transportation theories. Thus, the continuous time random walk (CTRW) theory [85] has been used to study the complicated process of light transportation in scattering media. This theory has been successfully applied in various anisotropic tissue structures such as the skin [86]. PMT have these advantages: (1) it is simple to implement, (2) the results have good immunity to light incident angle, (3) reflectance profile is not affected by the incident light intensity, and (4) no sample preparation is required during the measurements. With these advantages PMT can provide a real-time quality control and assurance device for products having fibrous structure in an industrial setting.

6.3 ADDITIVES AND PROCESSING OF EDIBLE ANIMAL BY-PRODUCTS

6.3.1 Color and Food Engineering

Descriptions of color in EABP an MBFs differ, depending on whether chemical, biochemical, sensorial or technological aspects are foremost in the observer's mind. Most studies have been directed to evaluate the effects of processing and shelf life on meat color and the effect of incorporating functional ingredients is gaining interest (Figure 6.3). For functional meat products to be successfully commercialized, they must be healthy, safe, and appetizing. This last characteristic is related with the external appearance of the product, to which cesía and color both contribute [81].

6.3.1.1 Technological Aspects

Meat-based foods (MBFs), in general, and meat products elaborated with offal and other edible animal by-products can be considered as multicomponent and, frequently, multiphase systems. Certain added ingredients (water, etc.) or components of the meat (e.g., fat) may undergo phase changes due to the temperature and pressure changes associated with processing. The color of meat products is generally developed in one or more stages of the manufacturing process (salting, smoking, marinating, drying, curing, etc.). Frequently, color development during processing can serve as an objective quality criterion.

The diversity and complexity of MBFs imply dramatic color effects on derived products, for example, (1) the relative amounts of different tissues (muscular adipose and connective), (2) the type

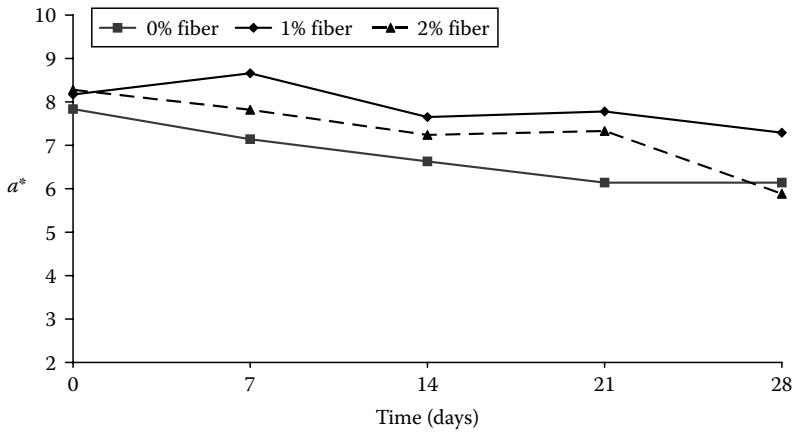

FIGURE 6.3 Redness (a^*) evolution during the shelflife of a Spanish blood sausage "botifarró" added with citrus dietary fiber.

and concentration of meat and nonmeat ingredients, (3) technological treatments, and (4) changes in the MBFs ultrastructure resulting from treatment.

Regarding the percentage of the different tissues used, the relation between muscle, conjunctive or adipose tissues, and the percentage of blood or viscera have a dramatic effect on color. Among nonmeat ingredients, salt, particularly, affects the final color of MBFs. The individual incorporation of curing agents even at different concentrations is not involved in generating the color associated with cured MBFs, for which it is necessary to add salt and nitrites jointly. Insofar as paprika (the most commonly used in MBFs) is concerned, the color of the MBFs (both during processing and shelf life) will depend on the content and stability of paprika carotenes.

A great variety of technological treatments are possible in the case of MBFs, ranging from the conventional (salting, cooking, smoking, or drying) to the new technologies (high pressures, lyophilization, etc.). The color of MBFs can be studied from different points of view: (1) physical, (2) chemical, (3) ultrastructural, and (4) microbiological.

From a physical standpoint, EABP and MBFs can be considered as opaque solids that disperse, reflect or transmit light, a property that results from the interaction of light with the macro and microscopic structure of the MP. Chemically, the property is related with the concentration and state of the pigments present in meat and meat products, whether of heminic origin (myoglobin, hemoglobin, and citochromes) or in carotenoid form (carotene and xanthophylls). The influence of ultrastructure is one of the most recent areas of interest and study. The changes that take place at macro and microscopic level strongly affect the color of MBFs, i.e., changes linked to the change from sol to gel in dry-cured MPs, the effect of phosphates and salt on the water holding capacity or even the inclusion of air in a meat emulsion can notably affect the color of the product. Lastly, microbiology plays a primordial role in the formation and stabilization of color, particularly in dry-cured products. However, microbiota can also harm MP color through generating substances that alter the stability of the nitrosated pigments, reducing their shelf life.

6.3.2 Additives and Processing

Meat color is closely related to its pH [87]. The state of the pigment is affected by the meat pH, and the reaction of the heme with other reactants such as oxygen on their effect on the state of the heme iron [88]. Faustman and coworkers [24] reported that the rate of autoxidation of OMb increases rapidly with increasing hydrogen ion concentration. From a technological point of view, the additives usually used in meat processing modify the myoglobin forms, especially producing metmyoglobin, and other factors (endogenous reductor muscle factors) take place to enhance final meat color.

6.3.2.1 Additives

To understand the color of MBFs, it is necessary to study the mobility of water in the product and its ultrastructure. Both criteria will enable the shelf life of the products to be ascertained. Understanding the fundamental aspects of treatments applied to meat products will contribute to the formation and stabilization of their color.

Color behavior in cured meat products (dry or cooked) is very different in relation to fresh meat products (without nitrite), because the presence of nitrite provokes the formation of other myoglobin states (nitrousmyoglobin, nitrousmetmyoglobin, etc.) that masks the effect of these additives on color. However, additives may also cause color problems in meat products since both may alter the behavior of proteins such as myoglobin [61]. In the dry-cured sausages formulated with EABP (ears, tails, snouts, blood, etc.) added with paprika (“Botillo del Bierzo,” “onion chorizo,” or “Galician Morcilla”), this spice masks reflectance meat spectra [15] and only paprika reflectance spectra can be observed (Figure 6.4). Also similar behavior is observed on color between paprika discoloration and dry-cured sausages paprika added [61].

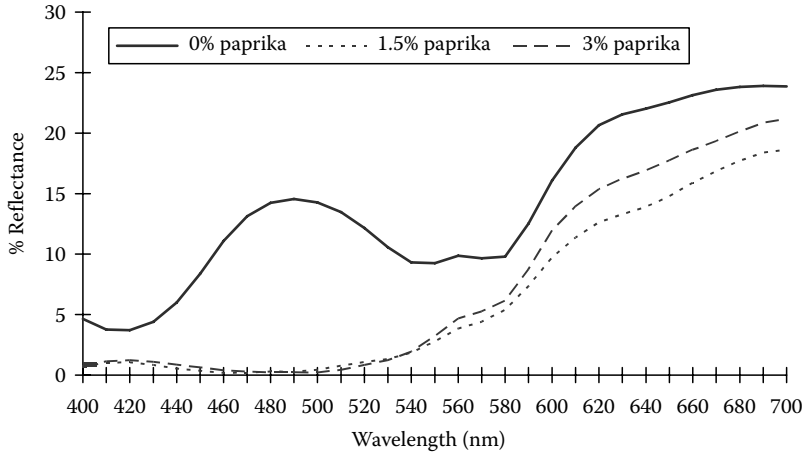

FIGURE 6.4 Reflectance spectra (400–700 nm) of a dry-cured meat product containing edible animal by-products (EABP) added with different paprika concentrations.

6.3.2.1.1 Salt

Salt as main ingredient for muscle-based products plays many important roles, one of them being color. All myoglobin states differed with the addition of different salt concentrations. Thus, the addition of salt to the meat favored MMb formation and decreased the proportion of OMb and DMb, due to its ability to oxidize the ferrous state of the heme group to the ferric state (MMb) [22]. According to the values reported by Fernández-López and coworkers [66] in a study of the color parameters of the pure states of Mb in pork meat, the increase in MMb after salt addition is accompanied by the increase in L^* values and decrease in a^* values. Fernández-López and coworkers [58] suggest that the meat color with added salt depends more on changes in the meat structure and free water content, together with their interaction with light, than the Mb state in the meat. Some other findings about the influence of sodium chloride and tripolyphosphate indicated that the addition of latter additive did not modify redness (a^*), yellowness (b^*), chroma (C^*), or hue (h°), but stabilized the percentage of oxymyoglobin. A decrease in pH and the addition of sodium chloride or sodium tripolyphosphate led to an increase in the metmyoglobin percentage.

6.3.2.2 Processing

Studying color formation mechanisms in MBFs during their manufacture is a complicated task because of the great variety of interactions that take place (physical, chemical, biochemical, enzymatic, and microbiological). In addition, the manufacturing processes are diverse and have very different effects on the muscle-based matrix and, therefore, on color. For example, smoking can be studied not only from a drying point of view but also as Maillard reaction induces through the interaction between aldehydes and ketones present in smoke and the amino acids in the MBFs [89].

Regarding the drying process in dry-salted and dry-cured meat products, dehydration leads the water to migrate along a concentration gradient from the inside to the outside of the product, thus creating objective color differences in wide diameter products (>55 mm diameter). These differences are visible to the human eye since the most external zones tend to have a more intense color than the inner parts. To optimize the drying process from a color point of view, the kinetic of the movement of water outward the product should be studied, and the gradients formed analyzed. Such studies are in the early stages and face the difficulty of studying water diffusion and its relation with color since both engineering aspects, as well as product microstructure must be considered. Microstructure of the food plays an important role in the color components of the final meat

product, particularly the yellow-blue coordinate (b^*). However, the instrumental study of color is complicated since it requires a wide surface for the measurements to be made while the ultrastructure presents no such "wide" surface.

Technological processing leaves "fingerprints" and the spectrum can be used to quantify the degree to which the MBF color is altered by such processes. However, upon reflectance spectra, other aspects have to be considered in the spectrum such as the nature of the surface (smooth, rouge) or the particles (nonmeat ingredients) or molecules dispersed or dissolved in the aqueous phase (salt, phosphates, etc.).

The study of how technological processes influence the color of MPs is fundamental for determining the mechanisms that control this physical property. For this reason, it is important to determine which color components are modified during processing and which ingredients/conditions are most favorable for maintaining a product's color.

From a technological point of view, the breakdown of the tissues by mincing facilitates the exit of the liquid remaining in the muscle tissue and intracellular space and is accompanied by soluble proteins such as myoglobin. Such modification of the muscle structure by mincing should influence how color develops in the final meat products. Thus, it is very important to know the time that the fatty tissue was cut (optical properties changes after cutting). Immediately after cutting adipose tissue had reflectance minima at about 430 and 555 nm showing DHb. But 1 h after cutting, the reflectance minima changed to about 420, 540, and 580 nm, characteristics of oxy-hemoglobin. When this tissue is analyzed 3 days after cutting, the spectra show slight reflectance minima at 640 nm characteristics of met-hemoglobin. When the adipose tissue is stored for large periods and then is minced to elaborate meat products, oxidized state of hemoglobin can be a catalyst of oxidation reactions. These oxidation reactions can modify meat product color [46].

On the other hand, the incorporation of air by the action of mincing should also be taken into account, since this increases L^*, a greater scattering of light being caused by the air bubbles trapped in the meat batter (cooked or dry-cured) [89].

In a study of the color parameters in different primary meat products, Perez-Alvarez and coworkers [17] reported that the concentration of Mb is not an influential factor for this coordinate. Johansson and coworkers [90] reported that the yellowness coordinate in fresh meat increased with more oxymyoglobin (OMb). Fernández-López [91] reported that both oxidation and Mb oxygenation affect the b^* values, by increasing them. Mincing destroys muscle structure, liberating the sarcoplasmic proteins, among them Mb. The mincing process per se facilitate the incorporation of air and the consequent formation of OMb, which contribute to the increase in b^* values [29]. When adipose tissue is grinded whiteness increase and may be useful for sausage production. But the cooking process can reduce L^* values as pork dewlap is scalded during paté elaboration processing. When meat rich in connective tissues are added in MBF products, lightness increased. When this collagen is heat treated, gelatin is produced and jellifies. This phenomena increases L^* by increasing light reflection.

In all MBF, fat characteristics are very important for textural and technological properties [86,92]. Fat hardness o softness can modify MBF product color by the oxidization of heme pigments through oxygen permeability [59].

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Part III

Nutritional Quality

7 Composition and Calories

Karl-Otto Honikel

CONTENTS

7.1 INTRODUCTION

In different countries, by tradition and culture, people eat meat of different species of animals and subsequently also different parts of the carcass that are not skeletal muscles or fatty (adipose) tissue. The latter are the by-products. In general, the expression "by-products" describes all parts, edible or inedible for human beings, but it varies which of the by-products are considered as edible [1]. Another expression of these parts of a carcass is "offal." By offal those parts of the carcass are meant that "fall of the butcher's block" [2]. The expression is used for inedible by-products. Regarded as inedible for all human beings are mineralized bones. Since the BSE crisis in most parts of the world, brains of grown up cattle and sheep have been taken away from the plate of consumers. In generalization, today, often brains of all species are regarded as inedible by-products. Nevertheless, the composition of brains of some species will be addressed in this chapter. Furthermore, blood, skin, and sexual organs of animals are often regarded by many consumers as offal. Due to the refusal of people to accept some by-products as edible, the compositional data of these "offal" are not mentioned in most data books and databanks. Literature data of these "offal" are also incomplete. Reported are only data of protein or fat, sometimes minerals and residues like hormones. These studies do not permit to list even the main constituents and to calculate energy values. Thus, only those by-products of the species mentioned in Table 7.1 are addressed in this chapter.

The decision about compiling the data for composition of the products in Table 7.1 was made after 11 data books and their relevant data banks—if the latter ones were accessible without fees—have been consulted [3–13]. Access to the data books and data banks is obtained easily via www.fao.org/infoods/directory-en.stm. After the data had been collected, the observation was made that the compositional data of major and minor constituents of by-products within the same species differed considerably between the sources. Even wider, the data of prepared (heated) by-products differed. Furthermore, the composition of heated by-products were not reported in all cases. Hence, it was decided to compile only the data of raw material.

TABLE 7.1
Addressed Compositional and Caloric Data of the Edible Animal By-Products and Species

By-Product	Porcine	Bovine	Small Bovine	Chicken	Lamb/Sheep	Other Species
Blood	x	x				
Liver	x	x	x	x	x	Duck
Heart	x	x	x	x	x	
Tongue	x	x	x			
Kidney	x	x	x		x	
Spleen	x	x	x		x	
Lung	x	x				
Tripe		x			x	
Chitterlings	x					
Stomach	x					
Thymus			x		x	
Pancreas	x	x				
Lard	x					
Tallow		x				
Suet		x				
Calf fat			x			
Cow udder		x				
Skin	x					
Brain	x		x		x	
Ear	x					
Feet	x					
Giblets/gizzard				x		

The most likely reason for the discrepancies in the analytical data is that the raw materials used were from animals of different breeds, gender, age, and feeding regimes. Differences of analytical methods with macro constituents may exist; but they will not lead to differences of 100% and more, e.g., in the fat content of bovine liver. These differences in the concentrations of macro constituents were also observed in minor constituents with the data of minerals and vitamins. It is interesting to note that in some minor components of the by-products the values of concentrations differed considerably, in other minor constituents the variation was rather small. In Table 7.2, values of 11 data books for bovine liver with regard to sodium, potassium, vitamin A, and vitamin B2 content are presented. I chose a by-product which is most often analyzed. The values of sodium varied from 65 to 116 mg Na/100 g, i.e., a factor of 1.8, the concentrations of potassium ranged from 230 to 330 mg/100 g, i.e., a 30% difference. Vitamin A ranged from 5100 µg RE/100 g to 22,000 µg RE/100 g, a factor of more than 4. The reason in this case may be that the figures were reported in different units (µg RE resp. IE), which the authors of the source did not take into account. With vitamin B2 the variation was between 2–3 (mean 2.5) and 4.2 mg/100 g, a difference of 40%.

As the author of this chapter I had to come to a decision which data I should use for the tables, the calculations, and discussions of composition and calories. In a handbook of analysis and within the subheading of "nutritional quality," I decided to analyze the nutritional composition in comparison of species and by-products in major and minor constituents and in relation to meat cuts. The choice in presentation was either presenting the range or a "mean" value. For calculations of energy, a mean value was needed in any case. After the compilation of the individually reported data of each by-product, species, and constituent I considered which the most likely concentration or range of the constituents would be. I decided to present one value only. A range would have not given any valuable further information except the large variation in data books. In some cases I chose the

TABLE 7.2
Values of Sodium, Potassium, Vitamin A and Vitamin B2 in Bovine Liver according to Handbooks

Reference	Sodium (mg/100 g)	Potassium (mg/100 g)	Vitamin A[a] (µg RE/100 g)	Vitamin B2 (mg/100 g)
[1]	81–136	281–320	3,700–32,000	2.8–3.3
[3]	115	290	15,000	2.9
[4]	115	330	18,000	2.9
[5]			15,300	2–3
[6]	81	320	5,100	3.1
[7]	96	325	10,000	2.9
[8]	116	292	6,000	2.9
[9]	81	320	16,500	3.1
[10]	65	300	22,000	3.0
[11]	82	285	14,200	4.2
[12]	73	323	10,500	2.8
[13]	—	—	10,800	3.0

[a] µg RE means µg retinol equivalent.

arithmetic mean, in some the median (50%) value. In a few cases I decided for smaller or bigger values of concentration than mean or median. In any case it is the personal decision as the author of this chapter which values appear in the tables. Most of the constituents will also be addressed in other chapters and most likely there the analytical methods of determination will be described. Thus, I decided not to go into the topic of chemical analysis.

Together with the major constituents and calories also the fatty acid groups and cholesterol are listed in Table 7.3. Tables with the concentrations of minerals and vitamins will follow.

7.2 COMPARING OF COMPOSITION OF MEAT AND ANIMAL BY-PRODUCTS

In foods, the contents of protein, fat, and degradable carbohydrates (monosaccharides or their multiples) have to be taken into account for the calculation of nutritional energy. The energy values are presented in Table 7.4. They are expressed in this table in kcal and kJ/100 g. Later on, only the more common calculation in kcal (1 kcal = 4.2 kJ) is used.

In meat (derived from skeletal muscles and adjacent fat) and fatty tissue, only protein and fat add to the energy content. In muscles of living animal, a polysaccharide made of glucose units, glycogen, may be stored up to 1.2% of the weight of the muscle. Rather minor concentrations of glucose and other monosaccharides exist in muscles of live animals [4]. In the postmortal anaerobic glycogenolysis, the glycogen is metabolized to lactic acid resp. lactate with different velocities depending on species and stress before and within the slaughter process [14]. With the lactic acid formation the pH falls. For instance, in stress susceptible pigs the final pH value of around 5.5 may be reached within 60 min. In grown-up cattle, it may take up to 36 h until the glycogenolysis finishes. Lactic acid normally lowers the pH value of muscular cells from about 7.0 in the live animal to 5.4–5.8 as the ultimate value. Animals that are stressed antemortem for a longer period of time get partially depleted of glycogen [15]. The postmortal pH fall is then lower and ends usually above 6.0 up to 6.8. The metabolic pathway of glycogenolysis involves more than a dozen enzymes, which with falling pH slow down in their velocity or even lose their activity. Thus, between 0.2 and 0.5 g carbohydrates (glycogen and glucose derivatives)/100 g remain in the meat. Around 0.2–1.0 g lactic acid/100 g will be formed. Calculating the nutritional energy of both carbohydrates and

TABLE 7.3
Selected Ingredients of By-Products

Main constituents
- Protein
- Fat

Elements or accompanying constituents of main compounds
- Fatty acids (SFA, MUFA, PUFA)[a]
- Cholesterol

Minerals	Vitamins
Macroelements	A
Sodium (Na)	B1
Potassium (K)	B2
Magnesium (Mg)	Niacin
Calcium (Ca)	B6
Phosphorous (P), presented as phosphate (HPO_4^{2-})	Folate
Trace elements	Pantothenate
Iron (Fe)	B12
Zinc (Zn)	C
Selenium (Se)	D
Copper (Cu)	E
Iodine (I)	H
Manganese (Mn)	K

[a] SFA, saturated fatty acid; MUFA, monounsaturated fatty acid; PUFA, polyunsaturated fatty acid.

TABLE 7.4
Range of Main Constituents and the Calculated Nutritional Energy in Meat of Skeletal Muscles at 48 h Postmortem

Constituents	Energy [18] (kcal/kJ)	Concentration (g/100 g)	Nutritional Energy Range (kcal/100 g)	Nutritional Energy Range (kJ/100 g)
Protein	4/17	15–23	60–92	255–391
Fat	9/37	1–30	9–270	37–1110
Carbohydrates	4/17	0.5–1.5	2–6	8–25
Lactic acid	3.6/15	0.2–1.0	0.7–3.6	3–15
Sum (range)			100–300	420–1260

lactic acid, a maximum of 6 kcal/100 g can be provided (Table 7.4). The leanest cuts of meat (<2% fat) deliver about 100 kcal nutritional/100 g. The overall mean of meat cuts of all common meat species contains about 20% protein and 10% fat [16]. This adds up to about 170 kcal/100 g, thus, the 6 kcal/100 g by carbohydrates and lactic acid can be neglected in meat. In fatty tissue, this energy calculation is even more applicable as the carbohydrate concentration is much lower than in muscular tissue [4] and no pH fall occurs [14]. Furthermore, in fatty tissues no postmortem energy delivering metabolism occurs from fat or fatty acids, as oxygen is needed for energy metabolizing breakdown, which does not exist in sufficient concentrations after death. In the usual time between slaughter and final consumption no fat metabolism occurs in meat of farm animals [17].

TABLE 7.5
Composition of a Bovine Muscle per 100 g Raw Material

Main Constituents	Unit	Beef Topside
Protein	g	20.7
Fat	g	4.5
Energy	kcal	122
Elements or accompanying constituents of main compounds		
Saturated fatty acids	g	1.9
Monounsaturated fatty acids	g	2.0
Polyunsaturated fatty acids	g	0.15
Cholesterol	mg	50
Minerals		
Macroelements		
Sodium (Na)	mg	65
Potassium (K)	mg	360
Magnesium (Mg)	mg	25
Calcium (Ca)	mg	6
Phosphorous (P) as phosphate	mg	205
Trace elements		
Iron (Fe)	mg	2.4
Zinc (Zn)	mg	3.7
Selenium (Se)	µg	7
Copper (Cu)	mg	0.1
Iodine (I)	µg	3
Manganese (Mn)	µg	11
Vitamins		
A	µg RE	5
B1	mg	0.05
B2	mg	0.1
Niacin	mg	4.7
B6	mg	0.35
Folate	µg	9
Pantothenate	mg	1
B12	µg	2
C	mg	<1
D	µg	tr
E	µg	0.5
H	µg	5
K	µg	15

Source: Souci, S.W. et al., Der kleine SFK, *Lebensmitteltabelle für die Praxis*, 3rd edn., Wiss. Verlagsgesellschaft, Stuttgart, Germany, 2004.

Most by-products differ in the postmortem metabolism from muscular tissue. Only muscle-like by-products like heart or tongue show some pH fall, usually smaller than in skeletal muscles. Other by-products like liver do not metabolize the main constituents. If carbohydrates are stored in higher concentrations, then they remain unchanged and they will add to the energy calculation.

As a reference and in comparison to the composition of by-products, Table 7.5 shows the composition of a bovine muscle (topside), which is a rather lean cut of bovine carcasses. It contains

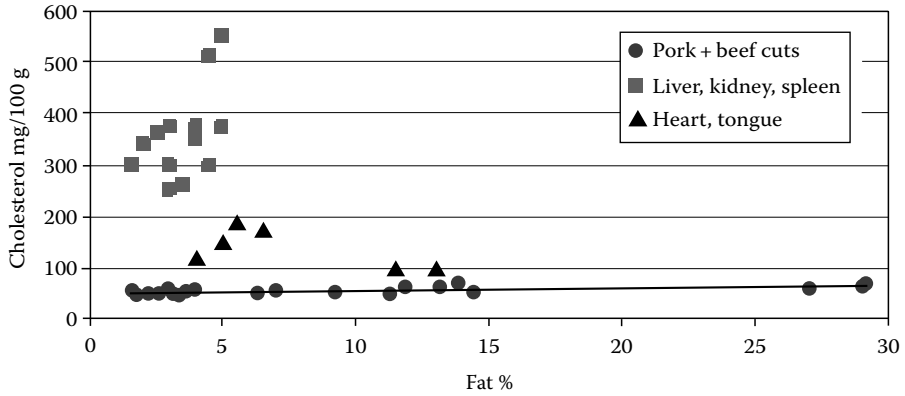

FIGURE 7.1 Cholesterol and fat content in different edible animal by-products.

20.7% protein and 4.5% fat. The carbohydrate/lactic acid contents are not mentioned due to their low values. But it should be emphasized that the sum of fatty acids in the fat is 4.05 g equivalent to 90% of the fat content as the glycerol moiety of the fat has a weight share of close to 10% of the fat weight. This calculation assumes that the triacylglycerols in fat of meat and also in animal by-products consists of 3 fatty acids with chain length of 16 or 18 C-atoms. For phospholipids the fatty acid weight share is even lower with 75%–90% of the fat value. But due to the low fat content of most by-products, the use of 0.9 for the fatty acid part of fat does not cause a big error. The concentrations of the saturated fatty acids group in the topside meat is slightly lower than the sum of monounsaturated fatty acids. The polyunsaturated fatty acids add up to small values (<5% of total fatty acids).

For meat cuts from skeletal muscle including the ones with adjacent fat and fatty tissue, the cholesterol content is in the range of beef topside. It ranges for lean to fat meat cuts from 45 to 70 mg cholesterol/100 g raw material (Figure 7.1). In unprocessed by-products cholesterol concentrations are always above this range.

The macro mineral concentrations in all lean meat cuts are high in potassium and phosphorus, low in sodium, calcium, and magnesium. With trace elements, there is none very high or low in meat cuts. The exceptions are iron and zinc content, which rise with the red color of the meat. The same as for minerals also applies to vitamins in meat, again with a few exceptions.

7.3 RECOMMENDED DAILY ALLOWANCES ACCORDING TO GUIDELINE 90/496 EEC AND D-A-CH (2002)

As a further reference the recommended daily allowances (RDA) are presented in Table 7.6. The values given there indicates that those amounts should be eaten regularly. Eating more in the range of two- to threefold does not cause any health problems [5]. Taking up only half of the daily recommended values may lead, in the long run, to deficiencies and maybe health problems. It must be realized, however, that some of the nutrients can be stored for some time. Others are excreted or used up in the body rather fast. For instance, calcium and phosphate are bound in bones and teeth and exhibit a half-life in the body of years; potassium and sodium are excreted via urine and perspiration within days or a few weeks. The fat soluble vitamins (A, D, E, and B12) are stored for months and years, vitamin C, however, only for a few days [19].

7.4 LOSSES OF CONSTITUENTS ON PREPARATION

The amounts of RDA values are related to the intake. This means as by-products are heated before consumption that the concentrations of many constituents are lower than they are reported in the data books and banks. The data of constituents in Tables 7.5 as well as 7.7 through 7.9 are those of raw materials. Minerals like potassium will be lost by cooking with the cookout, which may amount

TABLE 7.6
Recommended Daily Allowances (RDA) according to References [17,20]

Nutrient	Unit	RDA Male	RDA Female	Nutrient	Unit	RDA Male	RDA Female
Energy	kcal	2500	2000				
Protein	g	60	50				
Fat	g	90	70				
Fatty acids	% of total fatty acids						
SFA[a]	"	<35	<35				
MUFA	"	<50	>50				
PUFA	"	>15	>15				
Cholesterol	mg	300	300				
Minerals				Vitamins			
Macroelements				A	µg RE	1000	800
Na	mg	550	500	B1	mg	1.2	1.0
K	mg	2000	2000	B2	mg	1.4	1.2
Mg	mg	350	300	Niacin	mg	16	13
Ca	mg	1000	1000	B6	mg	1.5	1.2
Phosphate	mg	700	700	Folate	µg	400	400
Trace elements				Pantothenate	mg	6	6
Fe	mg	10	15	B12	µg	3	3
Zn	mg	10	7	C	mg	70	70
Se	µg	50	50	D	µg	5	5
Cu	mg	1.2	1.2	E	mg	14	12
I	µg	200	150	H	µg	40	40
Mn	µg	3000	3000	K	µg	70	60

[a] SFA, saturated fatty acid; MUFA, monounsaturated fatty acid; PUFA, polyunsaturated fatty acid.

to more than 30% of the raw weight. Sodium, on the other hand, is added to food during preparation and/or on the plate. Cations with 2 or 3 positive charges are lost in smaller amounts as they are bound to protein side chains. Vitamins are lost, on the one hand, with the cookout and, on the other hand, are inactivated/degraded by the heat. Losses of 10% to >50% are possible, depending on the process of preparation [4]. As the loss on preparation varies widely with vitamins, a direct calculation of intake from of the contents of raw materials and RDA values must be done with care.

Comparing Tables 7.5 and 7.6, it shows quite a difference of nutrients in meat/100 g and RDA values. For instance, a portion of 100 g raw meat provides 30% (for male) and 40% (for female) of protein requested in the RDA values, fat in 100 g topside adds up to 5%–6% of the RDA values only, and cholesterol in 100 g topside amounts to 17% of the RDA value. We can assume of the just mentioned ingredients that on cooking low amounts are lost and the amount in g/mg is present after preparation. It is different with the following constituents. Raw meat that weighs 100 g contains only 11% of the RDA of sodium. Prepared on the plate ready for consumption, 1% salt, i.e., 400 mg, is usually added and 400 mg exists naturally in meat (30% lost as cookout of 550 mg/100 g), and 800 mg sodium is present in the meat on the plate, which is about 150% RDA. Likewise, 100 g of raw topside cut provides 18% of potassium. This may end after 30% cookout at 12.5% of the RDA value. In the meat, 8% of the RDA of divalent magnesium and 0.6% (!) of calcium are provided. Iron, on the other hand, provides 25% (for male) and 16% (for female) of the RDA;, zinc is even with 30%–50% of the RDA value present in the beef cut and selenium with about 15% of RDA. We can assume that, of these di- and tri-valent cations, only small amounts are lost during cooking. Iodine, on the other hand, provides 1%–2% of RDA only.

With the vitamins the beef topside contains raw, e.g., about 30% of the niacin RDA value and 25% of vitamin B6, 16% of pantothenate and 20 resp. 25% of vitamin K. All other vitamins in bone muscles are low in comparison to the RDA values. We can assume that in prepared by-products about 25% of the vitamins are lost in cookout or degradation. For more details, it is recommended to consult the data books mentioned in the reference list [3–13].

7.5 MAJOR NUTRIENTS IN BY-PRODUCTS

Table 7.7 shows that the protein content of all by-products with few exceptions amounts to about 15%–22%. Exceptions are the rendered fats (lard, tallow, suet) but not the fresh calf fat (abdominal and kidney fat), tripe, chitterlings, skin, and brain. The protein content is near the values of a meat cut with a low or medium amount of adjacent fat.

The fat content is low (<5%) in liver heart, kidney, spleen, thymus, and lung of all species. These by-products are in their fat content comparable to lean meat cuts. Higher in fat are tongue, chitterlings, stomach, pancreas, brain, ear, cow udder, feet of swine, skin, and fat (unrendered or rendered).

In most by-products, the monounsaturated fatty acid group (MUFA) are higher than the sum of saturated fatty acids (SFA). Lowest are always the polyunsaturated fatty acids (PUFA). In all cases, the sum of MUFA+PUFA is higher than the SFA content. In other words, SFA contents are always <50% of all fatty acids. This result mirrors the composition of meat.

All species of meat animals contain in their fat more than 50% unsaturated fatty acids.

The carbohydrate content in nearly all by-products is below 1%, in giblets 2% are reported. Liver of all species contains, however, 3.5%–15% carbohydrates. This is mainly glycogen. The variation between the species is reported in all data books. It seems, without calling this a dogma, that younger animals have higher glycogen stores in the liver. As reported above, muscular tissue metabolizes the glycogen postmortem to lactic acid leading to a pH fall. In liver, the glycogen is not metabolized and the pH value remains around 7. The high pH value limits the shelf life of all by-products in comparison to meat. The chilling requirements of animal by-products is, therefore, lower (3°C and lower) than with meat at 7°C or lower [21].

Calculating the energy contents of the by-products all of those that are low in fat and carbohydrates provide 100 and less kcal/100 g raw material. The energy content of liver due to its carbohydrate content adds up to 130–190 kcal/100 g, heart provides 100–130 kcal/100 g, the fatter tongue 170–185 kcal/100 g. The fatty tissues of the by-products start with 280 kcal/100 g in fresh calf fat. The rendered fats come close to 900 kcal/100. Stomach, chitterlings, pancreas, cow udder, ear, fat, and skin are around or above 200 kcal/100 g. Hence, many of the animal by-products contain more nutritional energy in the range or just above the average meat cut of all species, which amounts to 10% fat and 170 kcal/100 g.

The cholesterol content of lean and fatter meat, as shown in Figure 7.1, is determined between 50 and 70 mg/100 g. All by-products contain higher cholesterol concentrations. The lowest cholesterol contents of the by-products have the rendered fats, the tissues of tongue, ear, feet, and skin. All other tissue are well above 150 mg cholesterol/100 g. Liver, kidney, and spleen are in the range of 250–550 mg cholesterol/100 g. Brain is the organ with cholesterol contents >1000 mg/100 g (Table 7.7). As has been shown in Figure 7.1 with meat, the cholesterol content does not depend strongly on the fat content. Cholesterol is a constituent of cellular membranes of animals necessary for their functionality. It is more dependent on size of cells (surface area) than on fat content.

7.6 MINERAL CONTENT OF BY-PRODUCTS

As already indicated above, cellular structures contain more potassium than sodium. In the Table 7.8, blood is the only by-product that contains more extra- than intracellular fluid. Thus, its sodium content is higher than the potassium concentration. Rendered fat contain only traces or small amounts of minerals as they consist of fat with >94% (Table 7.8).

TABLE 7.7
Major Constituents Including Fatty Acid Groups and Cholesterol in By-Products per 100 g Raw Material

By-Product	Species[a]	Energy (kcal)	Protein (g)	Fat (g)	SFA[b] (g)	MUFA[c] (g)	PUFA[d] (g)	Cholesterol (mg)	Carbohydrates (g)
Blood	b	70	16.5	0.4	0.1	0.1	0.1	90	0.1
	s	70	17	0.4	0.1	0.1	0.1	40	0.1
Liver	b	130	21	3	1.1	1.2	0.5	300	5
	yb	130	19	1.5	0.4	0.6	0.3	300	8.5
	s	140	22	4.5	1.6	1.7	0.5	300	3
	p	190	22	5	1.7	2.2	0.5	550	15
	l	150	21	5	1.6	2.3	0.7	375	5
	Duck	145	18.5	4.5	1.6	1.2	1.1	510	3.5
Heart	b	115	17	5	2	2.1	0.5	150	0.5
	yb	110	17	4	1.6	1.7	0.3	120	<0.5
	s	115	17	5	1.7	2.3	0.5	150	0.5
	p	135	19	6.5	2	2.8	0.7	175	0.5
	l	120	17	5.5	1.9	2.3	0.7	190	<0.5
Tongue	b	185	16.5	13	5	6	0.5	100	0.5
	yb	170	17	11.5	4	5.5	0.5	100	1
	s	180	16	13	6	7.5	1.8	100	0.5
Kidney	b	100	16	4	1.4	1.5	0.5	350	1
	yb	105	16.5	4	1.5	1.4	0.5	375	1
	s	90	16	3	1	1.2	0.5	375	1
	l	95	17	3	1.2	1.1	0.4	—	1
Spleen	b	110	18	3.5	1.3	1.2	0.5	260	1
	yb	100	18.5	2	—	—	—	340	0.5
	s	105	18	2.5	1	0.7	0.2	360	0
	l	95	17	3	—	—	—	250	
Lung	b	90	17	2.4	1	0.65	0.35	290	1
	s	85	15	2.5	1	0.6	0.35	320	—
Tripe	b	100	15	4	2	1.3	0.1	160	<0.5
	l	125	10	9.5	3.5	3.8	1	260	<0.5

(*continued*)

TABLE 7.7 (continued)
Major Constituents Including Fatty Acid Groups and Cholesterol in By-Products per 100 g Raw Material

By-Product	Species[a]	Energy (kcal)	Protein (g)	Fat (g)	SFA[b] (g)	MUFA[c] (g)	PUFA[d] (g)	Cholesterol (mg)	Carbohydrates (g)
Chitterlings	s	250	10	23.5	8	9	4	160	<0.5
Stomach	s	150	16.5	9.5	—	—	—	190	<0.5
Thymus	yb	105	19	3	1	1.54	0.2	280	tr
	l	125	18	6	2.3	2.5	0.4	300	tr
Pancreas	b	225	16	18	6.7	8	1.4	200	<0.5
	s	190	18.5	13	—	—	—	195	0.5
Lard	s	890	0	99	38	45	10	95	0
Tallow	b	870	4	95	42	42	3	110	0
Suet	b	860	2	94	42	39	3	80	2
Calf fat	yb	280	18.5	23	8.5	10	1.5	75	0
Cow udder	b	230	15.5	18.5	—	—	—	—	1
Brain	yb	120	10.5	8.5	3	2.5	1	1350	<1
	l	120	10.5	8	—	—	—	1600	<1
	s	125	10.5	9	3	2.5	1.5	2200	
Ear	s	225	22.5	15	—	—	—	82	<0.5
Feet	s	260	22	19	6.5	9	2	105	<0.5
Giblets gizzard	p	120	18	4.5	1.5	1.5	1	260	2
	p	110	18	4	1.2	1.5	1.2	130	0.5
Skin	s	350	13.5	32.5	9	13.5	6	110	<1

[a] b, bovine; s, porcine; p, chicken; l, lamb/sheep; yb, calf.
[b] SFA, saturated fatty acids.
[c] MUFA, monounsaturated fatty acids.
[d] PUFA, polyunsaturated fatty acids.

TABLE 7.8
Minerals in By-Products per 100 g Raw Material

By-Product	Species[a]	Na (mg)	K (mg)	Ca (mg)	Mg (mg)	P[b] (mg)	Fe (mg)	Zn (mg)	Se (µg)	Cu (mg)	I (µg)	Mn (µg)
Blood	b	330	43	7	3	50	50	0.5	15	0.1	—	8
	s	210	170	7	9	75	40	0.3	8	0.15	—	7
Liver	b	120	300	7	35	350	9	4	15	3.5	14	400
	yb	85	300	7.5	20	350	8.5	3	15	2.5	8	300
	s	80	300	8	30	300	20	7.5	50	1.5	10	400
	p	65	255	8	20	320	8	3	46	0.5	2.5	250
	l	85	300	7.5	20	250	8.5	4.5	55	7.5	—	200
	Duck	100	250	12	20	270	3	2	tr	6	2.5	—
Heart	b	90	250	5	17	210	5.5	1.5	15	0.3	30	50
	yb	105	260	5	22	200	4.5	1.5	15	0.3	30	50
	s	70	200	4.5	20	170	4	2	22	0.45	1	50
	p	65	175	12	17	175	6	6.5	—	0.35	—	100
	l	140	280	5	20	210	3.5	2	2	0.5	—	20
Tongue	b	75	220	7	18	175	2.5	3	2	0.1	2.5	50
	yb	95	240	9	17	185	2	3.5	2	0.15	2.5	50
	s	115	255	11	18	190	4.5	2.6	12	0.25	1.5	500
Kidney	b	180	230	10	20	330	9.5	2	115	0.4	4	100
	yb	190	225	10	15	275	4.5	2	200	0.4	4	100
	s	130	250	8	20	250	5	2.5	190	0.8	4	150
	l	150	270	9	15							
Spleen	b	80	320	6	20	360	44	4	30	2.5	—	100
	yb	95	360	6	17	340	9	2.5	—	—	—	100
	s	85	320	6	20	370	21	7	35	2.7	—	150
	l	85	360	—	—	—	42	3	—	0.1	—	250
Lung	b	200	340	12	15	195	6.5	1.5	30	0.25	—	20
	s	150	150	7	15	200	20	2	20	—	—	—
Tripe	b	45	270	75	8	80	0.5	1.5	2.5	0.1	—	—
	l	105	200	10	12	285	1.5	—	—	—	—	—

(*continued*)

TABLE 7.8 (continued)
Minerals in By-Products per 100 g Raw Material

By-Product	Species[a]	Na (mg)	K (mg)	Ca (mg)	Mg (mg)	P[b] (mg)	Fe (mg)	Zn (mg)	Se (µg)	Cu (mg)	I (µg)	Mn (µg)
Chitterlings	s	35	115	20	5	30	2	2	—	0.15	—	—
Stomach	s	52	200	10	—	155	2	2	—	0.35	—	—
Thymus	yb	75	290	10	18	400	2	2	2.5	0.2	4	150
	l	75	420	8	20	400	1.5	—	—	—	—	—
Pancreas	b	65	250	10	15	335	1.5	—	—	0.05	—	—
	s	45	200	10	15	235	2	2.5	—	0.1	—	150
Lard	s	2	1	1	1	3	0.05	0.1	7	0.02	2	—
Tallow	b	Nil	Nil	Nil	Nil	Nil	Nil	Nil	—	—	—	—
Suet	b	tr	16	tr	tr	tr	tr	tr	tr	tr	5	—
Calf fat	yb	65	280	10	20	170	1	3.5	3	0.05	1.5	—
Cow udder	b	—	—	70	—	160	2.5	—	—	—	—	—
Brain	yb	125	315	10	15	275	2	1	—	0.2	—	40
	l	110	300	10	12	270	2	1.5	—	0.25	—	50
	s	120	260	10	15	280	1.5	1.5	1.5	0.25	—	10
Ears	s	55	190	20	7	40	2.5	—	—	—	—	—
Feet	s	60	275	60	7	55	—	—	—	—	—	—
Giblets	p	75	230	10	20	200	6	3.5	—	0.25	—	150
Gizzard	p	75	235	10	15	135	3.5	1.0	—	0.1	—	100
Skin	s	65	105	11	15	100	1	1	—	0.05	—	20

[a] b, bovine; s, porcine; p, chicken; l, lamb/sheep; yb, calf.
[b] P means HPO_4^{2-}.

In cellular by-products the potassium content is about 2.5–4 times higher than the sodium content with the exception of kidneys in which the sodium content is rather high and varies between 60% and 90% of the potassium value. This is valid for all species. In muscular tissue and meat, potassium is about 5–6 times higher than the sodium content (Table 7.5). All by-products with the exception of tripe, chitterlings, cow udder, and feet of swine contain calcium concentration of 10 mg/100 g or lower equivalent to meat. In swine feet, some calcium of bones may have been brought into the edible part on dissection. With the exception of blood, magnesium is by a factor of 2–3 higher than the calcium concentration. Meat also has a 3–4 times higher magnesium concentrations in relation to calcium.

Phosphate is in nearly all cellular by-products in the concentration range of potassium, exceptions are chitterlings, ears, and feet. Phosphates are one of the main anions in the cells besides chloride. Due to its high molecular weight of 96 Da (HPO_4^{2-}) in comparison to potassium (molecular weight 39 Da), a molar ratio of about 2.5 potassium exists in the cells on equal amounts in grams. The phosphate anion exists at pH 7 near the equilibrium of $H_2PO_4^-$ and HPO_4^{2-}. The ionic strength of phosphate, therefore, is about twice that of the potassium ion. Thus, phosphate will cover about 70%–80% of necessary anionic strength to match the cation load. The same applies for meat cuts.

In the area of the microelements the iron concentration of by-products is usually higher than that of zinc. In meat, zinc concentrations are higher than the iron content. In blood, liver, kidney, spleen, and lung, the iron content exceeds the zinc content considerably. Selenium is high in kidney in comparison to all other by-products and higher than in meat. Copper accumulates in liver, heart, and kidney, all other by-products are low <0.5 g/100 g. Iodine is reported high for liver and heart, in many tissues no values are reported. It can be assumed that the concentrations in these products are low. Meat contains only a portion of the iodine concentration of liver and heart. Manganese is in the range of 50–400 µg/100 mL and is highest in liver. In most by-products manganese is higher than in meat.

By-products mirror meat in their overall concentration. However, differences in the concentrations of the single minerals exist.

7.7 VITAMIN CONTENTS OF BY-PRODUCTS

In Table 7.9, it becomes obvious at the first sight that liver contains the highest concentrations of many vitamins in the by-products. Vitamin A, folate, pantothenate, vitamin C, D, E, H, and K are highest in liver of the various species. Vitamin A occurs besides liver only in kidney giblets and skin. The concentrations of vitamin B1, B2, niacin, and B6 are rather uniform in liver, heart, tongue, kidney, and spleen, and their concentrations are similar to the ones in the bovine topside meat cut shown in Table 7.5. Folate, which is present in livers with high concentrations, is also found in heart, tongue, kidney, and giblets but in lower concentrations. The concentrations are still higher than in meat. Pantothenate is available in rather high concentrations in liver, heart, tongue, and kidney. The content in spleen is lower and similar to meat. Vitamin B12 is very high in many by-products. Bovine topside meat contains only one-tenth to one-fiftieth of the vitamin B12 of liver.

Also vitamin C is available in a number of by-products. Liver and spleen provide concentrations of vitamin C equivalent or higher than apples near the range of citrus fruits [4]. Also in kidney, the vitamin C concentration is high. In muscular tissue and meat, the vitamin C concentration is negligible and <1 mg/100 g. Vitamin D occurs only in liver and kidney in mentioning amounts. Meat contains only traces. The vitamin E concentrations of most by-products are similar to meat and rather low with regard to RDA values. Vitamin H (Biotin) concentrations are high in liver and kidney up to 10 times the concentrations in meat. The amounts in tongue and heart match the meat concentrations. Vitamin K is rarely reported with by-products. Only the values of liver are mentioned in some data books. Liver contains two- to fivefold the concentrations of bovine topside cuts.

TABLE 7.9
Vitamins in By-Products per 100 g Raw Material

By-Product	Species[a]	A (RE µg)	B1 (mg)	B2 (mg)	Niacin (mg)	B6 (mg)	Folate (µg)	Pantothenate (mg)	B12 (µg)	C (mg)	D (µg)	E (mg)	H (µg)	K (µg)
Blood	b	30	0.1	0.1	3.5	0.01	7	—	0.6	Nil	0.1	0.4	—	—
	s	25	0.1	<0.1	3.5	0.01	5	—	0.6	Nil	0.1	0.4	—	—
Liver	b	21,000	0.3	3.5	20	1	2,600	7.5	100	30	1.7	0.7	10	75
	yb	31,500	0.55	3	10	1	1,950	8	60	30	0.2	1	—	27
	s	20,000	0.3	3	21	0.7	1,000	7	40	25	5	0.7	50	30
	p	18,000	0.35	2.5	14	0.8	590	7.5	25	30	0.2	0.3	44	80
	l	50,000	0.35	3	14	0.4	220	8	85	35	0.6	0.4	25	—
	Duck	12,000	0.35	2.5	13.5	0.75	700	6	55	6	0.5	5	40	—
Heart	b	6	0.45	0.9	35	0.3	20	2.5	10	2	1	0.2	3.5	tr
	yb	10	0.4	0.65	35	0.3	20	3	12	5	1	0.8	3.5	tr
	s	5	0.45	1	10	0.45	9	2.5	2.5	3	0.7	0.2	—	tr
	p	30	0.3	0.75	9.5	0.25	75	2.5	7.5	3	—	—	—	tr
Tongue	b	Nil	0.1	0.4	6.5	0.15	20	2	5	3	tr	0.1	2	tr
	yb	Nil	0.1	0.3	8.5	0.1	20	2	5	2.5	tr	0.1	3.5	tr
	s	9	0.3	0.4	8	0.35	8	2	3.5	3.5	0.6	0.5	2	tr
Kidney	b	800	0.4	2	9.5	0.45	80	3.5	30	15	1	0.2	25	tr
	yb	300	0.4	2	9.5	0.5	30	4	30	15	1	0.2	25	tr
	s	150	0.35	1.7	13.5	0.6	42	3	10	12	1	0.2	30	tr
	Goose	310	0.6	2.2	7.5	0.2	30	4	52	10	—	—	—	—
Spleen	b	tr	0.15	0.3	8	0.12	—	1.2	5.5	45	—	—	—	—
	yb	tr	—	—	—	0.1	—	—	—	40	—	—	—	—
	s	tr	0.15	0.3	6	0.05	—	1	3.5	30	—	—	—	—
	p	tr	—	—	—	0.1	—	1	2.5	13	—	—	—	—

Lung	b	45	0.1	0.4	4	0.05	10	1.5	4	40	—	—	—	—
	s	tr	0.1	0.4	3.5	0.1	—	1	2.5	13	—	—	—	—
Tripe	b	tr	<0.1	0.15	0.1	tr	2	tr	1.5	3	tr	0.1	tr	—
	l	—	—	—	—	—	—	—	—	—	—	—	—	—
Chitterlings	s	tr	0.01	0.05	0.1	0.01	—	—	0.8	4.5	—	—	—	—
Stomach	s	—	0.1	0.1	4.5	0.05	—	—	1	—	—	—	—	—
Thymus	yb	tr	0.05	0.25	3.5	0.05	13	1	6	20	4	0.45	3	—
	l	tr	0.15	0.25	—	0.05	13	1	6	30	—	—	—	—
Pancreas	b	17	0.1	0.4	4.2	—	—	3.5	14	20	—	—	—	—
Lard	s	1.5	tr	tr	tr	0.02	—	tr	tr	tr	tr	0.4	—	—
Tallow	b	—	—	—	1.8	—	—	—	tr	tr	—	1	—	—
Suet	b	55	tr	tr	tr	tr	tr	tr	tr	tr	tr	1.5	—	—
Calf fat	yb	—	0.05	0.15	10	0.5	3	1	1.5	1	0.2	0.15	—	—
Cow udder	b	—	0.1	0.18	1.3	—	—	—	—	—	—	—	—	—
Brain	yb	—	0.15	0.25	4.5	0.3	3	2.5	12	15	—	—	—	—
	l	—	0.15	0.3	4	1	3	1	11.5	15	—	—	—	—
	s	—	0.15	0.25	4.5	0.2	—	3	2	15	—	—	—	—
Ear	s	—	0.1	0.1	0.8	—	—	—	—	—	—	—	—	—
Feet	s	—	0.05	0.1	1	—	—	—	12	—	—	—	—	—
Giblets	p	8900	0.05	1	6.5	0.4	345	3.2	11.5	16	—	—	—	—
Gizzard	p	215	0.1	0.2	4.5	0.15	50	0.75	2	3	—	—	—	—
Skin	s	265	0.5	0.05	4	0.1	3	0.5	0.6	—	0.1	0.1	—	—

[a] b, bovine; s, porcine; p, chicken; l, lamb/sheep; yb, calf; tr, traces.

7.8 CONTENTS OF BY-PRODUCT AND RDA VALUES

Most by-products except fatty tissue, rendered fat, pancreas, and tongue are rather lean with less than 200 kcal/100 g and by international accepted standards nutrient dense foods (Table 7.7). If we assume a portion of 100 g (USDA uses 3 ounces equivalent to 85 g for a portion), then the energy intake is less than 10% of the RDA value with 2000 kcal for a woman of 25–50 years of age and a normal physical activity (Table 7.6). Carbohydrates of accountable amounts are only present in liver. It means that many animal by-products are low energy foods with a high nutrient density. Due to their low carbohydrate content, they are very valid foods for people with diabetes.

Like in meat, all animal by-products exceed 50% of unsaturated fatty acids in their fat.

Cholesterol in animal by-products, however, is always present in higher concentrations than in skeletal muscle meat with or without adjacent fat with 50–70 mg/100 g, as shown in Figure 7.1. All by-products exceed 100 mg/100 g. Brains even exceed 1000 mg/100 g. The recommended amount is not more than 300 mg/day.

With minerals (Table 7.8), the native sodium content is low in many cases (<100 mg/100 g). A minimum of 500–600 mg/day is required and an intake above 2400 mg/day should be avoided. Salt added on preparation is just used for flavoring purposes and it could be reduced or even renounced. Thus, we can state that unprocessed animal by-products are low in "salt." Potassium and magnesium provide with 100 g raw material per day about 10%–15% of RDA. Calcium in by-products is as low as in meat with less than 1% RDA. Phosphate, on the other hand, provides 30%–40% of RDA with 100 g. Many by-products contain high concentrations of iron. The values shown in Table 7.8 exceed in blood, liver, and spleen by 100% of the RDA values. But one must keep in mind that all cations with 2 or 3 positive loads are only absorbed to a certain extent, which is higher for animal tissues than for plant tissue. It is discussed that from animal food about 20% of iron, zinc, magnesium, and other di- or tri-valent cations are absorbed in the human digestive tract [19]. Zinc is also present in significant amounts (20%–40%) and so are selenium (15%–200% of RDA, the latter in kidney) and copper (15%–70% of RDA). Iodine and manganese are low in by-products.

With vitamins liver and kidney provide more than 100% RDA of vitamin A. Vitamins B1 and B2 are present in significant amounts/100 g (30%–300% of RDA). Niacin is present in many by-products with 50%–150% of RDA values. Vitamin B6 often covers 15%–50% RDA. Folate is very high in liver up to seven times of the RDA value, in all other by-products the concentration is low in relation to RDA. Pantothenate again is present in a number of by-products by 15%–200% of RDA; the highest again is in the liver. With vitamin B12 content, most by-products well exceed 100% of RDA values by an intake of 100 g. Even Vitamin C covers in liver and spleen about 30%–40% and in kidney with 15%–30% of RDA values. Vitamins D and E concentrations are low in all by-products, except liver of porcine with vitamin D and duck liver with vitamin E. Vitamin H is again present in significant amounts in liver (20%–120% RDA) and in kidney (60%–70% RDA) with an intake of 100 g. Vitamin K is only high in liver with 35%–110% RDA.

7.9 CONCLUSION

Animal by-products are nutrient dense foods; many are low in fat content. They provide significant amount of minerals and vitamins. On the other hand the cholesterol content is higher than in meat. The contaminants and residues are addressed in other chapters of this book. They will show that the unwanted substances were a problem of the 1970s and 1980s. They have been reduced in the last decades to low concentrations [22–24].

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8 Essential Amino Acids

María-Concepción Aristoy and Fidel Toldrá

CONTENTS

8.1 INTRODUCTION

A large proportion of the total animal live weight is made up of by-products that are biologically edible. Thus, organs, fatty tissues, bones, and blood, represent 39% of the live weight of cattle, 30% of the live weight of pigs, and 35% of the live weight of lambs.[1] Edible meat by-products are recognized as a source of high-quality nutrients like essential amino acids and among them organ meat proteins have higher biological value than other edible by-products (lard, blood, etc.) being rich in protein content (by 20%) with a balanced essential amino acids profile similar to that of muscle proteins. As an example, taking the essential amino acid contents of chicken muscle proteins as 100, corresponding values were 105 for liver, 100 for heart, and 92 for gizzard.[2] Owing to their large content of these amino acids organ proteins are almost 100% digestible. Exceptions are those organs rich in connective tissue, mainly collagen, that have their composition especially rich in hydroxyproline, proline, hydroxylysine, and glycine, being low in tyrosine and devoid of tryptophane.[3] This is the case with tripe, stomach, lungs, chitterlings and other by-products like comb, tongue, ears, snouts or feet. It is clear that it is economically advantageous to make profit of these products, and, for this purpose, the nutritive quality must be demonstrated by adequate analytical techniques. Furthermore, the importance of essential amino acids in nutrition and health makes their analysis of high relevance.

The total amino acid content of fresh liver is about 19%, essential amino acids being 42% of them.[4] Essential amino acids in meat by-products are a very important group of nutrients, which are affected only to a very little extent by processing and home cooking because the low-carbohydrate content of these products does not cause secondary degradation reactions like maillard or racemization and cross-linking reactions, which diminish the amino acids availability. Figure 8.1 shows the chromatograms of fresh and roasted pork liver free amino acids in which a good recovery of essential amino acids (written in bold), after cooking can be observed. Only a considerable decrease in available cysteine was described during sterilization of canned beef liver[5] and after alkali treatment of blood plasma.[6]

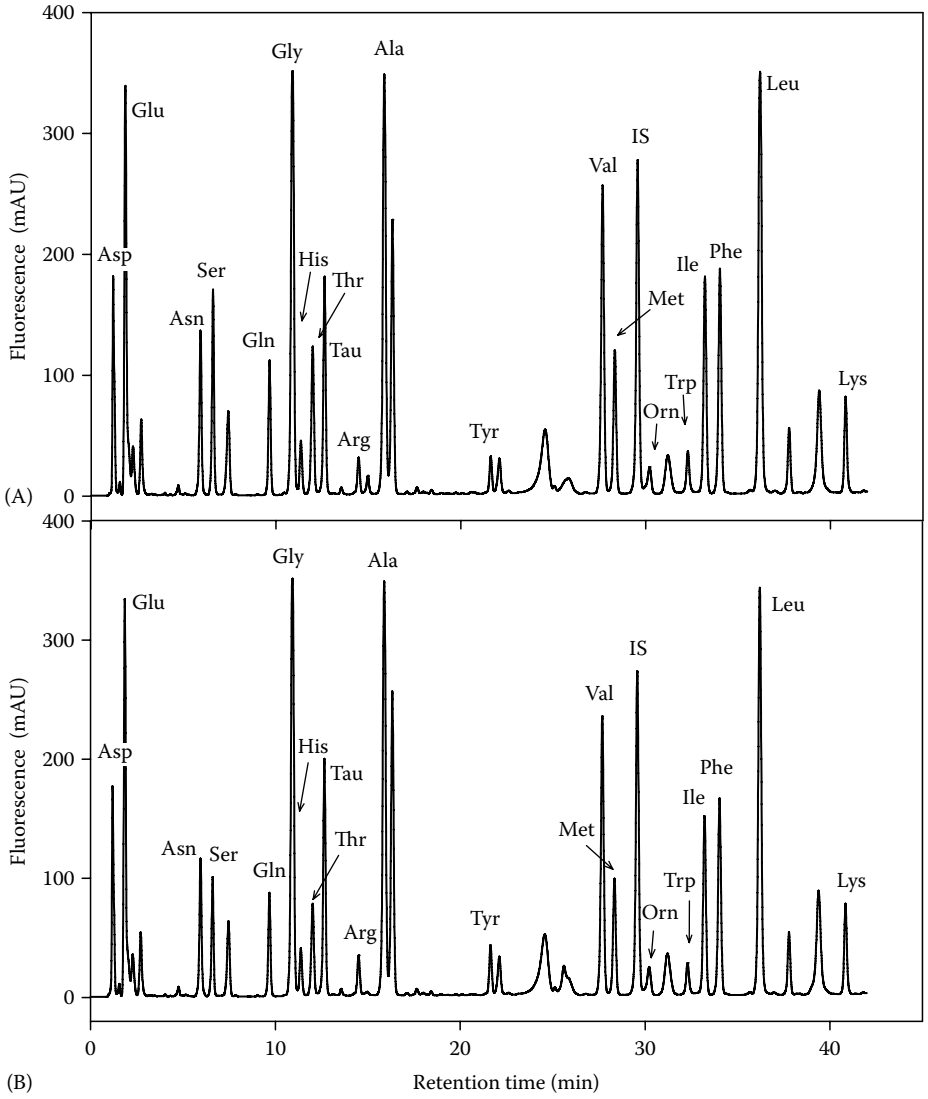

FIGURE 8.1 Free amino acids from fresh (A) and roasted (B) pork liver, after derivatization with OPA. IS, internal standard nor-valine; Tau, taurine; Orn, ornithine; OPA, *o*-phthaldialdehyde.

Valine, leucine, isoleucine, phenylalanine, tryptophan, threonine, methionine, lysine, and histidine are essential amino acids because they cannot be synthesized de novo by the organism and therefore, must be supplied in the diet. However, other amino acids may be considered "semiessential" or "conditionally essential amino acids" depending on the developmental stage and health status of the individual. This is the case of arginine or glutamine whose intake under exceptional severe stress conditions (intense exercise, infectious disease, surgery, burn injury) may turn out to be decisive, and tyrosine, which is formed from phenylalanine, may be essential in the diet of those patients suffering from phenylketonuria. Cysteine is not classified as an essential amino acid because it can be usually synthesized by the human body under normal physiological conditions if sufficient amounts of other sulfur-amino acids (i.e., methionine and homocysteine) are available. So, sulfur-containing amino acids are sometimes considered for convenience as a single pool of nutritionally equivalent amino acids.

TABLE 8.1
Essential Amino Acid Composition of Pork Meat By-Products and Lean Tissue (mg/g Protein)[a]

	Liver	Kidney	Tongue	Feet	Brain	Lean Tissue
Valine	61.8	57.6	52.0	23.0	57.0	53.6
Leucine	89.1	89.8	80.2	42.1	87.2	81.3
Isoleucine	50.7	53.4	45.6	16.0	46.2	48.3
Phenylalanine	49.0	47.2	41.4	26.9	50.9	40.0
Tyrosine	34.1	36.0	—	15.8	41.9	35.7
Tryptophan	14.1	13.0	11.5	1.9	12.8	13.4
Threonine	42.6	41.4	42.2	26.1	46.7	47.0
Methionine	24.8	21.4	22.4	10.1	19.8	24.6
Lysine	77.1	72.0	81.8	43.0	78.6	98.6
Histidine	27.2	24.0	25.1	12.0	26.9	50.7
Cystine	18.9	21.9	—	—	—	13.0
Total	489.3	477.8	402.2	217.0	468.0	506.1

Source: Data from *Edible Meat By-Products. Advances in Meat Research,* Vol. 5, Anderson, B.A., Composition and nutritional value of edible meat by-products, 15–45, copyright (1988), with permission from Elsevier.

[a] Data were extracted and calculated from Ref. [1] by considering g protein = gN × 6.25.

The content of essential amino acids from edible animal by-products is scarcely described in the literature. Dvorak and Vognarova[7] published the values for the contents of essential amino acids from calf, beef and pig liver, heart, tongue, spleen, kidneys, lungs and brain, and for cow udder, tripe, calf sweetbreads, and pork crackling. Savran and Pavlova[2] analyzed these contents in heart, liver, and gizzard of various poultry species (hens, chickens, geese, and ducks). Zarkadas and Maloney[8] assessed the nutritional quality of chicken gizzard. Anderson[1] reviewed in a table the amino acids content (mg/gN) of edible meat by-products from a great variety of mammalian species, together with that of the same species lean tissue. These data were collected from USDA reports. Data from some edible pork tissues extracted from this review were converted into mg of essentials amino acids per gram of protein and presented in Table 8.1. The nutritive value, with respect to the essential amino acids content, of these products in relation to the lean tissue is evident.

Essential amino acids in animal by-products can be analyzed following the typical procedures described for amino acids in general.[9,10] Nevertheless, some extra careful attention must be paid to some of them, especially when hydrolysis is required.[11,12] Furthermore, cysteine requires specific methodologies for its analysis. All these methods are briefly described in this chapter.

8.2 SAMPLE PREPARATION

Edible animal by-products range over a miscellaneous number of products including organs (normally constituted by smooth proteins), mixed tissues (muscular and adipose tissues or colagen), fluids (blood), etc. Therefore, each kind of sample will require a different initial manipulation for its analysis.

In general, solid samples must be freed from visible fat and ground with a grinder or mortar, and a representative portion weighed to be analyzed. If total amino acids are required, sample will be submitted to hydrolysis to get their protein amino acids free and added up to the total pool together with the free amino acids content. If only free amino acids is the matter, a sample

extract will be deproteinized by any of the methods described in the literature. Some methodologies for these analyses are summarized below.

8.2.1 Sample Hydrolysis for Total Amino Acid Analysis

The hydrolysis is the Achilles heel of some essential amino acid analysis, which is especially sensible to the most used hydrolysis methods, which are acid hydrolysis. Indeed, the acid digestion with constant boiling 6 N hydrochloric acid in an oven at around 110°C for 20–96 h is the preferred method to hydrolyze food proteins for analytic purposes. This hydrolysis must be carried out in sealed vials under nitrogen atmosphere and presence of antioxidants/scavengers in order to minimize the degradation suffered by some especially labile amino acids (tyrosine, threonine, serine, methionine, and tryptophan) in such acidic and oxidative medium. Phenol (up to 1%) or sodium sulfite (0.1%) is a typical protective agent and is effective for nearly all amino acids except for tryptophan and cysteine. The hydrolysis may be carried out in liquid or vapor-phase depending if the hydrochloric acid is in contact with the sample or only the vapor reaches the sample when heating. Hydrolysis may be improved by optimizing the temperature and time of incubation.[13] After the hydrolysis time, the excess of hydrochloric acid is evaporated by vacuum.

Alternative reagents for acid hydrolysis are 4 M methanesulfonic acid (115°C for 22–72 h or 160°C for 45 min, under vacuum) and 3 M mercaptoethanesulfonic acid (160°C–170°C for 15–30 min), which have been described to improve tryptophan and methionine recoveries.[14–19] These acids possess a high boiling point and thus, only the liquid-phase hydrolysis is possible and after hydrolysis, the hydrolyzed sample will have to be submitted to pH adjustment before analysis. The use of protective reagents like tryptamine[17,18,20] or thioglycolic acid[21,22] is also advisable to prevent oxidation.

The alkaline hydrolysis with 4.2 M of either NaOH, KOH, LiOH or BaOH, with or without the addition of 1% (w/v) thiodiglycol for 18 h at 110°C is recommended by some authors,[18,23–27] for a better tryptophan determination. This would be the method of choice in food samples containing high sugar concentration.

The hydrolysis of cystine and/or cysteine presents some additional problems due to the reactiveness of their sulfhydryl group. Cyst(e)ine is partially oxidized during acid hydrolysis yielding several adducts: cystine, cysteine, cystein sulfinic acid, and cysteic acid making its analysis rather difficult. Several procedures have been proposed to analyze cyst(e)ine after acid hydrolysis. The simplest method consists in submitting the sample to a performic acid oxidation, previous to the acid hydrolysis.[28–34] This process transforms the cyst(e)ine to cysteic acid, which is acid-resistant and will be analyzed together with at least nine amino acids plus methionine (transformed into methionine sulfone) and lysine with optimized recoveries, as proposed by Gehrke et al.[30] Nevertheless, tyrosine and phenylalanine will be markedly destroyed by this oxidation procedure.[30]

As can be observed in this section, no single set of conditions will yield the accurate determination of all amino acids. In fact, it is a compromise of conditions that offer the best overall estimation for the largest number of amino acids. In general, the 22–24 h acid hydrolysis at 110°C (vapor-phase hydrolysis, preferably), or 2 h at 145°C,[29] with the addition of a protective agent like phenol, yields acceptable results for the majority of essential amino acids, being enough for the requirements of any food control laboratory. However, when the analysis of tryptophan and/or cyst(e)ine is necessary, adequate special hydrolysis procedures as those described above should be performed.

8.2.2 Sample Preparation for Free Essential Amino Acids Analysis

The analysis of free amino acids requires having them in solution. Thus, they must be extracted from insoluble material and deproteinized to eliminate possible interfering proteins.

8.2.2.1 Extraction

The extraction consists in the separation of the free amino acids fraction from the insoluble portion of the sample. It is usually achieved by homogenization of the ground sample in an appropriate solvent by using a Stomacher™, Polytron™, Ultra Turrax™ or by means of a simple stirring in warm solvent. The extraction solvent can be hot water, 0.01–0.10 N hydrochloric acid solution, or diluted phosphate buffers. In some cases, concentrated strong acid solutions such as 4%–5% of 5-sulfosalycylic acid,[35–37] 2%–5% of TCA acid,[38,39] or rich alcohol-containing solution (>75%) such as ethanol[40–43] or methanol[44] have been successfully used as extraction solvents with the additional advantage that proteins are not extracted and, then, there is no need for further cleaning up of the sample.

Once homogenized, the solution is centrifuged at least at 10,000 g under refrigeration to separate the supernatant from the nonextracted materials (pellet) and filtered through glass-wool to retain any fat material remaining on the surface of the supernatant.

8.2.2.2 Deproteinization

The deproteinization process can be achieved through different chemical or physical procedures. Chemical methods include the use of concentrated strong acids like 3.5% sulfosalicylic (SSA), 0.6 N perchloric (PCA), 10%–12% trichloroacetic (TCA) acids or organic solvents like methanol, ethanol or acetonitrile. Under these conditions, proteins precipitate by denaturation while free amino acids remain in solution. Physical methods consist in the forced filtration (mainly by centrifugation) through cut-off membrane filters (1,000, 5,000, 10,000, 30,000 Da) that allow free amino acids through while retaining large compounds. All these methods give a sample solution rich in free amino acids and free of proteins.

Differences among all these chemical and physical methods are ascribed to several aspects like the differences in the recovery of amino acids, compatibility with derivatization (pH, presence of salts,…) or separation methods (interferences in the chromatogram,…), etc. It is important to consider that strong acids exert a very low pH in the medium that can interfere with the precolumn derivatization[45] where high pH is necessary to accomplish the majority of the derivatization reactions. Thus, it is essential either, to completely eliminate this acid by evaporation or extraction or adjust the pH of the sample solution. This is not a problem when the amino acids have to be analyzed by ion-exchange chromatography and postcolumn derivatization; indeed, SSA has been commonly used prior to ion-exchange amino acid analysis because it gives an appropriate pH for the chromatographic separation,[37] and a good choice for other derivatization procedures is the use of 0.6 N PCA, which is easily neutralized by the addition of KOH or potassium bicarbonate to form the insoluble potassium perchlorate with no described interferences.

The use of organic solvents, by mixing two or three volumes of organic solvent with one volume of extract, has given very good results[46–48] with amino acid recoveries around 100% for all them[48,49] with the additional advantage of simplicity and cleaning. Even it can be easily concentrated by evaporation if necessary. Some comparative studies on these deproteinization techniques have been published.[49–51]

8.3 ANALYSIS

After sample preparation, target essential amino acids are currently analyzed by chromatographic (high-performance liquid chromatography (HPLC) or gas liquid chromatography(GLC)) or capillary electrophoresis methods, which are very efficient separation techniques. The choice mainly depends on the available equipment or personal preferences, because each methodology has its advantages and drawbacks.

8.3.1 High-Performance Liquid Chromatography

HPLC is the most versatile and wide widespread technique to separate amino acids. Before or after this separation, amino acids are derivatized to allow their separation and/or to enhance their detection.

8.3.1.1 Derivatization

Derivatization is an usual practice in the amino acid analysis. Some reports comparing amino acid derivatization methods for the HPLC[9,46,52–54] analysis of biological samples have been published. Pre- or postcolumn derivatization reagents used in the analysis of free amino acids are also useful for essential amino acids, with some exceptions. Essential amino acids like histidine, lysine, tryptophan, and cysteine, present some difficulties. The most-used derivatization methods are described below:

Ninhydrin: This reagent is used in postcolumn derivatization after amino acid cation-(CE) chromatographic analysis. The reaction takes place in hot (at pH 6) and rends colored derivatives detectable at 570 (primary amino acids) and 440 nm (secondary amino acids).

4-Dimethyl-Aminoazobenzene-4′-Sulfonyl Chloride (Dabsyl-Cl, DBS-): This reagent forms stable (for weeks) derivatives with primary and secondary amino acids, which are detectable in the visible range presenting a maximum from 448 to 468 nm. The high wavelength of absorption makes the baseline chromatogram very stable with a large variety of solvents and gradient systems. Detection limits are in the low picomole range.[18] The reaction time is around 15 min at 70°C and takes place in a basic medium with an excess of reagent. The major disadvantage is that the reaction efficiency is highly matrix dependent and variable for different amino acids, being especially affected by the presence of high levels of some chloride salts.[48] To overcome this problem and obtain an accurate calibration, standard amino acids solution should be derivatized under similar conditions. By-products originating from an excess of reagent absorb at the same wavelength and appear in the chromatogram. Nevertheless, Stocchi et al.[18] obtained a good separation of 35 DBS-amino acids and by-products in a 15 cm C18 column packed with 3 µm particle size.

Phenylisothiocyanate (PITC): The methodology involves the conversion of primary and secondary amino acids to their phenylthiocarbamyl (PTC-) derivatives, which are detectable at ultraviolet (UV) (254 nm). The PTC-amino acids are moderately stable at room temperature for 1 day and much longer, especially in the dryfreezer. The methodology is well described in the literature.[55–57] Sample preparation is quite laborious; requires a basic medium (pH = 10.5) with triethylamine and includes several drying steps, being the last one necessary to eliminate the excess of reagent, which may cause some damage to the chromatographic column. Derivatization reaction is complete at 20 min at room temperature. The chromatographic separation takes around 20 min for hydrolyzed amino acids and 65 min for physiological amino acids.

The reproducibility of the method is very good, ranging from 2.6% to 5.5% for all amino acids except for histidine (6.3%) and cystine (10.0%). PTC-cystine shows a poor linearity that makes the quantitation of free cystine nonfeasible with this method.[54] Detection limits are in the high picomole range. The hydrolyzed PITC-amino acids are easily separated with any C18 column, while the selection of the column is critical to get a good resolved physiological amino acids chromatogram. Figure 8.2 shows a chromatogram of chicken comb total PTC-amino acids. The high abundance of hydroxyproline and glycine, and the presence of the two diastereomers of Hyl (Hyl 1 and 2) reveal the high content of connective tissue (collagen) in comb. Nevertheless, as appreciated in the figure, essential amino acids, including Tyr, are well-balanced in such product.

The reliability of the method has been tested on food samples[58] and compared with the traditional ion-exchange chromatography and postcolumn derivatization.[50,57,59]

1-Dimethylamino-Naphtalene-5-Sulfonyl Chloride (Dansyl-Cl): Dansyl-Cl reacts with both primary and secondary amines to give a highly fluorescent derivative (λ_{ex} 350, λ_{em} 510 nm). The dansylated amino acids are stable until 7 days at –4°C,[6] if protected from light. The sample derivatization

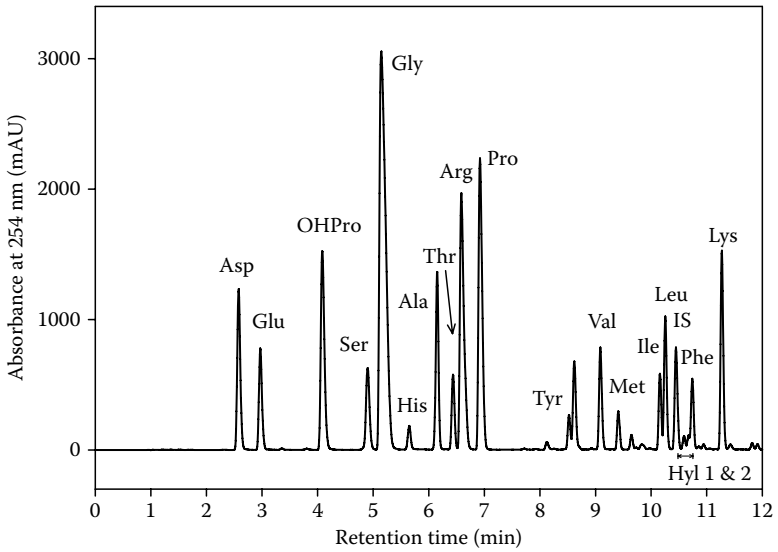

FIGURE 8.2 Chicken comb hydrolyzed amino acids after derivatization with PITC. IS, internal standard nor-leucine; Hyl 1 and 2, hydroxylysines 1 and 2.

appears as simple, only needs a basic pH, around 9.5, and a reaction time of 1 h at room temperature (in the dark), or 15 min at 60°C or even 2 min at 100°C. However, the reaction conditions (pH, temperature, and excess of reagent) must be carefully fixed to optimize the product yield and to minimize secondary reactions.[60,61] Even so, this chemical will commonly form multiple derivatives with histidine, lysine, and tyrosine. Histidine gives a very poor fluorescence response (10% of the other amino acids), reinforcing the poor reproducibility of its results.[54] Another problem is that the excess of reagent (needed to assure a quantitative reaction) is hydrolyzed to dansyl sulfonic acid, which is highly fluorescent and may interfere into the chromatogram as a huge peak. On the contrary, this methodology reveals excellent linearity for cystine and also cystine-containing short chain peptides.[54,62,63] This derivative has been also used to analyze taurine.[64]

o-Phthaldialdehyde (OPA): This reagent reacts with primary amino acids in the presence of a mercaptan cofactor to give a highly fluorescent adduct. The fluorescence is recorded at 455 or 470 nm after excitation at 230 or 330 nm, respectively and the reagent itself is not fluorescent. OPA derivatives can be detected by UV absorption (338 nm) as well. It may be used either for precolumn- or postcolumn derivatization. The last one used to be coupled with CE HPLC.[21,22] The choice of mercaptan (2-mercaptoethanol, ethanethiol or 3-mercaptopropionic acid) can affect derivative stability, chromatographic selectivity and fluorescent intensity.[21,22,37,61,65,66] The derivatization is fast (1–3 min) and is performed at room temperature in alkaline buffer, pH 9.5–10.5. OPA amino acids are not stable; this problem is overcome by standardizing the time between sample derivatization and column injection by automation. The major disadvantage when applied to essential amino acids could be that the yield with lysine and cysteine is low and variable but the addition of detergents like Brij 35 to the derivatization reagent seems to increase the fluorescence response of lysine.[67–69] Figure 8.3 shows a chromatogram of hydrolyzed chicken gizzard OPA-amino acids.

Cyst(e)ine forms very weakly fluorescent derivatives and thus the conversion of cysteine and cystine to cysteic acid by oxidation with performic acid (see Section 8.2.1) or carboxymethylation[70] of the sulfhydryl residues with iodoacetic[71] or the formation of the mixed disulfide *S*-2-carboxyethylthiocysteine from cysteine and cystine, using 3,3′-dithiodipropionic acid[72] and incorporated by Godel et al.[73] into the automatic sample preparation protocol described by Schuster[47] will be necessary to analyze them. In these methods, cysteine and cystine are quantified together.

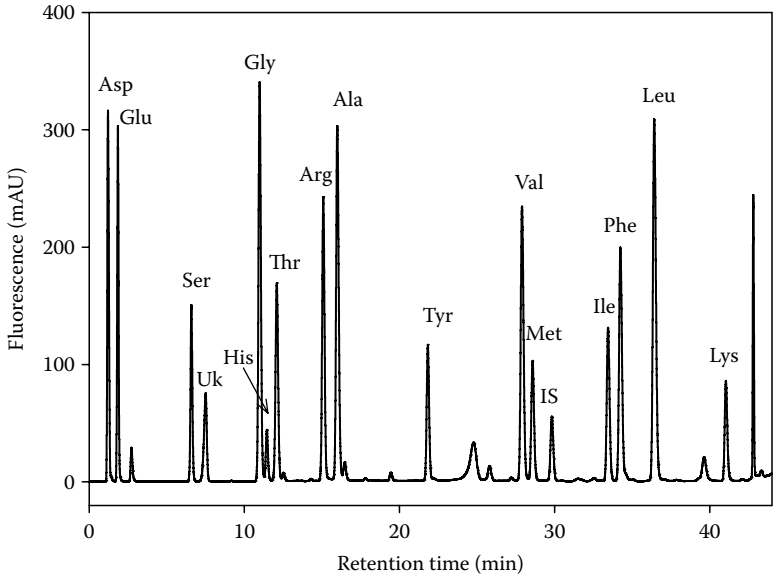

FIGURE 8.3 Chicken gizzard hydrolyzed amino acids after derivatization with OPA. IS, internal standard nor-valine; Uk, unknown.

6-Aminoquinolyl-N-Hydroxysuccinimidyl Carbamate (AQC): It reacts with primary and secondary amines from amino acids yielding very stable derivatives (1 week at room temperature) with fluorescent properties (λ_{ex} 250 nm; λ_{em} 395 nm). UV detection (254 nm) may also be used. Sensitivity is in the fmol range. The main advantage of this reagent is that the yield and reproducibility of the derivatization reaction is scarcely interfered by the presence of salts, detergents, lipids, and other compounds naturally occurring in meat products. Furthermore, the optimum pH for the reaction is in a broad range, from 8.2 to 10.0, that facilitates sample preparation. The excess of reagent is consumed during the reaction to form aminoquinoline (AMQ), which is only weakly fluorescent at the amino acid derivatives detection conditions and does not interfere in the chromatogram. Reaction time is short, 1 min, but 10 min at 55°C would be necessary if tyrosine monoderivative is required, because both mono and di-derivatives are the initially adducts from tyrosine. Fluorescence of tryptophan derivative is very poor and UV detection at 254 nm may be used to analyze it. In this case, the AMQ appears as a big peak at the beginning of the chromatogram, and may interfere with the first eluting peak (see Ref. [74]). The chromatographic separation of these derivatives has been optimized for the amino acids from hydrolyzed proteins but, the resolution of physiological amino acids is still incomplete, and needs to be improved,[75] which is the main drawback of this method.

Cysteic acid and methionine sulfone which are the adducts after performic acid oxidation of cystine/cysteine and methionine, respectively, are well separated inside the chromatogram[74] as shown in Figure 8.4A. Two chromatograms of hydrolyzed pork liver AQC-amino acids without (Figure 8.4A) and with (Figure 8.4B) previous performic acid oxidation can be compared. This oxidative treatment also affects tyrosine.

Some special derivatives are also proposed to determine cyst(e)ine:7-halogenated-4-nitrobenzo-2-oxa-1,3-diazoles can be used in the quantitative estimation of thiols and amines. For instance, 7-chloro-4-nitrobenzo-2-oxa-1,3-diazole (NBD-Cl) has been used for the analysis of cysteine and cystine in foods by Akinyele et al.[28] This reagent reacts with cyst(e)ine in acidic medium (0.2 M sodium acetate/HCl buffer, pH 2.0) giving a greenish product showing a maximum of absorbance at 410 nm. This method is highly specific for cyst(e)ine, and does not need a posterior chromatographic separation.

5,5′-Dithio-bis-nitrobenzoic acid (DTNB) is used for the precolumn derivatization of sulfydryl and disulfide amino acids.[76]

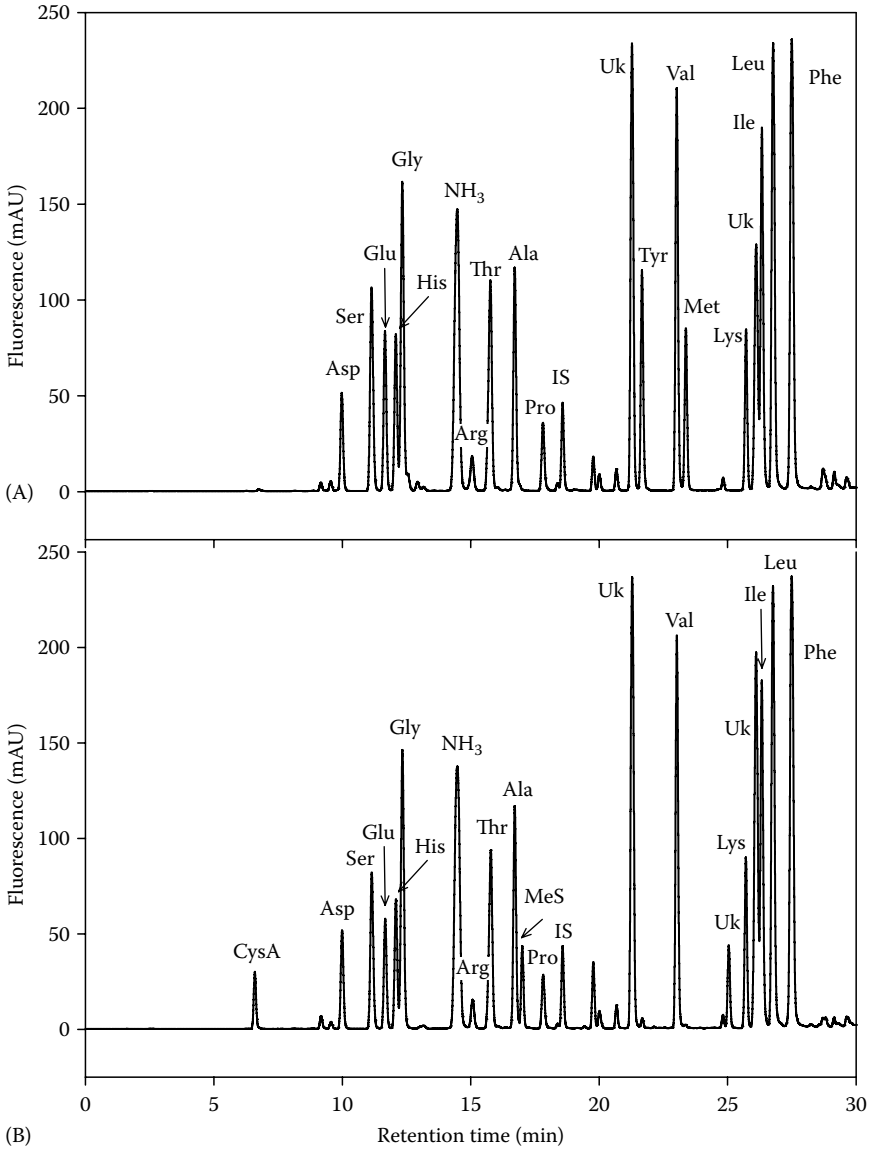

FIGURE 8.4 Pork liver hydrolyzed AQC-amino acids without (A) and with (B) after performic acid oxidation. IS, internal standard α-amino butyric acid; CysA, cysteic acid; MeS, methionine sulfone; Uk, unknown; AQC: 6-aminoquinolyl-*N*-hydrosysuccinimidyl carbamate.

Fluorescamine, which rends fluorescent derivatives with primary amino acids has been used in precolumn derivatization of taurine from plasma. The analysis was performed by using an RP-column and the eluent was monitored at 480 nm (emission) after excitation at 400 nm.[77]

8.3.1.2 Separation and Detection

The HPLC separation techniques most used for the analysis of essential amino acids are CE-HPLC and reversed-phase (RP-HPLC). CE-HPLC is used for the separation of nonderivatized amino acids which are then postcolumn (ninhydrin or OPA) derivatized, while RP-HPLC is mainly used to separate precolumn derivatized amino acids (see the above described reagents). Many peaks appear in an amino acid analysis chromatogram and thus, the choice of the RP column is very important, especially in the case of free or physiological amino acids. In the case of hydrolyzates, the sample is

simpler and the use of shorter columns is advisable to reduce the time of analysis. RP has also been used to separate some underivatized amino acids like Met, which is further detected at 214 nm[78] or the aromatic amino acids Tyr, Phe, and Trp that can be detected at 214 nm but also at 260 or 280 nm. Indeed, Phe presents a maximum of absorption at 260.0 nm; Tyr at 274.6 and Trp at 280.0 nm.[11] For the rest of amino acids, the detector used depends on the chosen derivative, and it is worthwhile to take into account the previous section about derivatization (Section 8.3.1.1), because certain derivatives from some specific amino acids have a poor response. Also, it may be taken into account that fluorescence is much more sensible than UV detection.

There are several different techniques for the analysis of any amino acid. A good example is tryptophan, which was analyzed by CE chromatography with postcolumn derivatization with OPA and fluorescence detection[79] or byRP-HPLC without derivatization and UV or fluorescence detection[80] or even byRP-HPLC previous derivatization.

8.3.2 Gas–Liquid Chromatography

GLC technique is in general not recommended for some of the essential amino acids like cysteine, tryptophane or methionine. Nevertheless, a method of analysis for tryptophan in proteins based upon the GLC separation of skatole produced by pyrolysis of tryptophan at 850°C was developed by Danielson and Rogers.[81] Sample pretreatment for this method is limited to only sample lyophilization to form a dry solid, and hydrolysis is not required.

General GLC methods to analyze amino acids include their previous derivatization to enhance volatility and thermal stability and thus improve their chromatographic behavior. Main drawback is the different derivatives and derivatization conditions need to accomplish an only derivative for each essential amino acid.[82] Some proposals are the kit offered by Supelco (Sigma-Aldrich, Bellefonte, PA) which uses *N*-methyl-*N*-(*t*-butyldimethylsilyl)trifluoroacetamide (MTBSTFA) as derivatizing reagent and a short (20 m) capillary column (they give the conditions to separate 24 amino acids in 8 min) or the method EZ:fast, which is a patent-pending method (Phenomenex, Torrance, CA) to analyze protein hydrolyzates and physiological amino acids from serum, urine, beer, wine, feeds, fermentation broths, and foodstuffs. This method includes a derivatization reaction (proprietary) in which both the amine and carboxyl groups of amino acids are derivatized. Derivatives are stable for up to one day at room temperature and for several days if refrigerated and are further analyzed by GC/FID, GC/NPD, GC/MS, and LC/MS. Results (50 amino acids and related compounds) are obtained in about 15 min (sample preparation included) when using the GC method or 24 min by using the LC method.

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9 Fatty Acids

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CONTENTS

9.1 INTRODUCTION

The specific guidelines for fat (20%–35% of caloric intake) and fatty acids in human nutrition were reviewed recently (November 2008) by the Food and Agriculture Organization of the United Nations (FAO) and the World Health Organization (WHO) [1]. Among the recommendations for fatty acids, reducing the intake of saturated fatty acids (SFA; <10% of caloric intake) and *trans* fatty acids (TFA; <1%) and increasing the intake of n-3 polyunsaturated fatty acids (PUFA; 0.5%–2%), especially eicosapentaenoic acid (EPA, 20:5n-3) and docosahexaenoic acid (DHA, 22:6n-3; 0.25–2 g/day), are particularly encouraged.

The fatty acid content and composition of meat and milk, in contrast to those of meat by-products, have been extensively studied due to its implications for human health. In fact, the high contents of SFA and TFA and the low levels of n-3 PUFA of some foods from animal origin may contribute to the imbalance in the fatty acid intake of today's consumers, which is prejudicial for human health (see Section 9.4.1). By contrast, some conjugated linoleic acid (CLA) isomers may have beneficial effects on the prevention and treatment of several chronic diseases (see Section 9.4.2). However, the major TFA in ruminant-derived foods, vaccenic acid (VA, 18:1t11), is the precursor of the most prevalent CLA isomer, rumenic acid (*cis*9,*trans*11

(c9,t11)), in animal tissues. The information about TFA and CLA isomeric distribution is of utmost importance since isomer-specific biological effects have been reported.

This chapter provides an overview of the techniques and methodologies available for the analysis of fatty acids (see Section 9.2.1), including CLA isomers (see Section 9.2.2), in the most relevant edible animal by-products (brain, heart, kidney, liver, pancreas, thymus, tongue, and tripe, mainly from beef and pork origin; see Chapter 1 for greater detail). The most common methodologies used for the analysis of fatty acids in these by-products (gas chromatography [GC], silver ion chromatography and spectroscopy) are described. A summary of complementary GC and silver ion high-performance liquid chromatography (Ag^+-HPLC) required for the analysis of individual CLA isomers, as well as some additional methodologies, is presented. Although CLA isomers are fatty acids with a conjugated double bond system, they are studied separately in this chapter due to their specific analytical methodologies and biological effects. TFA and CLA in foods are of rising importance, and knowledge of the differential biological effects of these individual isomers is increasing. The particularities of fatty acid analysis for the most relevant edible animal by-products, when applicable, are considered. Moreover, examples of the application of these methodologies for the characterization of fatty acid composition in edible by-products are provided.

In Section 9.3, the fatty acid composition (see Section 9.3.1), including CLA isomers (see Section 9.3.2), of the most eaten beef and pork by-products are reviewed from the literature. Since the data published are very scarce, this information was complemented with an original and comprehensive characterization of fatty acids in some of the most important meat by-products (brain, heart, kidney, liver, pancreas, and tongue).

Finally, the nutritional quality of fat from the organ meats most eaten will be discussed and compared regarding their composition in fatty acids (see Section 9.4.1), including CLA isomers (see Section 9.4.2). Due to the above mentioned imbalance in the fatty acids of human diet, considerable attention has been placed on improving the nutritional and health value of fats of animal origin. Some of the strategies used for this purpose are illustrated here. In ruminant animals, the reduction of SFA and n-6 PUFA levels as well as the increase of n-3 PUFA and CLA contents, mainly through the supplementation of their diets with PUFA oils, are major targets in ruminant fats research. In monogastric animals, the drive has been to increase n-3 PUFA and CLA contents in their fats by direct diet supplementation with these fatty acids.

9.2 ANALYTICAL METHODOLOGY OF EDIBLE ANIMAL BY-PRODUCTS

9.2.1 Fatty Acid Composition

Animal-derived products are composed of polar lipids, mainly phospholipids from the cell membranes, and neutral lipids, mainly triacylglycerols from adipocytes and cytosolic droplets from other cells. Phospholipids are richer in PUFA and triacylglycerols richer in SFA and monounsaturated fatty acids (MUFA). Thus, the fatty acid composition depends largely on the fat level and tissue, in addition to genetic and nutritional factors [2]. The animal species is a major source of variation of fatty acid composition, with foods from ruminant animals having in general lower amounts of PUFA and higher amounts of SFA and TFA due to the biohydrogenation of unsaturated fatty acids conducted by the rumen microbial ecosystem.

The most used strategy for fatty acid determination of animal-derived foods is the extraction of lipids with organic solvents, followed by their transesterification and subsequent chromatographic analysis. As lipids from animal tissues are both polar and nonpolar compounds, a combination of organic solvents with different polarities is needed. Although some solvents or solvent combinations have been suggested as extractants for lipids, the best and most used solvent mixture is chloroform/methanol (2:1 V/V), equilibrated with one fourth of its volume of a saline solution (e.g., 0.88% potassium chloride in water), as proposed by Folch et al. [3]. However, chloroform exhibits some toxicity if inhaled in large amounts. Methylene dichloride (or dichloromethane) may be a good alternative

to chloroform with less toxic and oxidizable properties [4]. Usually, the tissue is homogenized in the presence of both solvents, but better results may be obtained if the tissue is first extracted with methanol alone before chloroform is added to the mixture. With difficult samples, more than one extraction may be needed, and with lyophilized tissues, it may be necessary to rehydrate prior to carrying out the extraction [4]. Moreover, the extractability of lipids from tissues is variable and, therefore, alternative or modified procedures are sometimes needed.

Extract concentration may be required before esterification or separation of the lipid fractions by thin-layer chromatography (TLC) and/or solid-phase extraction (SPE); keep the temperature below 40°C during the concentration under vacuum and store the concentrated extracts at −20°C. In order to prevent oxidation of PUFA, a synthetic antioxidant, such as butylated hydroxytoluene (BHT), should be added to the extraction solvents. The most common derivatives used to analyze fatty acids in offal by-products, either by GC or HPLC, are methyl esters prepared by reaction with an excess of methanol in the presence of catalytic amounts of acid or base [5,6]. Base-catalyzed transesterification is very useful in the methylation of O-acyl lipids, although neither free fatty acids nor N-acyl lipids are usually esterified in these conditions. Moreover, aldehydes are not liberated from plasmalogens with basic reagents and cholesterol esters are transesterified very slowly. Acidic catalysts are probably the best esterifying agents since they methylate free fatty acids and O-acyl lipids very efficiently and are also used to prepare dimethylacetals from aliphatic aldehydes or plasmalogens [5]. However, acid-catalyzed methylation isomerizes CLA isomers. Therefore, a basic methylation followed by a mild acid transesterification must be used for the analysis of CLA isomers. However, care should be taken in analyzing fatty acids, such as those that are amide-bound, like in sphingolipids, as they are transesterified very slowly with these reagents. Sphingolipids are important components of the lipids in the brain, the cerebrosides being the major brain sphingolipid (up to 10% total brain lipids) [5,7].

The usual analytical techniques for the determination of total and individual fatty acids include GC, HPLC, TLC, infrared spectroscopy (IR), ^{13}C nuclear magnetic resonance (NMR) spectroscopy, attenuated total reflectance (ATR), mass spectrometry (MS), and a combination of hyphenated techniques.

9.2.1.1 Gas Chromatography

Capillary GC with flame ionization detection (GC-FID) is by far the most used method for the analysis of fatty acids, as methyl esters (FAME), in meat and meat products, including edible by-products [8]. Most detailed reports on the chromatographic resolution of animal-derived foods use meat and milk matrices, but they are generally applicable to meat by-products. Although fatty acids are generally analyzed as their methyl ester derivatives, occasionally they can also be analyzed by 4,4-dimethyloxazoline (DMOX) or picolinyl derivatives by GC in combination with mass spectrometry (GC-MS).

Animal fats usually have around 20 major fatty acids with a chain length from C12 to C22. However, ruminant-derived fats also show a large number of minor fatty acids (*trans*, conjugated, and odd- and branched-chain fatty acids) derived from rumen microbial metabolism. The analysis of TFA, in particular, is extremely challenging and complex because of the wide range of positional mono-, di-, and triene isomers. Thus, for ruminant-derived products, it is necessary to use long capillary columns to identify also the minor fatty acids.

The fatty acid profile of beef by-products is usually determined using long highly polar fused silica capillary columns coated with cyanoalkyl polysiloxane stationary phases. The 100 m CP-Sil 88™ column is most commonly used for *trans/cis* FAME separation (Figure 9.1), although SP-2380™, SP-2560™, BPX-70™, and HP-88™ columns have been frequently used [5,9]. However, even with long polar capillary columns, some fatty acids are not completely separated (e.g., 18:1 and CLA isomers). The complete resolution of *trans* 18:1 isomers as well as of *trans* 16:1, *trans* 18:2, and *trans* 18:3 isomers is rather complex. Depending on the analytical conditions, numerous overlaps may occur leading to either underestimations of *trans* 18:1 and 18:2 contents [10] or overestimations of

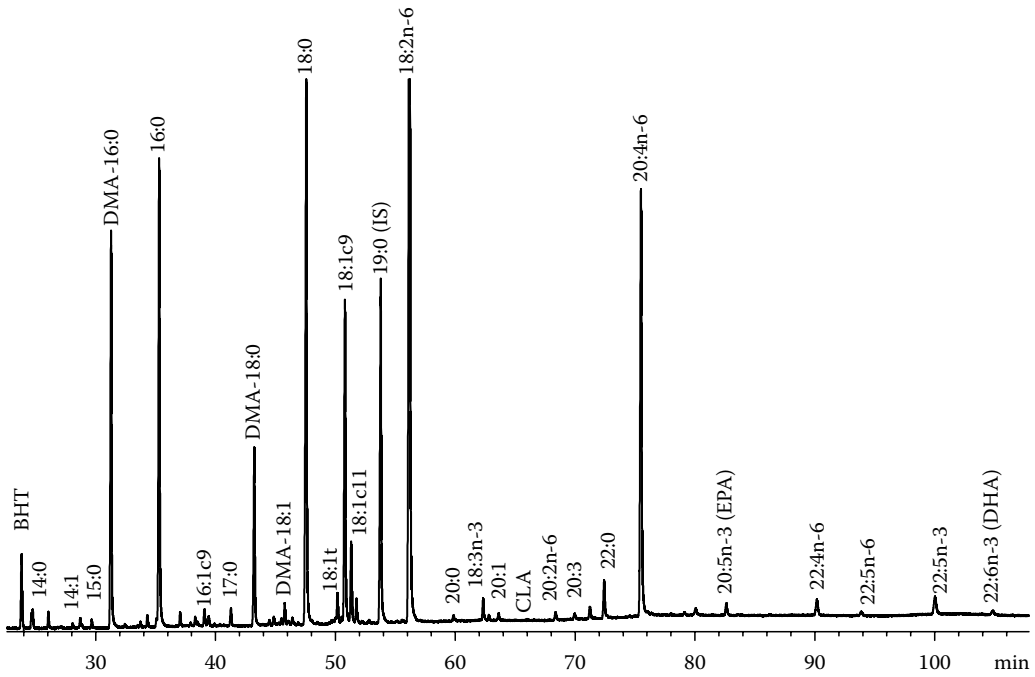

FIGURE 9.1 GC-FID chromatogram of a total fatty acid methyl ester (FAME) mixture from a typical beef heart sample, obtained using a CP-Sil 88 capillary column (100 m × 0.25 mm i.d., 0.25 µm film thickness) in the conditions described by Alves and Bessa [57]. The abbreviations mean the following: BHT, butylated hydroxytoluene, used as an antioxidant additive; CLA, conjugated linoleic acid (18:2c9,t11 co-elutes with the 18:2t7,c9 and 18:2t8,c10 isomers); DHA, docosahexaenoic acid; DMA, dimethylacetal; EPA, eicosapentaenoic acid; IS, internal standard.

trans 16:1 levels [11]. In addition, on a CP-Sil 88 18:3 isomers can overlap with 20:0 or 20:1, a finding that does not occur with SP-2340™ columns. The elution times for both *trans* and *cis* 18:1 isomers increase with the position of the double bond along the carbon chain, eluting the *trans* isomers before the *cis* isomers on cyanoalkyl polysiloxane stationary phases. The main overlaps take place in the 18:1 region, where *trans* 12 to *trans* 16 isomers co-elute with the 18:1 isomers of *cis* configuration. Column overloaded or large amounts of *cis* isomers affect the resolution and quantification of *trans* isomers. Moreover, column temperature affects greatly the *cis/trans* isomer resolution [12]. To maximize the separation of the TFA, low-temperature isothermical programs have been applied successfully by several authors [13,14]. Kramer et al. [14] used isothermal temperature at 120°C to separate the 16:1 isomers, 150°C to 18:1 isomers, 175°C to 20:1 isomers, and 220°C to 22:1 and 24:1 isomers, using a CP-Sil 88 capillary column. However, even when using stepwise isothermal temperature conditions, the resolution of all 18:1 isomers could not be achieved accurately without a prior separation of *cis* and *trans* isomers by silver ion chromatography. Recently, Kramer et al. [15] reported that a combination of two separate GC analyses may be successfully used in the determination of the most geometric and positional isomers of 16:1, 18:1, 20:1, 18:2, and 18:3, without a prior silver ion separation. Only CLA isomers still required the complementary Ag^{+}-HPLC separation. Finally, a GC method from the American Oil Chemist Society (AOCS) for measuring TFA in animal and vegetable fats that meets the current Food and Drug Administration (FDA) regulations has been reported (AOCS Method Ce1h-05).

Lipids from monogastric animals, relative to those from ruminant animals, have a much simpler fatty acid profile (mainly linear even-chain fatty acids) with higher PUFA and lower SFA contents. GC-FID is also the method of choice for the analysis of fatty acids in pork by-products.

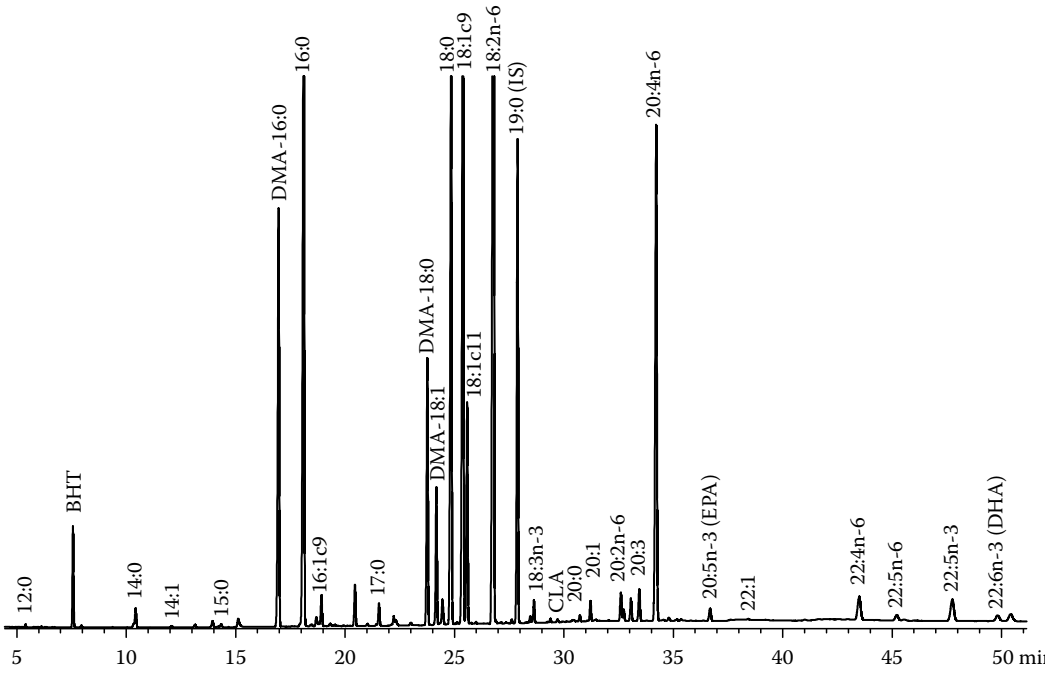

FIGURE 9.2 GC-FID chromatogram of a total fatty acid methyl ester (FAME) mixture from a typical pork heart sample, obtained using an OmegaWax 250 capillary column (30 m × 0.25 mm i.d., 0.25 µm film thickness) in the conditions described by Alves and Bessa [57]. The abbreviations mean the following: BHT, butylated hydroxytoluene, used as an antioxidant additive; CLA, conjugated linoleic acid (18:2c9,t11 co-elutes with the 18:2t7,c9 and 18:2t8,c10 isomers); DHA, docosahexaenoic acid; DMA, dimethylacetal; EPA, eicosapentaenoic acid; IS, internal standard.

Several capillary columns have been developed especially for the separation and analysis of long-chain (>C18) unsaturated fatty acids, such as DB-Wax™, CarboWax™, SupelcoWax™, and OmegaWax™. Official Methods of analysis for long-chain n-3 PUFA recommend capillary columns with polyethylene glycol stationary phases, such as SupelcoWax or OmegaWax columns (AOCS Ce1i-07 and Ce1b-89). In pork by-products, good resolution of the major fatty acids can be achieved with 30 m capillary columns (Figure 9.2). These polar polyethylene glycol stationary phases combine the advantage of a relative high resolution capability with those of a relatively high thermal stability [16], although highly polar cyanoalkyl polysiloxane stationary phases have been also frequently used. The nonadecanoic acid (19:0) and tricosanoic acid (23:0) methyl esters have been extensively used as internal standards for the analysis of the highly unsaturated n-3 fatty acids EPA, DHA, and docosapentaenoic acid (22:5n-3) [16]. The chromatographic conditions for long-chain PUFA analysis usually require high oven temperatures (above 200°C). However, the damage of DHA by the use of high temperatures in GC analysis was recently reported [17]. It was recommended by the same author that the FID response correction factors for these analytical conditions should be used for the accurate determination of long-chain n-3 PUFA. Bragagnolo and Rodriguez-Amaya [18] determined the fatty acid composition in the skin and backfat of suckling and adult pigs by GC-FID using a 30 m capillary column (DB-Wax, 0.30 mm i.d., 0.25 µm film thickness).

Recent developments in GC instrumentation (fast, very fast, and ultrafast GC, with runtimes of 5–10 min, a few minutes, and less than 1 min, respectively) allow the full exploitation of narrow-bore capillary columns (fast columns up to 0.05 mm of i.d.), which are able to obtain very fast temperature programming rates [19]. The chromatographs equipped with the ultrafast mode allows the operator to achieve the determination of FAME with up to a 20-fold speed increase over conventional GC methods with excellent accuracy and precision of both peak areas and retention times.

The columns for fast GC are shorter and have a much smaller internal diameter than a standard GC column. For instance, a CarboWax capillary column, 5–10 m long (0.1 mm i.d., 0.1 µm film thickness), is suitable for FAME analysis in meat by-products [19]. The transesterification of fatty acids with methanolic KOH was recently reported as the most suitable process for the ultrafast GC analysis of pork fat [20]. Finally, Destaillats and Cruz-Hernandez [21] reported the separation of complex FAME matrices (e.g., milk fat and tuna oil) using a short and highly polar BPX-70 (10 m × 0.1 mm i.d., 0.2 µm film thickness) fast capillary column. The GC parameters were optimized to achieve the separation of FAME ranging from butyric acid (4:0) to nervonic acid (24:1) with a total runtime of 4.9 min. From the results obtained so far, it is clear that the use of fast columns will significantly impact the analysis of lipids.

9.2.1.2 Silver Ion Chromatography

The complete analysis of all TFA can only be accomplished by the previous separation of *cis* and *trans* isomers. Silver ion chromatography has been applied in the separation of fatty acids according to both the number and the configuration of their double bond [5]. Ag^+-TLC, Ag^+-SPE, or Ag^+-HPLC fractionation, followed by analysis of the fraction by GC, allows a complete and accurate analysis of TFA, especially the mono- and diethylenic isomers [22]. Common TLC solvent systems are hexane:diethyl ether (90:10 V/V), benzene for *cis/trans* methyl linolenate, and chloroform:acetone:acetic acid (96:5:0.5 V/V) for *cis/trans* methyl arachidonate fractionation [5,23]. When samples are analyzed by GC before and after silver ion separation, the amount of TFA can be determined. The quantification of *trans* 18:1 isomers may be easily achieved by using the area of the *trans* 18:1 isomer eluting before *trans* 12 in the nonfractionated chromatogram and the total *trans* 18:1 area in silver ion fractionated chromatogram, as reported by Kramer et al. [24]. Otherwise, the incorporation of a known amount of methyl penta- or heptadecanoate as an internal standard to *trans* and *cis* 18:1 fractions allows quantification by GC [22]. This procedure was adopted by the International Union of Pure and Applied Chemistry (IUPAC) for the quantification of TFA in natural and hydrogenated animal and vegetable oils and fats (IUPAC Method 2.302). Christie [5] proposed a method where fractions containing both *trans* monoenes and saturated FAME are collected together, while the proportion of the *trans* monoenes is calculated by reference to one or all of the saturated compounds.

Cruz-Hernandez et al. [12] reported a complete methodology using argentation TLC and GC in the analysis of *trans* 18:1 isomers in dairy fats. The quantification of the different *trans* isomers of 18:1, 18:2, and 18:3 and their overestimation have been widely studied by Precht and Molkentin [10] in milk fat. These authors proposed a GC isothermical at a low temperature (125°C) to achieve the separation of *trans* 13 and *trans* 14 18:1 isomers, as well as *trans* 11 and *trans* 12 16:1 isomers [25].

9.2.1.3 Spectroscopy

The measurement of the intensity of a characteristic absorption band, at 966 cm^{-1}, under a defined set of analytical conditions constitutes the basis for the various IR methods used for the determination of total *trans* unsaturation in fats [22]. The older standard official methods have been subjected to modifications in order to improve its detected limitations. Improvements were made at correcting the various background interferences, calibrations, and reference materials in AOCS and Association of Analytical Communities (AOAC) International Methods. However, results from IR are biased, in particular those concerning fat products with little TFA [26].

Advances in equipment technology introduced the Fourier-transform infrared (FT-IR) and near-infrared (FT-NIR), as well as the ATR-IR spectroscopic instruments. TFA formed during the biohydrogenation of ruminant animals were quantified by Ulberth and Henninger [27] in milk fat using the FT-IR methodology and are only slightly higher than those obtained by the same authors using the Ag^+-TLC-GC technique. An ATR-IR method has been widely studied for the determination of total TFA content in food products [28,29], which has been adopted by AOCS (Official Method AOCS Cd 14d-99) and AOAC International (Official Method AOAC 2000.10). Recently, Azizian

and Kramer [30] described a rapid FT-NIR method coupled to chemometric calibration techniques that allows qualitative and quantitative information of most fatty acids present in oils and fats, including TFA. None of these spectroscopic methods are directly applied to meat matrices but only to oils, extracted fats, or FAME mixtures.

9.2.1.4 Other Methods

Few hyphenated techniques have been developed for the analysis of fatty acids. Coupling capillary GC to FT-IR spectrometers led to some advantages in the determination of *trans* monounsaturated FAME, particularly the measurement of subnanogram quantities of compound, in contrast to those required for the standard IR equipment [31]. The online measurement of FT-IR spectra of compounds eluting from the capillary column allows the lack of interferences, particularly due to the partial overlap of adjacent *trans* and *cis* isomers [23]. Although the GC-FT-IR technique can provide information about the double bond geometry of fatty acids, it is relatively expensive for routine analysis [31].

Other common analytical approaches could also be applied for the determination of fatty acids in fats, particularly the FT-NIR coupled with chemometric calibration procedures, as described by Azizian and Kramer [30].

Ultra-performance liquid chromatography (UPLC), one of the most significant developments in separations science in the past decade, is the result of holistic innovation that simultaneously reengineered LC particle technology, column design, injectors, pumps, and detectors. The increased performance of sub-2 µm hybrid columns, used in conjunction with the ability to deliver mobile phase at high pressures with low dispersion, provide significantly more resolution and sensitivity, while reducing runtimes. The UPLC seems to have a great potential for the analysis of fatty acids. For instance, it was recently used in the determination of a mixture of 11 fatty acids (e.g., oleic, linoleic, methyl linoleate, stearic, and palmitic acids) (reviewed recently in [32]). The separation column was an UPLC bridged ethylsiloxane/silica hybrid (BEH) Phenyl C18™ (10 cm × 2.1 mm i.d., 1.7 µm particle size) and the mobile phase consisted of acetonitrile–water (3:1 V/V) in the isocratic mode. The flow rate was of 0.3 mL/min, the column was heated at 40°C, and the run was completed in 5 min.

9.2.2 Profile of Conjugated Isomers of Linoleic Acid

Conjugated isomers of linoleic acid, commonly referred to as CLA, are a group of fatty acids, composed of positional (from carbons 6,8 to 12,14) and geometric (*trans*,*trans*, *cis/trans*-*cis,trans* and *trans,cis*- and *cis*,*cis*) isomers of linoleic acid (18:2n-6), containing a conjugated double-bond system [33]. Twenty four different CLA isomers have been reported as occurring naturally in food, especially in ruminant fat [12]. The major CLA isomer in ruminant meat (rumenic acid, 18:2c9,t11), like the usual second-most prevalent isomer (18:2t7,c9), is mainly produced in the tissues through Δ9 desaturation of VA [34]. The origin of all other CLA isomers is the ruminal biohydrogenation of dietary C18 PUFA [35]. Interest in these compounds has expanded since CLA was found to be a naturally occurring compound, present in animal-derived foods, and associated with a multitude of health benefits.

In animal tissues, natural CLA is in esterified form and is present at relatively low levels. Thus, a pre-concentration step is required, which can be accomplished either by reversed-phase HPLC or by silver ion chromatography or by improving the efficacy of the process, by using the two techniques sequentially [5]. In addition, there is no single methylation procedure adequate for CLA analysis in meat lipids: base-catalyzed methylation transesterifies acyl lipids but not N-acyl or alk-1-enyl acyl lipids; acid-catalyzed methylation isomerizes *cis/trans* to *trans,trans* CLA and produces methoxy artifacts [12]. Therefore, base- and acid-catalyzed methylations must be used in sequence or separated for the derivatization of meat lipids, including those of meat by-products [36]. Finally, recent reviews on CLA isomer analysis have been reported elsewhere [32,36].

9.2.2.1 Gas Chromatography

GC-FID, the standard technique in most laboratories for the routine analysis of fatty acids, is the most widely used methodology for the quantification of total CLA methyl esters. However, this technique is of limited value for the analysis of individual CLA isomers. The various types of geometrical isomers give distinct peaks, but within these groups, positional isomers are not fully resolved. GC with 100 m highly polar capillary columns (e.g., CP-Sil 88, SP-2560, HP-88, and BPX-70 columns) must be used in order to achieve improved separations of CLA methyl esters [14]. However, some FAME (e.g., 21:0 and 20:2 isomers) elute in the same region as the CLA isomers even when these highly polar GC columns are used [12]. Moreover, the relative elution of these FAME differ slightly between columns, even from the same supplier, and depends on the age of the column [9]. A quantitative GC method for both bioactive c9,t11 and t10,c12 CLA isomers as nonesterified fatty acids and triacylglycerols (previously fractionated by TLC) was developed (100 m SP-2380 capillary column, 0.32 mm i.d., 0.20 µm film thickness) and applied to liver from hamsters fed a diet supplemented with both CLA isomers [37]. Recently, both c9,t11 and t10,c12 CLA isomers in human plasma have been quantified by fast GC (Rtx-2330™ capillary column, 40 m × 0.18 mm i.d., 0.10 µm film thickness) [38]. Some individual (c9,t11, t9,c11, c11,t13, t10,c12, and t9,t11) and mixtures (from Δ8,10 to Δ11,13) of CLA isomers, as free acids or methyl esters, are commercially available from Matreya Inc. (Pleasant Gap, PA, USA), Nu-Check Prep (Elysian, MN) or Sigma Inc. (St. Louis, MO). Additional standards of mixtures (*trans,trans*, *cis/trans*, and *cis,cis* from carbons 7,9 to 12,14) of CLA isomers, as methyl esters, can be prepared according to the procedures described by Destaillats and Angers [39], Delmonte et al. [40], or Eulitz et al. [41]. A definitive identification of the interfering FAME requires, instead of GC-FID, GC-MS using the DMOX derivatives and/or 4-methyl-1,2,4-triazoline-3,5-dione (MTAD) adducts as invaluable adjuncts [42].

9.2.2.2 Silver Ion High-Performance Liquid Chromatography

Ag^+-HPLC, which uses columns packed with silver ions (ChromSpher 5 Lipids™), has proved to be very useful for the separation of geometrical and positional isomers of CLA [43]. The chromatographic separation is based on the interaction between the π-electrons of double bonds and the empty *d* orbitals on the silver. Improved separations of CLA isomers have been obtained using three of these argentated columns in series [44]. The mobile phase is *n*-hexane containing 0.1% acetonitrile and 0.5% diethyl ether, with specific detection of the conjugated double bonds of CLA methyl esters at 233 nm. *Trans,trans* isomers elute first, followed by *cis/trans*, and then *cis,cis*, and within each group most positional isomers are clearly resolved. As an example, Figure 9.3 illustrates a separation of CLA methyl ester isomers in a typical bovine pancreas sample. However, the separation of CLA isomers using Ag^+-HPLC still has two major drawbacks: the reproducibility of the retention times among chromatographic runs and the lack of an appropriate internal standard for the quantification of CLA isomers.

Therefore, the best strategy for the analysis of the individual CLA isomers in ruminant fats is the combination of GC, using 100 m highly polar capillary columns, and Ag^+-HPLC, using three 25 cm columns in series [45]. The CLA peaks are quantified by GC analysis of total FAME and the relative concentrations obtained by Ag^+-HPLC are used to calculate the unresolved peaks in the GC chromatogram. Kraft et al. [46] performed the calculation by comparing the HPLC areas of c9,t11, t7,c9, and t8,c10 isomer peaks with the peak area of the three co-eluted isomers from the GC chromatogram. The quantification of the other CLA isomers was assessed from their Ag^+-HPLC areas relative to the area of the main isomer c9,t11. A detailed description of the quantification process of individual CLA isomers using these two complementary methods was described by Cruz-Hernandez et al. [36]. Finally, the determination of underivatized individual CLA isomers by Ag^+-HPLC, with two columns in tandem, was applied to muscle, adipose tissue, liver, brain, and pancreas from rats fed a diet enriched with a mixture of c9,t11 and t10,c12 CLA isomers [47].

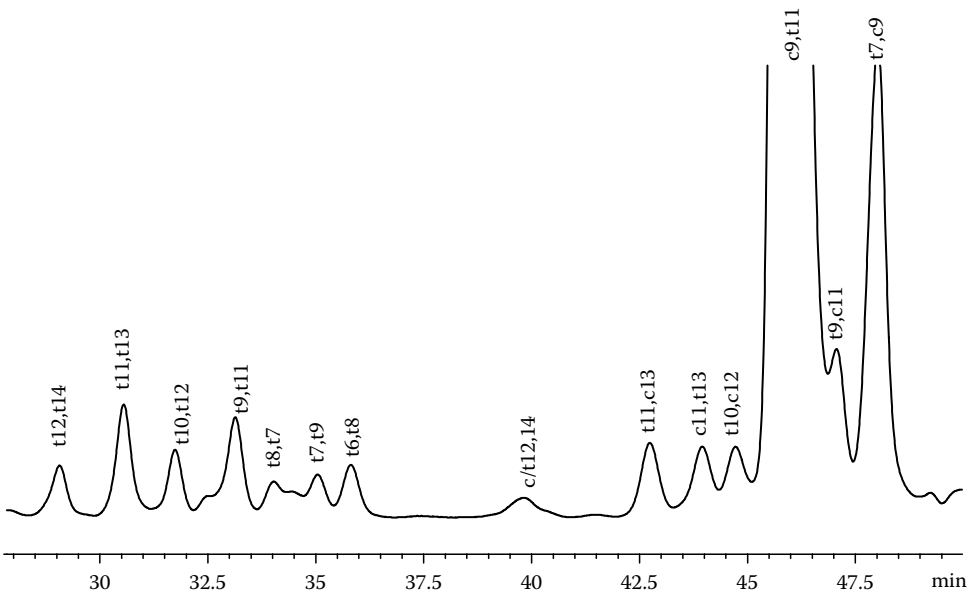

FIGURE 9.3 Partial Ag^+-HPLC chromatogram (*trans,trans* and *cis/trans* regions) of a total conjugated linoleic acid (CLA) methyl ester mixture from a typical beef pancreas sample, obtained using three ChromSpher 5 Lipids columns (25 cm × 4.6 mm i.d., 5 µm particle size) in tandem and the conditions described by Alfaia et al. [66].

9.2.2.3 Other Methods

^{13}C NMR spectroscopy, the most complete single analysis for commercial CLA preparations, requires a substantial amount of sample and is not likely to be applicable to tissue extracts at natural levels [48]. Reversed-phase HPLC can also be used for CLA analysis, although it is mainly useful for the analysis of CLA metabolites [49]. However, when combined with the second derivative of UV absorbance, it is possible to analyze CLA and its metabolites [50]. FT-NIR requires the extraction of fat from meat and does not distinguish between *cis,trans* and *trans,cis* isomers [30]. Finally, chemical ionization tandem mass spectrometry (CI-MS and CI-MS/MS) with acetonitrile as the reagent of chemical ionization provides a rapid alternative to conventional CLA analysis. This technique, in combination with GC, enables the rapid and positive identification of the double bond position and geometry in most CLA isomer methyl esters [51].

9.3 LIPID COMPOSITION OF EDIBLE ANIMAL BY-PRODUCTS

9.3.1 Fatty Acid Composition

Beef liver, tail, ears, and feet have a protein level close to that of lean meat (<5% fat, according to the Food Advisory Committee criteria [52]), but a large amount of collagen is found in the ears and feet. The lowest protein level is found in the brain, chitterlings, and fatty tissue (see Chapter 2 for greater detail). Pork tail has the highest fat content and the lowest moisture content of all meat by-products [53]. There is three to five times more cholesterol (0.26–0.41 g/100 g product) in organ meats than in lean meat [54], as well as large quantities of phospholipids. Brain has the highest contents of cholesterol (1.3–2.2 g/100 g) and phospholipids compared to other meat by-products (see Chapter 5 for greater detail).

Table 9.1 shows the contents of total fatty acids and fatty acid composition of some of the most eaten beef by-products (brain, heart, kidney, liver, pancreas, and tongue). Since the information regarding the fatty acid composition of these edible by-products is very scarce in the literature it was

TABLE 9.1
Fatty Acid Content and Composition of Some Beef By-Products

	Brain	Heart	Kidney	Liver	Pancreas	Tongue
Total fatty acids (g/100 g product)	1.9–6.4	1.0–3.2	0.94–2.1	1.5–2.3	1.9–16	1.1–15
Fatty acid composition (% FA)						
10:0	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.–0.13
12:0	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.–0.59
14:0	0.41–0.72	0.27–2.1	0.41–1.0	0.18–0.64	0.92–2.0	1.9–3.8
14:1	0.11–0.19	0.22–0.34	0.16–0.29	0.07–0.35	0.11–0.20	0.65–1.1
15:0	0.11–0.18	0.17–0.49	0.34–0.51	0.15–0.30	0.24–0.62	0.32–0.45
16:0	14–19	12–19	16–19	7.2–13	21–24	17–28
16:1 isomers	0.33–0.44	0.07–1.8	0.09–1.9	0.06–1.5	0.08–6.8	0.04–5.5
17:0	0.47–0.56	0.40–1.6	0.62–1.1	0.78–1.3	0.55–1.1	0.71–1.0
18:0	20–21	16–19	13–18	31–37	14–24	11–16
18:1c9	22–26	8.2–33	16–26	8.8–18	28–35	34–43
18:1t isomers	0.03–5.6	0.06–3.3	0.09–4.7	0.06–1.8	0.14–2.7	0.11–3.4
18:2n-6	0.42–0.64	12–39	14–24	13–19	7.9–15	3.8–10
18:2c9,t11[a]	0.03–0.06	0.16-.030	0.31–0.64	0.39–0.75	0.29–0.60	0.37–0.63
18:3n-3	0.10–0.16	0.34–1.1	0.62–1.2	0.30–1.4	0.58–0.95	0.36–0.78
18:4	0.44–0.60	n.d.	n.d.	0.05–0.07	n.d.	n.d.
20:0	0.18–0.27	0.10–0.20	0.28–0.57	0.05–0.07	0.09–0.16	0.06–0.11
20:1	2.1–3.5	0.08–0.34	0.31–0.57	0.07–0.30	0.14–0.29	0.13–0.92
20:2n-6	0.09–0.12	0.16–0.28	0.42–0.72	0.19–0.43	0.05–0.08	0.04–0.12
20:3 isomers	0.08–0.24	n.d.–0.13	n.d.–0.31	n.d.–0.12	0.03–0.06	n.d.
20:4n-6	5.0–7.8	3.9–14	11–16	6.0–12	1.8–5.1	1.1–4.6
20:5n-3	n.d.	0.33–0.73	0.34–0.59	0.31–0.52	0.10–0.32	0.06–0.20
22:0	0.53–0.60	0.85–1.7	0.65–1.7	0.38–4.8	0.13–0.45	0.17–0.69
22:4n-6	4.6–5.2	0.39–0.67	0.60–0.87	1.7–3.4	0.14–0.32	0.19–0.65
22:5n-6	1.8–3.4	0.08–0.20	0.08–0.21	0.41–0.83	0.03–0.08	0.03–0.16
22:5n-3	0.39–5.9	0.67–1.0	1.0–1.5	2.4–4.0	0.37–0.88	0.19–0.91
22:6n-3	7.4–13	0.12–0.19	0.28–0.56	0.72–1.6	0.05–0.11	0.04–0.17
Partial sums of fatty acids (% FA)						
Total saturated fatty acids	36–42	30–43	32–41	46–52	39–51	36–46
Total monounsaturated fatty acids	26–30	10–35	18–28	10–20	31–40	39–48
Total polyunsaturated fatty acids	24–26	17–55	26–43	19–39	11–22	5.9–18
Total *trans* fatty acids	5.9–9.6	3.9–5.6	4.8–7.4	3.0–7.2	4.9–5.9	4.9–6.2

[a] This CLA isomer co-eluted with minor amounts of the 18:2t7,c9 and 18:2t8,c10 isomers.

The ranges of values (minimum–maximum) shown were obtained from USDA National Nutrient Database for Standard Reference (Release 22, 2009 [99]) and from our data (unpublished results, n = 9).

n.d., not detected.

complemented with original data obtained by our research group. The methodology used for the determination of fatty acid composition in organ meats was based on the method of Folch et al. [3]. Briefly, representative samples of the tissues (brain, heart, kidney, liver, pancreas, and tongue) were lyophilized (−60°C and 2.0 hPa) to constant weight, the lipids were extracted with dichloromethane/methanol (2:1 V/V), and the solvent was evaporated to dryness. For both FAME and CLA methyl ester profile analysis, fatty acids and CLA isomers were converted to methyl esters by a combined transesterification procedure with NaOH in anhydrous methanol (0.5 M), followed by HCl:methanol (1:1 V/V), at 50°C for 30 and 10 min, respectively, according to Raes et al. [55] and Alfaia et al. [56].

Fatty acid composition of beef by-products was analyzed by GC-FID (chromatograph HP 6890; Hewlett–Packard, Avondale, PA) using a fused-silica 100 m capillary column (CP-Sil 88; 0.25 mm i.d., 0.2 µm film thickness; Chrompack, Varian Inc., Walnut Creek, CA), as described by Alves and Bessa [57]. Briefly, the initial oven temperature was 100°C (held for 1 min), then increased by 50°C/min to 150°C (held for 20 min), then increased by 1°C/min to 190°C (held for 5 min), and finally increased by 1°C/min to 200°C (held for 35 min). Ultrapure helium was used as the carrier gas, at a flow rate of 1 mL/min, and the split ratio was 1:50. The injector and detector temperatures were maintained at 250°C and 280°C, respectively. The identification of common fatty acids was accomplished by comparison of sample peak retention times with those of FAME standard mixtures (Sigma) and by using published chromatograms obtained with similar analytic conditions [15,58]. Structural analyses of some unknown peaks were conducted by the GC-MS/MS technique, using a Varian Saturn 2200 system (Varian Inc.) equipped with a CP-Sil 88 capillary column (100 m × 0.25 mm i.d., 0.2 µm film thickness). The quantification of total FAME was done using nonadecanoic acid as the internal standard. The results for each fatty acid were expressed as a percentage of the sum of identified fatty acids (total fatty acids).

The fatty acid content and the composition of some of the most eaten pork by-products (brain, heart, kidney, liver, pancreas, and tongue) are shown in Table 9.2. The data concerning the fatty acid content and the composition of beef and pork is abundant in the literature and was reviewed elsewhere [2,59]. In contrast, the information published regarding fatty acid composition of edible pork by-products is very limited, and therefore, it was complemented with original data obtained by our research group. The methodology used for the lipid extraction and transesterification of pork by-products was similar to that described above for beef by-products. The fatty acid composition of pork by-products was analyzed by GC-FID (chromatograph HP 6890, Hewlett-Packard) using a 30 m capillary column (OmegaWax 250; 0.25 mm i.d., 0.25 µm film thickness; Supelco, Bellefont, CA), as described by Alves and Bessa [57]. In brief, the initial column temperature of 150°C was held for 11 min, then increased to 210°C at a rate of 3°C/min and maintained for 30 min. Ultrapure helium was used as the carrier gas at a flow rate of 1.3 mL/min, and the split ratio was 1:50. The injector and detector temperatures were set at 250°C and 280°C, respectively. The nonadecanoic acid was used as the internal standard. Results for each FA were expressed as a percentage of the sum of the identified fatty acids (total fatty acids).

The range (minimum–maximum) of contents of total fatty acids for each by-product is similar for beef and pork (see Tables 9.1 and 9.2). Total fatty acids (g/100 g product) are relatively low (<5% fat, as in lean meat) in heart (1.0%–3.2% for beef and 1.0%–3.3% for pork), kidney (0.94%–2.1% for beef and 1.2%–2.4% for pork), and liver (1.5%–2.3% for beef and 2.4%–4.8% for pork), intermediate in brain (1.9%–6.4% for beef and 2.0%–5.2% for pork) and vary widely in pancreas (1.9%–16% for beef and 1.9%–12% for pork) and tongue (1.1%–15% for beef and 4.0%–16% for pork). Only brain, pancreas, and tongue may be fatter than lean meat.

It is well established that animal species is the major source of variation in meat fatty acid composition. Due to the biohydrogenation of unsaturated fatty acids in the rumen, lipids from monogastric animals, relative to those from ruminant animals, have a much simpler fatty acid profile with higher percentages of PUFA and lower relative proportions of SFA and TFA. In addition, the level of fatness also has an effect on the meat fatty acid composition by changing the triacylglycerol/phospholipid ratio. However, the percentages of SFA, MUFA, and PUFA are similar between the homologues beef and pork by-products (see Tables 9.1 and 9.2). The lipids of the beef and pork by-products reviewed are relatively saturated since the SFA 16:0 and 18:0 (and oleic acid, 18:1c9) predominates (with the exception of heart), which suggest a strong contribution of *de novo* synthesis of fatty acids in these organs. In addition, the detailed fatty acid composition, including the percentages of SFA (32%–34% for skin and 34%–41% for backfat), MUFA (48%–52% for skin and 47%–49% for backfat), and PUFA (17%–18% for skin and 11%–18% for backfat) in suckling and adult pigs has been described [18].

Arachidonic acid (20:4n-6) is by far the main long-chain PUFA in beef and pork by-products reviewed, excluding beef brain, in which DHA predominates. The percentages of n-3 PUFA are

TABLE 9.2
Fatty Acid Content and Composition of Some Pork By-Products

	Brain	Heart	Kidney	Liver	Pancreas	Tongue
Total fatty acids (g/100 g product)	2.0–5.2	1.0–3.3	1.2–2.4	2.4–4.8	1.9–12	4.0–16
Fatty acid composition (% FA)						
10:0	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.–0.13
12:0	n.d.–0.01	0.02–0.30	0.02–0.42	0.03–0.17	0.05–0.08	0.06–0.57
14:0	0.35–0.77	0.23–2.4	0.49–1.7	0.48–1.7	0.94–1.4	1.1–2.0
14:1	n.d.	n.d.	0.01–0.02	n.d.–0.01	n.d.–0.01	0.01–0.02
15:0	0.06–0.08	0.03–0.09	0.07–0.12	0.07–0.17	0.06–0.15	0.05–0.09
16:0	18–20	15–18	22–29	16–27	21–30	24–27
16:1 isomers	0.82–2.3	0.15–3.0	0.47–3.8	0.39–2.8	0.31–6.8	0.32–4.0
17:0	0.26–0.31	0.19–0.54	0.30–0.67	0.37–0.72	0.30–0.65	0.33–0.55
17:1c9	0.12–0.15	0.12–0.22	0.06–0.32	0.18–0.59	0.15–0.38	0.33–0.54
18:0	19–23	12–14	14–17	15–27	15–24	11–13
18:1c9	21–28	12–27	17–41	17–34	24–33	39–46
18:2n-6	0.60–1.7	23–35	7.2–17	12–16	8.6–16	7.9–11
18:2c9,t11[a]	0.01–0.03	0.03–0.08	0.01–0.06	0.05–0.12	0.03–0.08	0.10–0.23
18:3n-3	0.02–2.3	0.37–2.4	0.17–0.42	0.32–1.2	0.36–0.77	0.33–0.50
20:0	0.23–0.31	0.04–0.11	0.15–0.22	0.02–0.05	0.19–0.35	0.11–0.20
20:1	1.2–1.8	0.21–0.55	0.44–0.84	0.22–0.35	0.46–0.75	1.3–1.6
20:2n-6	0.10–0.14	0.59–0.89	0.69–0.92	0.23–0.45	0.25–0.35	0.53–0.64
20:3 isomers	0.04–0.96	0.07–0.99	0.11–1.4	0.03–0.74	0.04–0.41	0.07–0.35
20:4n-6	9.1–11	8.2–20	3.4–19	3.1–17	1.8–4.9	1.1–2.2
20:5n-3	0.07–0.10	0.35–0.49	0.17–0.62	0.07–0.48	0.07–0.20	0.03–0.06
22:1	0.30–0.51	0.02–0.09	0.04–0.10	0.03–0.79	0.01–0.04	0.02–0.08
22:4n-6	4.2–5.3	0.88–1.3	0.87–1.8	0.30–1.4	0.13–0.31	0.36–0.65
22:5n-6	2.2–3.3	0.18–0.32	0.06–0.15	0.05–0.33	0.02–0.05	0.03–0.07
22:5n-3	0.26–4.3	1.1–1.9	0.53–0.97	0.32–2.4	0.15–0.38	0.15–0.29
22:6n-3	6.6–8.7	0.20–0.56	0.27–1.0	0.10–1.4	0.04–0.11	0.05–0.08
Partial sums of fatty acids (% FA)						
Total saturated fatty acids	40–43	29–35	38–46	41–46	39–55	38–42
Total monounsaturated fatty acids	28–32	13–31	19–45	19–40	27–40	44–51
Total polyunsaturated fatty acids	26–29	34–57	11–43	17–39	12–22	11–15
Total *trans* fatty acids	0.01–0.03	0.03–0.08	0.01–0.06	0.05–0.12	0.03–0.08	0.10–0.23

[a] This CLA isomer co-eluted with minor amounts of the 18:2t7,c9 and 18:2t8,c10 isomers.
The ranges of values (minimum–maximum) shown were obtained from USDA National Nutrient Database for Standard Reference (Release 22, 2009 [99]) and from our data (unpublished results, n = 11).
n.d., not detected.

usually very low in beef and pork by-products, with the exception of DHA in brain (7.4%–13% for beef and 6.6%–8.7% for pork). In line with this, Cordain et al. [60] reported that brain has the highest percentages of DHA, relative to other organ meats, in wild ruminant animals (8.9% in elk, 9.6% in deer, and 9.2% in antelope). The docosapentaenoic acid may also be present in brain beef (0.38%–5.9%) and pork (0.26%–4.3%) at relatively high levels. In contrast to the other fatty acid classes, the percentages of TFA in the beef by-products vary up to 9.6% of total fatty acids, while the TFA percentages in pork by-products is only residual (<0.23%). TFA are unsaturated fatty acids that have at least one double bond in the *trans* configuration. However, the chemical definition of FDA is more limited, considering TFA as unsaturated fatty acids that contain one or more isolated

(i.e., nonconjugated) double bound in the *trans* configuration [61]. In ruminant fats, the main TFA are *trans* octadecenoates (*trans* 18:1), with VA predominating, although in animals fed diets with high starch contents, the 18:1t10 may predominate [62,63]. In addition, *trans* 16:1 and *trans* PUFA (18:2 and 18:3) isomers are also usually present.

The methodologies reviewed here may be used, for instance, to assess the content and profile of TFA and PUFA in meat by-products, especially those from ruminant animals, fed diets supplemented with polyunsaturated oils. In addition, these analytical techniques may be of great value for the determination of the n-3 PUFA profile in meat fats from monogastric animals fed different diets. It is well established that the content of n-3 fatty acids in meat fat can be readily improved by increasing the levels of n-3 PUFA in monogastric animal diets through the incorporation of vegetable oils [64] and/or oily fish by-products [65].

9.3.2 Conjugated Isomers of Linoleic Acid

Total CLA content and its isomeric distribution in some of the most eaten beef and pork by-products (brain, heart, kidney, liver, pancreas, and tongue) are shown in Tables 9.3 and 9.4, respectively. Since this information was not available in the literature, the results depicted here were obtained by our research group. For this, the same extracts used for fatty acid analysis, obtained as described above in FAME preparation (Section 9.3.1), were also used for the determination of the CLA isomeric profile as the CLA methyl esters.

In order to obtain a detailed CLA isomeric distribution, the isomers were individually separated by triple silver-ion columns in series (ChromSpher 5 Lipids, 25 cm × 4.6 mm i.d., 5 µm particle size;

TABLE 9.3
Conjugated Linoleic Acid Content and Isomeric Distribution of Some Beef By-Products

	Brain	Heart	Kidney	Liver	Pancreas	Tongue
Total CLA (mg/100 g product)[a]	1.1–2.5	2.1–4.3	3.9–8.4	6.7–18	8.7–18	4.8–32
CLA profile (% CLA)						
t12,t14	0.57–2.4	0.60–1.7	0.25–0.88	0.51–1.3	0.41–1.2	0.42–0.71
t11,t13	2.5–3.2	1.6–4.4	0.87–1.8	1.0–2.8	0.69–3.0	0.44–1.3
t10,t12	0.26–0.55	0.49–1.3	0.13–0.42	0.13–0.41	0.34–0.91	0.24–0.47
t9,t11	2.5–3.9	1.4–2.4	1.6–3.4	1.5–6.2	1.2–2.9	0.91–1.4
t8,t7	0.22–1.1	n.d.–0.51	0.14–0.67	0.18–0.52	0.24–0.75	0.21–0.56
t7,t9	1.3–2.9	0.51–2.1	1.0–2.2	0.55–2.0	0.51–1.3	0.44–1.3
t6,t8	n.d.–1.6	n.d.–1.5	0.27–0.75	n.d.–0.73	n.d.–0.86	0.19–0.71
c/t12,14	1.0–1.7	0.49–0.84	0.29–0.57	0.19–0.56	0.35–0.65	0.54–1.4
t11,c13	18–23	1.5–4.3	4.0–5.5	0.48–1.0	0.80–2.6	2.1–2.7
c11,t13	1.7–2.5	1.6–2.4	0.38–1.1	0.50–1.1	0.64–1.4	0.38–1.3
t10,c12[b]	n.d.	0.68–1.7	n.d.	0.13–0.83	0.47–1.3	0.73–2.6
c9,t11[c]	60–64	74–85	76–84	82–89	78–87	81–86
t7,c9	2.9–3.7	3.1–5.5	6.2–10	3.8–6.2	5.2–8.8	5.6–7.9
Partial sums of isomers (% CLA)						
Total *trans,trans*	9.0–13	5.8–12	4.6–9.4	4.4–12	4.1–10	3.1–6.1
Total *cis/trans*	87–91	88–94	91–95	88–96	90–96	94–97

[a] Total CLA was determined by the combination of GC-FID and Ag^+-HPLC techniques, as described in the text.

[b] In brain and kidney samples, this minor CLA isomer co-eluted with the major c9,t11 isomer.

[c] This CLA isomer co-eluted with minor amounts of the t9,c11 isomer.

The ranges of values (minimum–maximum) shown were obtained from our data (Unpublished Results, n = 9).

n.d., not detected.

TABLE 9.4
Conjugated Linoleic Acid Content and Isomeric Distribution of Some Pork By-Products

	Brain	Heart	Kidney	Liver	Pancreas	Tongue
Total CLA (mg/100 g product)[a]	0.5–1.4	0.5–1.0	0.2–1.1	2.1–4.8	0.9–3.5	5.5–21
CLA profile (% CLA)						
t12,t14	0.88–2.0	n.d.–1.8	0.43–1.7	0.35–0.72	0.36–1.1	0.21–1.5
t11,t13	5.4–9.8	1.1–2.2	1.4–2.7	0.69–1.5	0.87–1.7	0.55–1.5
t10,t12	0.26–0.87	n.d.–1.5	0.53–1.2	0.25–1.1	0.68–1.6	0.54–0.7
t9,t11	4.5–6.8	2.2–3.7	2.9–5.9	2.0–7.6	3.7–6.8	4.2–6.6
t8,t7	n.d.–1.9	n.d.–3.5	n.d.–1.0	0.41–7.1	0.45–1.9	0.62–1.8
t7,t9	n.d.–7.1	1.7–5.0	2.3–4.3	1.0–5.6	1.1–3.9	0.51–1.5
t6,t8	n.d.–3.8	n.d.–5.2	1.9–4.9	1.1–3.7	n.d.–3.0	n.d.–0.41
c/t12,14	3.3–7.4	n.d.–1.8	0.54–2.2	0.52–2.4	0.35–1.4	0.44–1.4
c/t11,13	8.7–11	1.4–2.9	1.7–4.3	0.52–1.1	0.92–1.7	3.2–4.3
t10,c12	0.73–1.7	2.0–6.2	1.6–7.0	1.5–4.0	2.0–8.3	1.3–4.2
c9,t11[b]	54–64	67–84	67–81	71–87	72–85	79–86
t7,c9	1.4–2.0	0.76–9.0	1.3–3.1	1.6–4.3	1.6–4.2	0.25–0.47
Partial sums of isomers (% CLA)						
Total *trans,trans*	18–26	8.8–16	12–18	6.5–23	9.4–15	7.2–12
Total *cis/trans*	74–82	84–91	82–88	77–94	85–91	88–93

[a] Total CLA was determined by the combination of GC-FID and Ag^+-HPLC techniques, as described in the text.

[b] This CLA isomer co-eluted with minor amounts of the t9,c11 isomer.

The ranges of values (minimum–maximum) shown were obtained from our data (Unpublished Results, n = 11).

n.d., not detected.

Chrompack), using an HPLC system (Agilent 1100 Series, Agilent Technologies Inc., Palo Alto, CA) equipped with an autosampler and a diode array detector adjusted at 233 nm, as described by Alfaia et al. [66]. In brief, the mobile phase was composed of 0.1% acetonitrile and 0.5% diethyl ether in *n*-hexane, maintained at a flow rate of 1 mL/min, and injection volumes of 20 µL were used. The identification of CLA isomers was achieved by spectral analysis and by comparison of their retention times with commercial standards and with data published in the literature [67,68]. Standards of CLA isomers (c9,t11, t10,c12, c11,t13, c9,c11, and t9,t11) were purchased from Matreya Inc. (Pleasant Gap, PA) or prepared (*cis/trans* and *trans,trans* from carbons 7,9 to 12,14) by the procedure reported by Destaillats and Angers [39]. The amounts of CLA isomers were calculated from their Ag^+-HPLC areas relative to the area of the main isomer c9,t11 identified by GC-FID (which comprises also the t7,c9 and t8,c10 isomers) as described by Kraft et al. [46]. A detailed description of the quantification process of individual CLA isomers using these two complementary methods was reported by Cruz-Hernandez et al. [36]. The total CLA content was calculated as the sum of its main isomer c9,t11 (plus t7,c9 and t8,c10) determined by GC-FID with the other identified minor isomers quantified by Ag^+-HPLC analysis. The individual CLA isomers were expressed as a percentage of the sum of identified *trans,trans* and *cis/trans* CLA isomers (% total CLA).

As expected, the range of contents of total CLA is much higher in beef by-products than in pork by-products (see Tables 9.3 and 9.4). In fact, CLA isomers are produced in the tissues through Δ9 desaturation of *trans* monoenes (c9,t11 and t7,c9), whereas the other isomers derive from the ruminal biohydrogenation of dietary C18 PUFA [34]. Regarding beef by-products, the range of values for total CLA (mg/100 g product) are relatively low in brain (1.1–2.5), heart (2.1–4.3), and kidney (3.9–8.4), and relatively high in liver (6.7–18), pancreas (8.7–18), and tongue (4.8–32). The same profile of range values for total CLA is presented by the pork by-products analyzed. These

differences may be explained, at least in part, by the distinct concentration of triacylglycerols in these organs (see Tables 9.1 and 9.2).

The diet is the major factor affecting the content and profile of CLA in ruminant meats [55,69]. Furthermore, it is also well known that many of the differences in the CLA profile appear to be related to pasture *vs.* concentrate feeding. French et al. [70] reported that meat fat from grazing steers has greater CLA contents (10.8 mg/g FAME) than that from animals fed concentrate (3.7 mg/g FAME). Regarding the CLA profile, pasture feeding when compared with concentrate feeding mainly increases the proportion of the t11,c13 isomer in beef lipids (up to 18.5% total CLA), with a decrease of the t7,c9 isomer (down to 4.1% total CLA), while increasing the percentages of t11,t13 and t12,t14 isomers [45]. These results suggest that the t11,c13, t12,t14, and t11,t13 CLA isomers are sensitive indicators of grass intake. The c9,t11 is the major CLA isomer in all beef (60%–89% total CLA) and pork (54%–87%) by-products analyzed (see Tables 9.3 and 9.4). The t11,c13 and the c/t11,13 (t11,c13 + c11,t13) are clearly the second-most prevalent CLA isomers in beef (18%–23%) and pork (8.7%–11%) brain, respectively. In general, the t7,c9 and t11,c13, or t9,t11 are the second- and third-most predominant CLA isomers, respectively, in the remaining beef by-products analyzed. In contrast, the percentages of the several minor CLA isomers in the pork by-products analyzed, with the exception of brain, are much more similar. However, since the content of some *cis/trans* CLA isomers in pork by-products is residual, their correct identification may require further analytic work.

Although the CLA isomeric distribution in ruminant meat is well known [71,72], to our knowledge, no CLA content or isomeric distribution was reported in the literature for any beef or pork by-product from animals fed a diet unsupplemented with CLA isomers. However, it is increasingly evident that different CLA isomers have distinct physiologic properties and that diet intake should increase in order to achieve the potential beneficial values. Thus, these methodologies are essential to evaluate meat by-products for its CLA content, to design experimental diets to increase the amount of CLA in meat fats, and to determine the CLA profile in these CLA-enriched meat by-products. For instance, these methodologies may be used to assess the effect of PUFA oil supplementation of ruminant diets, the most straightforward method to modify CLA content and proportion in ruminant meats [55,69], on CLA content and the isomeric profile in ruminant by-products. These analytical techniques may also be used to evaluate the CLA profile in monogastric animals fed commercial CLA preparations for enrichment of animal by-products in these fatty acids. For instance, Martin et al. [73] assessed the fatty acid composition of liver from pigs fed CLA in combination with MUFA by GC-FID using a 60 m SupelcoWax-10™ capillary column. Moreover, the influence of diet supplementation with CLA isomers (mixture of c9,t11 and t10,c12) on the lipid weight and fatty acid composition of lipid metabolizing (liver and retroperitoneal adipose) and lipid sensitive (spleen and heart) tissues of mice have been determined by GC-FID using a 100 m SP-2380 capillary column [74]. Finally, Kramer et al. [75] assessed the distribution of CLA isomers in tissue (liver, heart, backfat, and omental fat) lipid classes of pigs fed a CLA mixture by GC-FID (100 m CP-Sil 88 capillary column) and Ag^+-HPLC (one 25 cm ChromSpher 5 Lipids column). Ten and twelve CLA isomers in the diet and in pig tissue lipids were separated by GC-FID and Ag^+-HPLC, respectively.

9.4 NUTRITIONAL QUALITY OF FAT FROM EDIBLE ANIMAL BY-PRODUCTS

9.4.1 Fatty Acid Composition

The consumption of dietary fats has long been associated to chronic pathologies, such as obesity, diabetes, cancer, arthritis, asthma, and cardiovascular diseases (CVD). Although some controversy still exists in the role of dietary fats in human health, certain fatty acids have demonstrated their positive or negative effect on several chronic diseases. Thus, the current recommendations of the FAO/WHO for adult humans include intakes of 20%–35% of diet energy of total fat, <10% SFA,

15%–20% MUFA, 6%–11% PUFA, 2.5%–9% n-6 PUFA, 0.5%–2% n-3 PUFA, <1% TFA, and <300 mg/day cholesterol [1]. Moreover, an intake of 0.5–2 g/day EPA plus DHA is recommended for the prevention of CVD, and possibly, some cancers and degenerative diseases of ageing. It was also concluded that there is no scientific rationale for the continued recommendation of the specific ratio of n-6 to n-3 PUFA, or linoleic acid to α-linolenic acid (ALA, 18:3n-3) [1,76] described in the 1994 FAO/WHO report [77].

The 2008 FAO/WHO Expert Consultation [1] concluded that some individual SFA (lauric to palmitic acids, 12:0–16:0) increase LDL-cholesterol levels when compared with stearic acid (18:0). In addition, there is convincing evidence that substituting 12:0–16:0 with PUFA reduces LDL-cholesterol levels, the TC/HDL-cholesterol ratio, and the risk of CVD. This effect can also be observed, although to a lesser extent, by substituting these SFA with MUFA.

Dietary n-3 PUFA have effects on diverse physiological processes impacting normal health and chronic diseases, such as the regulation of plasma lipid levels, cardiovascular and immune function, insulin action, and neuronal development and visual function [78]. In recent years, there has been much interest in the beneficial effects of the long-chain n-3 PUFA, in particular EPA and DHA. In addition, it was found that their consumption in most Western populations is suboptimal for protection against the most prevalent chronic diseases. The principal biological role of ALA seems to be as a precursor for EPA synthesis [79]. Crucially, it is now evident that the in vivo synthesis of EPA and DHA from dietary ALA is very limited in adult humans. However, several studies suggest that high intakes of ALA can beneficially affect a number of CVD risk factors, including LDL-cholesterol [80]. Thus, more studies are needed in order to determine the potential health benefits of ALA intake.

Arachidonic acid, a precursor of eicosanoids with potent bioactivities, is usually the main long-chain PUFA in animal tissues. Although arachidonic acid can be synthesized from linoleic acid, meat and meat products are the main dietary sources of this fatty acid. In spite of this, there are a very limited number of studies on arachidonic acid nutrition in adult humans. Even in pregnant women, there are no studies that specifically show benefit to dietary levels of arachidonic acid apart from DHA and EPA [81]. However, since dietary arachidonic acid bypasses some of the regulatory rate-limiting enzymatic steps that control its incorporation into membranes (Δ6 and Δ5 desaturase), it can unbalance the level of long-chain n-3 PUFA in membranes and thus reduce their health beneficial effects [81].

The association between the intake of TFA (4%–6% of diet energy) with CVD risk has been indicated in five prospective cohort studies [82,83]. Evidence is accumulating that different *trans* 18:1 isomers have differential effects on the plasma ratio of LDL-cholesterol/HDL-cholesterol, and this is an area of active investigation [84]. For instance, there is support that *trans* 9 and *trans* 10 18:1 isomers are more powerful in increasing the plasma cholesterol ratio than VA [84]. VA, the major TFA in most ruminant meats, and the precursor for the tissue c9,t11 CLA isomer in both animals and man, should be considered as a neutral or beneficial *trans* isomer [85]. However, the effects of TFA from ruminant fats and from hydrogenated vegetable oils on metabolic risk parameters remain to be established. The opinion of the European Food Safety Authority (EFSA) about the presence of TFA in foods and their effect on human health was reported in 2004 [82]. Most health professional organizations and some governments now recommend that the consumption of foods containing TFA [83] be reduced.

Attending to the imbalance of fatty acids in the typical Western diets, it is widely acknowledged that the nutritional trend should be to reduce the intakes of SFA, TFA, and n-6 PUFA, and to increase the intakes of n-3 PUFA, particularly EPA and DHA. A survey of European diets revealed that 21% of total fat intake comes from meat and meat products [86]. It is believed that the lower total and n-3 PUFA, as well as the higher SFA and n-6 PUFA of some meats contribute to the imbalance in the fatty acid intake of today's consumers [59,87]. As described before, the range of contents of total fatty acids for each by-product is similar for beef and pork but depends on the type of meat by-product (relatively low in heart, kidney and liver, intermediate in brain and varies

widely in pancreas and tongue). In spite of the biohydrogenation of unsaturated fatty acids in the rumen, the percentages of SFA, MUFA, and PUFA are similar between the homologues beef and pork by-products. The lipids of the beef and pork by-products reviewed are relatively saturated. The percentages of n-3 PUFA are relatively low in beef and pork by-products, with the exception of DHA (and docosapentaenoic acid) in the brain of both species. The contents of n-3 PUFA in meat and meat products were reviewed by Givens et al. [87]. Animal tissue lipids are important sources of n-3 PUFA in human diets [85]. Moreover, although the main source of EPA and DHA (and of total long-chain n-3 PUFA) is fish and seafood, the major sources of docosapentaenoic acid are ruminant and poultry meats. Finally, for people who do not consume fish, meat and meat products are the only source of EPA and DHA in the diet.

As expected, the relative proportions of TFA in all beef by-products reviewed are relatively high, while in pork by-products they are only residual. The TFA contents in meat and meat products were reviewed by Fritsche and Steinhart [23] and Valsta et al. [88]. The major sources of TFA in human diet are industrial and ruminant fats. The contribution of TFA from ruminant fat ranges 30%–80% of the total TFA, corresponding to 0.3%–0.8% of diet energy [89]. It was estimated that meat and meat products may contribute from 10% to 30% of TFA (higher in Mediterranean countries than in other countries) for human diet [90].

From a nutritional point of view, these animal by-products are, in general, relatively rich in SFA, cholesterol, and TFA (from ruminant origin), and therefore, should be part of a balanced diet only in small amounts. Beef by-products, relative to the homologues pork by-products, not only have high contents of TFA (prejudicial) but also high levels of CLA isomers and its endogenous precursor VA (beneficial). Some meat by-products may be excellent sources of long-chain PUFA. Brain has very high contents of long-chain n-3 PUFA (mainly DHA but also docosapentaenoic acid), although also very high levels of cholesterol. Based on these nutritional characteristics, in addition to the possible accumulation of pesticides, drug residues, and toxic heavy metals, some health organizations (e.g., United States Department of Health) recommend that only limited amounts of meat by-products be eaten [53].

9.4.2 Conjugated Isomers of Linoleic Acid

Many experimental studies, using mainly laboratory animals and cell-culture systems, suggest that some CLA isomers exert potentially positive effects on cancer, CVD, diabetes, body composition, inflammation, the immune system, and bone health (reviewed by Wahle et al. [33] and Bhattacharya et al. [91]). The National Academy of Sciences of the United States has recognized CLA as the only fatty acid that has been shown unequivocally to inhibit carcinogenesis in experimental animals [92]. The mechanism of carcinogenesis modulation by CLA is not completely understood, although it may be related to its anti-oxidative properties or with the induction of apoptotic cell death and cell cycle regulation [93]. However, specific physiological effects have been linked to individual CLA isomers. The t10,c12 isomer may play an important role on lipid metabolism, while the c9,t11 and the t10,c12 isomers seem to be equally effective in anticarcinogenesis [94]. However, the biological effects of these CLA isomers in humans remains to be established [93]. Few studies have investigated the health effects in humans of naturally occurring CLA from foods and evidence is weak and conflicting with respect to any health effects at current levels of intake. CLA research in humans has focused mainly on the effects of the t10,c12 isomer, which is a minor isomer in foodstuffs, rather than on the major isomer c9,t11. This is due to the circumstance that commercial CLA preparations consist of an equal mixture of t10,c12 and c9,t11 isomers. Thus, research to assess the benefits of CLA isomers for humans will constitute a unique challenge in the coming years.

Although optimal dietary intake in humans are not known, on the basis of the anticancer effects of CLA in rats, a daily consumption of 0.8–3.0 g of CLA might provide a significant health benefit to humans [69]. Depending on the country, the estimation so far of average total CLA or c9,t11 isomer consumption ranges between 95 and 440 mg (reviewed by Collomb et al. [95] and Martins

et al. [71]). However, in some countries (e.g., Australia) consumption of CLA may reach 1.5 g/day [96]. Regarding this, it is necessary to increase CLA intake in order to achieve its potential benefits on the prevention and treatment of several pathologies. Since meat and meat products contribute about 25%–30% of the total human CLA intake in Western populations, the inclusion in the diet of ruminant-derived foods rich in CLA may be very desirable. As described above, the range of contents of total CLA is much higher in the beef by-products than in the pork by-products reviewed. Regarding beef by-products, the range of values for total CLA are relatively low in brain, heart, and kidney, and relatively high in liver, pancreas, and tongue. A similar profile of range values for total CLA is presented by the pork by-products analyzed. These differences may be explained, at least in part, by the level of fatness of these organs. The CLA contents in meat and meat products were reviewed by Parodi [97] and Schmid et al. [69]. The highest CLA concentrations were found in lamb (4.3–19.0 mg/g lipid), with slightly lower concentrations in beef (1.2–10.0 mg/g lipid), representing 0.5%–2% of fatty acids. The CLA content of pork, chicken, and meat from horses is usually lower than 1 mg/g lipid. Furthermore, CLA contents (mg/g fat) vary substantially not only between species but also from animal to animal and within an animal in different tissues.

Given that individual CLA isomers have different biological activities, the determination of the CLA isomeric profile in ruminant-derived fat is required. As expected, the bioactive c9,t11 isomer is the main CLA isomer in all beef and pork by-products, being lower in the brain of both species. The main CLA isomer in meat and meat products, c9,t11 (~80% total CLA), is mainly associated with the triacylglycerol lipid fraction, and therefore, is positively correlated with the level of fatness. The t11,c13 isomer (beef) and c/t11,13 (pork) are clearly the second-most predominant CLA isomers in brain but not in the remaining meat by-products analyzed, where the t7,c9 CLA isomer is the second-most prevalent. The biological significance of the preferential deposition of c/t11,13 CLA isomers in brain is completely unknown. However, a preferential accumulation of the c11,t13 CLA isomer into the inner mitochondrial membrane of a pig heart and liver has been described before by Kramer et al. [75]. These authors hypothesized that the c11,t13 CLA isomer may have some biological activity because it is a structural analogue of 20:2c11,t13, a metabolite of c9,t11 CLA isomer, with similar biological activity to that of CLA isomeric mixtures.

The other bioactive CLA isomer, t10,c12, is only present in residual percentages in the beef and pork by-products analyzed. Concerning the estimation of average CLA daily intake, the above proposed range should only be achieved through diet supplementation. It is of note that dietary supplements have different CLA isomeric profiles compared to foodstuffs. The main difference concerns the high percentage of t10,c12 in supplements, which may have adverse effects on human health, as was suggested by Zee et al. [98].

9.5 CONCLUSIONS

An overview of the analytical techniques and methodologies available for the analysis of fatty acids, including those necessary for the individual separation CLA isomers in edible animal by-products, was presented in this chapter. For the detailed analysis of fatty acid composition in ruminant-derived by-products, which include a large number of minor fatty acids (*trans*, conjugated, and odd- and branched-chain fatty acids), a previous silver ion chromatographic *cis/trans* fractionation followed by GC analysis with long (100 m) highly polar capillary columns is required. The determination of the much simpler fatty acid profile in monogastric-derived by-products, mainly composed by linear even-chain fatty acids, can be performed by the straight GC separation of FAME with short (30 m) polyethylene glycol capillary columns. Regarding the analysis of total and individual CLA isomers in animal by-products, a combination of GC-FID and Ag^+-HPLC should be used. The CLA peaks are quantified by the GC analysis of the total FAME and the relative concentrations obtained by Ag^+-HPLC (three 25 cm columns in tandem) are used to calculate the unresolved peaks in the GC chromatogram. The fatty acid composition compiled from the very limited information published in the literature for some of the most eaten animal by-products (beef and pork brain, heart, kidney,

liver, pancreas, and tongue) was complemented with original data. Finally, the most used analytical methodologies were illustrated with possible applications to assess the nutritional quality of fat in some of the meat relevant by-products.

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10 Vitamins

Young-Nam Kim

CONTENTS

10.1 INTRODUCTION

Vitamins are a group of complex organic compounds that are essential to normal functioning and essential metabolic reactions in the body. Vitamins are not utilized as a source of energy or as a source of structural tissue components, but rather as cofactors or coenzymes in biochemical reactions. Vitamins are divided into two categories based on their solubility—those soluble in fat organic solvents are known as fat-soluble vitamins and those soluble in water are known as water-soluble vitamins.

Tables 10.1 and 10.2 list the concentrations of fat-soluble vitamins and water-soluble vitamins, respectively, in selected edible animal by-products according to the U.S. Department of Agriculture National Nutrient Database [1]. The percentages of the daily value (DV) of the vitamin estimated to be in 100 g of edible animal by-products are included in Tables 10.1 and 10.2. The DV, established by the U.S. Food and Drug Administration (FDA), is a nutrient reference value intended to help consumers understand how foods fit into their overall diets [2]. Foods containing 20% or more of the DV of nutrients per reference amount are indicated to be "high," "rich," or "excellent" sources of the nutrients. Foods containing 10%–19% of the DV are categorized as "good" sources. Most of edible animal by-products such as heart, kidneys, liver, and pancreas are good to excellent sources of most of the B-vitamins as defined by the U.S. FDA [3]. Fat-soluble vitamin A is present in edible animal by-products in varying amounts, often in concentrations higher than those in other meats. Considerable amounts of vitamin C are found in kidneys, liver, pancreas, and spleen of animals. Vitamins D and K contents of only several edible animal by-products have been provided by U.S. FDA.

TABLE 10.1
Concentrations of Fat-Soluble Vitamins in Selected Edible Animal By-Products[a]

	Vitamin A		Vitamin E		Vitamin D		Vitamin K	
Food Product	**IU/100 g[b]**	**% DV[c]**	**mg/100 g[d]**	**% DV**	**IU/100 g[e]**	**% DV**	**µg/100 g**	**% DV**
Pork								
Brain, raw	0	0	—[f]	—	—	—	—	—
Chitterlings, raw	0	0	0.18	1	—	—	0	0
Ears, frozen, raw	0	0	—	—	—	—	—	—
Feet, raw	0	0	0.02	<1	—	—	—	—
Heart, raw	25	<1	0.63	3	—	—	—	—
Jowl, raw	3	<1	0.29	2	—	—	—	—
Kidneys, raw	198	4	—	—	—	—	—	—
Leaf fat, raw	0	0	—	—	—	—	—	—
Liver, raw	21,650	433	—	—	—	—	—	—
Lungs, raw	0	0	—	—	—	—	—	—
Pancreas, raw	0	0	—	—	—	—	—	—
Spleen, raw	0	0	—	—	—	—	—	—
Stomach, raw	0	0	0.04	<1	0	0	0	0
Tail, raw	0	0	—	—	—	—	—	—
Tongue, raw	0	0	0.29	2	—	—	—	—
Beef								
Brain, raw	147	3	0.99	4	—	—	0	0
Heart, raw	0	0	0.22	1	—	—	0	0
Kidneys, raw	1,379	28	0.22	1	45	11	0	0
Liver, raw	16,898	338	0.38	2	49	12	3.1	3.9
Lungs, raw	46	<1	—	—	—	—	—	—
Pancreas, raw	0	0	—	—	—	—	—	—
Spleen, raw	0	0	—	—	—	—	—	—
Suet, raw	0	0	1.50	8	—	—	3.6	4.5
Thymus, raw	0	0	—	—	—	—	—	—
Tongue, raw	0	0	—	—	—	—	—	—
Tripe, raw	0	0	0.09	<1	0	0	0	0
Veal								
Brain, raw	0	0	—	—	—	—	—	—
Heart, raw	0	0	—	—	—	—	—	—
Kidneys, raw	308	6	—	—	—	—	—	—
Liver, raw	39,056	781	0.37	2	—	—	0.9	1.1
Lungs, raw	0	0	—	—	—	—	—	—
Pancreas, raw	0	0	—	—	—	—	—	—
Spleen, raw	0	0	—	—	—	—	—	—
Thymus, raw	0	0	0.09	<1	—	—	0	0
Tongue, raw	1	<1	—	—	—	—	—	—
Lamb								
Brain, raw	0	0	—	—	—	—	—	—
Heart, raw	0	0	—	—	—	—	—	—
Kidneys, raw	316	6.3	—	—	—	—	—	—
Liver, raw	24,612	492	—	—	—	—	—	—

TABLE 10.1 (continued)
Concentrations of Fat-Soluble Vitamins in Selected Edible Animal By-Products[a]

	Vitamin A		Vitamin E		Vitamin D		Vitamin K	
Food Product	**IU/100 g[b]**	**% DV[c]**	**mg/100 g[d]**	**% DV**	**IU/100 g[e]**	**% DV**	**μg/100 g**	**% DV**
Lungs, raw	89	2	—	—	—	—	—	—
Pancreas, raw	0	0	—	—	—	—	—	—
Spleen, raw	0	0	—	—	—	—	—	—
Tongue, raw	0	0	—	—	—	—	—	—

[a] Data obtained from U.S. Department of Agriculture National Nutrient Database for Standard Reference, Release 22 [1].
[b] International Unit (IU) vitamin A = 0.3 μg of all-trans-retinol or 0.6 μg of β-carotene.
[c] Percent Daily Value (DV), established by the U.S. Food and Drug Administration [2].
[d] As mg α-tocopherol.
[e] IU vitamin D = 0.025 μg cholecalciferol or ergocalciferol.
[f] No composition data provided by U.S. Department of Agriculture.

Generally, the methodologies used for determining the composition of the various vitamins in edible animal by-products are the same as those used for other foods. Several high-performance liquid chromatography (HPLC) methods using various detections [4–8] have been proposed to determine several vitamins simultaneously. Internal standards are frequently used in the analytical methods for determination of the vitamins. Several vitamins are lost or interconverted to their isomers in extraction and purification procedures during analyses. Various methods have been used for the determination of vitamins in foods. In-depth recent reviews of the methodologies for measuring vitamins are available [9,10].

10.2 FAT-SOLUBLE VITAMINS

10.2.1 Vitamin A and Carotenoids

Vitamin A is a fat-soluble vitamin that is essential for humans and other vertebrates. Vitamin A as retinoids, primarily retinyl esters, is abundant in some animal-derived foods, whereas carotenoids are abundant in plant foods as pigments. Carotenoids cannot be synthesized by the live animals but are obtained from their feeds. Because vitamin A and carotenoids are fat-soluble, they are associated with the fat portion of foods. Because most of vitamin A is metabolized and stored in an animal's liver, it is highly concentrated in the liver. In foods, retinyl esters and carotenoids are vulnerable to oxidation. Exposure to air, heat, and storage time also affect the destruction of vitamin A compounds. Thus, overcooking can cause loss of retinyl esters and provitamin A in foods. Reversed-phase HPLC followed by ultraviolet (UV) detection for retinoids and carotenoids is the most common method of analysis. HPLC methodologies are given in AOAC Official Methods 2001.13 and 2005.07 [11]. However, the analysis methods have not been presented yet for determination of retinoids and in edible animal by-products. During sample preparation and analysis, samples should be protected from heat, light, and oxidizing substances to avoid destructions and isomerizations of the retinoids and carotenoids. Antioxidants such as butylated hydroxytoluene (BHT), pyrogallol, or ascorbyl palmitate are used to prevent oxidation of retinoids and carotenoids.

Alkali hydrolysis (saponification) is routinely used to extract retinoids and carotenoids from foods. Saponification removes chlorophylls, unwanted lipids, and other materials, which may interfere with the chromatographic separation. Retinyl esters and carotenoid esters in foods are converted to retinol and carotenoids during saponification. However, the degradation and isomerization of retinol and carotenoids may occur during saponification. This is greater with higher concentrations of alkali and higher temperatures [12]. Hexane, petroleum ether, diethyl ether, dichloromethane, or

TABLE 10.2
Concentrations of Water-Soluble Vitamins in Selected Edible Animal By-Products[a]

	Thiamin		Riboflavin		Niacin		Vitamin B_6		Folate		Vitamin B_{12}		Pantothenic Acid		Vitamin C	
Food Product	**mg/100 g**	**% DV[b]**	**mg/100 g**	**% DV**	**mg/100 g**	**% DV**	**mg/100 g**	**% DV**	**µg/100 g**	**% DV**	**µg/100 g**	**% DV**	**mg/100 g**	**% DV**	**mg/100 g**	**% DV**
Pork																
Brain, raw	0.155	10	0.275	16	4.275	22	0.190	10	6	2	2.19	37	2.800	28	13.5	23
Chitterlings, raw	0.020	4	0.091	5	0.215	1	0.014	<1	3	<1	0.82	14	0.227	2	1.1	2
Ears, frozen, raw	0.080	5	0.110	6	0.780	4	0.020	1	0	0	0.07	<1	0.068	<1	0.0	0
Feet, raw	0.026	2	0.106	6	1.130	6	0.053	3	10	3	0.52	9	0.303	3	0.0	0
Heart, raw	0.613	41	1.185	70	6.765	34	0.390	20	4	1	3.79	63	2.515	25	5.3	9
Jowl, raw	0.368	25	0.236	14	4.536	23	0.090	5	1	<1	0.82	14	0.250	3	0.0	0
Kidneys, raw	0.340	23	1.697	100	8.207	41	0.440	22	42	11	8.79	147	3.130	31	13.3	22
Leaf fat, raw	0.106	7	0.065	4	1.249	6	0.030	2	0	0	0.23	4	0.000	0	0.0	0
Liver, raw	0.283	19	3.005	177	15.301	77	0.690	35	212	53	26.00	433	6.650	67	25.3	42
Lungs, raw	0.085	6	0.430	25	3.345	17	0.100	5	3	<1	2.75	46	0.900	9	12.3	21
Pancreas, raw	0.105	7	0.460	27	3.450	17	0.460	23	3	<1	16.40	273	4.555	46	15.3	26
Spleen, raw	0.130	9	0.300	18	5.867	29	0.060	3	4	1	3.26	54	1.055	11	28.5	48
Stomach, raw	0.051	3	0.201	12	2.480	12	0.034	2	3	<1	0.30	5	1.220	12	0.0	0
Tail, raw	0.210	14	0.110	6	2.060	10	0.370	19	5	1	0.88	15	0.673	7	0.0	0
Tongue, raw	0.490	33	0.485	29	5.300	27	0.240	12	4	1	2.84	47	0.641	6	4.4	7
Beef																
Brain, raw	0.092	6	0.199	12	3.550	18	0.226	11	3	<1	9.51	159	2.010	20	10.7	18
Heart, raw	0.238	16	0.906	53	7.530	38	0.279	14	3	<1	8.55	143	1.790	18	2.0	3
Kidneys, raw	0.357	24	2.840	167	8.030	40	0.665	33	98	25	27.50	458	3.970	40	9.4	16
Liver, raw	0.189	13	2.755	162	13.175	66	1.083	54	290	73	59.30	988	7.173	72	1.3	2

Lungs, raw	0.047	3	0.230	14	4.000	20	0.040	2	11	3	3.81	64	1.000	10	38.5	64
Pancreas, raw	0.140	9	0.445	26	4.450	22	0.200	10	3	<1	14.00	233	3.900	39	13.7	23
Spleen, raw	0.050	3	0.370	22	8.400	42	0.070	4	4	1	5.68	95	1.081	11	45.5	76
Suet, raw	0.007	<1	0.013	<1	0.259	1	0.030	2	1	<1	0.27	5	0.025	<1	0.0	0
Thymus, raw	0.109	7	0.345	20	3.452	17	0.160	8	2	<1	2.13	36	3.026	30	34.0	57
Tongue, raw	0.125	8	0.340	20	4.240	21	0.310	16	7	2	3.79	63	0.653	7	3.1	5
Veal																
Brain, raw	0.130	9	0.260	15	4.300	22	0.280	14	3	<1	12.20	203	2.720	27	14.0	23
Heart, raw	0.520	35	1.000	59	6.400	32	0.430	22	2	<1	13.76	229	2.780	28	8.0	13
Kidneys, raw	0.320	21	1.900	112	6.990	35	0.370	19	21	5	28.20	470	3.300	33	5.0	8
Liver, raw	0.173	12	2.440	144	10.550	53	0.957	48	125	31	59.85	998	6.065	61	0.7	1
Lungs, raw	0.047	3	0.231	14	4.025	20	0.110	6	11	3	3.83	64	—[c]	—	39.0	65
Pancreas, raw	0.134	9	0.425	25	4.252	21	0.190	10	3	<1	13.83	231	—	—	16.0	27
Spleen, raw	0.047	3	0.348	2	7.895	39	0.110	6	4	1	5.34	89	—	—	41.0	68
Thymus, raw	0.069	5	0.188	11	4.770	24	0.035	2	22	6	3.33	56	1.230	12	49.2	82
Tongue, raw	0.170	11	0.410	24	2.220	11	0.190	10	5	1	6.10	102	1.200	12	5.0	8
Lamb																
Brain, raw	0.130	9	0.300	18	3.900	20	0.290	15	3	<1	11.30	188	0.920	9	16.0	27
Heart, raw	0.370	25	0.990	58	6.140	31	0.390	20	2	<1	10.25	171	2.630	26	5.0	8
Kidneys, raw	0.620	41	2.240	13	7.510	38	0.220	11	28	7	52.41	874	4.220	42	11.0	18
Liver, raw	0.340	23	3.630	21	16.110	81	0.900	45	230	58	90.05	150	6.130	61	4.0	7
Lungs, raw	0.048	3	0.237	14	4.214	21	0.110	6	12	3	3.93	66	—	—	31.0	52
Pancreas, raw	0.030	2	0.250	15	3.700	19	0.070	4	13	3	6.00	100	1.000	10	18	30
Spleen, raw	0.047	3	0.348	20	7.895	39	0.110	6	4	1	5.34	89	—	—	23.0	38
Tongue, raw	0.150	10	0.380	22	4.650	23	0.180	9	4	1	7.20	120	0.970	10	6.0	10

[a] Data obtained from U.S. Department of Agriculture National Nutrient Database for Standard Reference, Release 22 [1].

[b] Percent Daily Value (DV), established by the U.S. Food and Drug Administration [2].

[c] No composition data provided by U.S. Department of Agriculture.

mixtures of these solvents are common extracting solvents. The reverse-phase C_{18} column is commonly used to resolve retinoids and carotenoids in foods. The polymeric C_{30} column designed at the U.S. National Institute of Standards and Technology [13] provides high absolute retention and resolution of *cis* and *trans* isomers of carotenoids [14]. Acetonitrile- or methanol-based mobile phases are used by the addition of 1% or 0.1 M ammonium acetate or acetic acid [15]. Maximum absorbance is 320–380 nm for retinoids and 400–500 nm for carotenoids. Thus, to perform simultaneous HPLC analysis of retinoids and carotenoids, a photodiode array detector is essential to establish the identity of the compound in each peak and validate homogeneity.

There are several units used for expressing vitamin A contents in foods. International units (IU) are defined by the relationship of 1 IU = 0.3 μg of all-*trans*-retinol or 0.6 μg of β-carotene. The biological activity of vitamin A is quantified by conversion of retinol and provitamin A carotenoids to retinol equivalents (RE). One RE is defined as 1 μg of retinol, 6 μg of β-carotene, or 12 μg of other provitamin A carotenoids. Therefore, 1 RE is equal to 3.33 IU based on retinol. In 2001, the U.S. Institute of Medicine (IOM) [16] proposed the new vitamin A unit, retinol activity equivalents (RAE). One RAE is equivalent to 1 μg of retinol, which is nutritionally equivalent to 12 μg of β-carotene or 24 μg of other provitamin A carotenoids.

10.2.2 Vitamin D

Vitamin D is a fat-soluble vitamin found in foods and also synthesized in the body after exposure to UV rays from the sun. Several forms of vitamin D have been described, but the two major physiologically relevant ones are vitamin D_2 and vitamin D_3 [17]. Vitamin D_2 (ergocalciferol) is a synthetic form of vitamin D that is produced by irradiation of plant and yeast steroid ergosterol. Vitamin D_3 (cholecalciferol) is the naturally occurring form of vitamin D produced from 7-dehydrocholesterol when the skin of animals and humans is exposed to sunlight, specifically UV-B radiation. Vitamin D is biologically inactive and is metabolized to 25-hydroxyvitamin D (25(OH)D) in the liver, which is the most abundant form of vitamin D in the circulatory system. This circulating metabolite is hydroxylated again to form its biologically active hormone, 1,25-dihydroxyvitamin D ($1,25(OH)_2D$), which acts as a hormone in controlling calcium homeostasis and regulating the growth of various cell types [18]. Most of vitamin D in animal foods is vitamin D_3 and $25(OH)D_3$, and liver and kidneys among edible animal by-products are good sources of vitamin D (Table 15.1). Therefore, to estimate vitamin D values in edible animal by-products, analyses of $25(OH)D_3$ should be included. Food processing, cooking, and storage of foods do not generally affect the concentration of vitamin D [18]. HPLC methods using an UV absorbance detector are available for the quantitation of vitamin D from most food matrices. Several HPLC methodologies for vitamin D analyses are provided in published articles [19–24], including AOAC Official Method 995.05, which is the method for determination of vitamin D in infant formulas and enteral products [11]. Vitamin D oxidation can occur during the oxidation of fats. Thus, an antioxidant such as pyrogallol or ascorbic acid is added when analyzing food samples.

Estimation of the low concentrations of vitamin D in edible animal by-products is often difficult due to interfering substances such as fats, cholesterol, vitamin A, and vitamin E. To remove fats, saponification and cleanup procedures should be applied. Hot saponification promotes thermal isomerization of vitamin D with the formation of previtamin D. Hence, several methods [21,25] have been used for saponification at ambient temperature overnight. After saponification, unsaponified lipids including vitamin D are extracted with diethyl ether:petroleum ether, 1:1 [21–23]. Vitamin A, vitamin E, sterols, and other interfering components in the unsaponified fraction are removed using a silica solid-phase extraction [20–27]. Both reversed-phase and normal-phase systems offer efficient resolution of vitamin D, 7-dehydrocholesterol, and hydroxylated metabolites. However, reversed-phase chromatography (RPC) can separate vitamin D_2 from vitamin D_3 [22,28]. Vitamin D shows identical UV absorption spectra with λ_{max} at 265 nm, which is sensitive enough for the detection of vitamin D_2, vitamin D_3, and their metabolites.

Vitamin D content in foods is expressed in either IU or μg of vitamin D. One IU of vitamin D is the activity obtained from 0.025 μg of cholecalciferol in bioassays. The activity of 25(OH)D is five times more potent than cholecalciferol. Therefore, the biological activity of 1 μg of vitamin D is 40 IU, and 1 IU is 0.005 μg of 25(OH)D or 0.025 μg D_2 or D_3 [17].

10.2.3 Vitamin E

Vitamin E is the most effective fat-soluble antioxidant known to occur in the human body. Natural vitamin E exists in eight different forms, four tocopherols (α-, β-, γ-, and δ-tocopherols) and four tocotrienols (α-, β-, γ-, and δ-tocotrienols). Vitamin E is found in plant and animal foods. Edible animal by-products provide small amounts of vitamin E. During processing, losses of vitamin E can occur quite rapidly. Losses are accelerated by oxygen, light, heat, and various metals, primarily iron and copper, and by the presence of free radicals in the fat that can initiate autoxidation.

For vitamin E assay in foods, HPLC methods using fluorescence or UV detection have largely replaced the colorimetric and polarimetric procedures of AOAC Official Methods 948.26, 971.30, and 975.45 [11]. Although gas chromatography (GC) methodologies (AOAC Official Methods 988.14 and 989.09) were developed to increase the precision of vitamin E quantification before the advent of HPLC procedures, HPLC is considered a suitable measurement of the individual tocopherols and tocotrienols as GC methodologies can be time consuming. HPLC methodologies are provided in AOAC Official Method 992.03 [11] and other published articles [4,29–31].

Quantification of vitamin E in oils can be directly injected onto a normal-phase liquid chromatography column after dilution with *n*-hexane or mobile phase [32]. However, alkaline hydrolysis for edible animal by-products is usually required to release the α-tocopherol [33]. Hydrolysis results in cleavage of the ester linkages of lipids in food samples, destroys pigments, and disrupts the sample matrix, which facilitates vitamin E extraction. Following saponification, the digest is extracted with ether, petroleum ether, hexane, ethyl acetate in hexane, or other organic solvent mixtures. During saponification, addition of antioxidants, pyrogallol, ascorbic acid, or BHT in extraction solvents, and protection from light are required to prevent losses of vitamin E. Both reversed-phase and normal-phase systems are useful for the resolution of vitamin E. The advantage of normal-phase HPLC systems is the ability to separate the eight tocopherols and tocotrienols that occur in nature. Reversed-phase systems cannot resolve the β- and γ-isomers [34]. However, reversed-phase HPLC is the preferred system for the determination of α-tocopherol with retinol and carotenoids in foods. Fluorescence detection provides sensitivity, specificity, and cleaner chromatograms compared to UV detection [35]. Although the UV absorbance of tocopherols and tocotrienols is relatively weak, detection at the absorption maximum (292–298 nm) using a variable wavelength detector affords sufficient sensitivity for most applications [36]. For simultaneous detection of vitamin E with other fat-soluble vitamins and carotenoids, a multichannel UV detector or a photodiode array detector may be useful in a single sample assay.

IUs of vitamin E activity (1 mg of α-tocopherol = 1.49 IU) may be used in food composition tables, but the IU is not commonly used. The vitamin E composition in foods is often expressed as mg of α-tocopherol equivalents based on the biological activity of the various forms of vitamin E [37]. However, the U.S. IOM [38] has indicated that the only form of the tocopherols and tocotrienols, which has vitamin E activity in humans, is α-tocopherol.

10.2.4 Vitamin K

Vitamin K is a fat-soluble vitamin. Two forms of vitamin K exist in nature: phylloquinone and menaquinones. Phylloquinone, known as vitamin K_1, is synthesized by plants. Menaquinones, known as vitamin K_2, are produced by bacteria and contain a polyisoprenyl side chain at the 3 position [39]. However, one of menaquinones, menaquinone (MK)-4, is not a major bacterial product, but is

synthesized by animals from phylloquinone [40,41]. Menadione, vitamin K_3, is a synthetic form of vitamin K. Menadione and its derivatives are used as additives in the feed industry. Menadione can be also converted to MK-4 in animal tissues [40,42]. There are only minute amounts of vitamin K in most edible animal by-products. Vitamin K is quite stable to oxidation and most food processing and food preparation procedures, while it is unstable to light, alkali, strong acid, and reducing agents [43].

Current methods to determine vitamin K in foods are HPLC procedures using fluorescence or electrochemical detection systems. AOAC Official Methods 992.27 and 999.15 [11] for phylloquinone determination in infant formulas and several HPLC methodologies [44–47] determining simultaneously phylloquinone and menaquinones have been developed. GC procedures have been described, but are not routinely used due to long retention times and the potential for on-column degradation from high column temperatures.

When analyzing vitamin K, saponification cannot be applied to remove fats and other components because of the instability of vitamin K under alkaline conditions. Vitamin K is extracted from foods with organic solvents such as ethanol, isopropanol, and acetonitrile, and then purified by solid-phase extraction with silica cartridges before the resolution of vitamin K by reversed-phase HPLC. Phylloquinone and menaquinones are detected by UV detection, but lipids and other interfering compounds remaining in extract solutions make UV detection unworkable for foods containing high fats. Although vitamin K does not fluoresce, the quinones are reduced to hydroquinones by the addition of zinc chloride using a post-column zinc metal reduction column, so that fluorescence detection provides a highly specific detection system for vitamin K determination in foods. Electrochemical detection is also used by adding an electrolyte such as sodium acetate or perchlorate in the mobile phase to support the conductivity. For the determination of phylloquinone and menaquinones in animal products, Koivu-Tikkanen et al. [44] extracted the samples with 2-propanol/hexane after adding internal standards. Sample extracts were purified by normal-phase HPLC and the fraction containing vitamin K was analyzed by reversed-phase HPLC using post-column reduction and fluorescence detection. The vitamin K composition of foods is commonly expressed as μg of vitamin K.

10.3 WATER-SOLUBLE VITAMINS

10.3.1 Thiamin (Vitamin B_1)

Thiamin, known as vitamin B_1, is one of the B-vitamins. Thiamin exists in interconvertible phosphorylated forms in nature: thiamin monophosphate, thiamin pyrophosphate, and thiamin triphosphate. Organs of animals are good to excellent sources of thiamin. Thiamin is most stable between pH 2 and 4, and unstable at alkaline pH [48]. In alkaline solution, thiamin is readily oxidized, even at room temperature. It is the most heat-labile of the B-vitamins with its decomposition dependent on pH and exposure time to heat. Also, thiamin is known as the most radiation-sensitive, water-soluble vitamin [49].

Several different analytical methodologies have been used to determine the thiamin content of foods. These methodologies include the fluorometric methods of AOAC Official Methods 942.23, 953.17, 957.17, and 986.27 [11], microbiological analyses [50–52], HPLC methods [6,7,53–55], and GC procedures [56,57].

Extraction procedures for the microbiological, HPLC, and GC analyses generally follow the thiochrome analysis procedures of AOAC Official Method 942.23 [11]. Because thiamin is stable under acidic conditions, hot acid hydrolysis with HCl is used to release the thiamin and thiamin phosphate esters from their associations with proteins, followed by enzyme hydrolysis of the phosphorylated thiamin to free thiamin using takadiastase, Mylase 100® (U.S. Biochemical Corp.), or α-amylase. The use of the same extraction procedure allows both thiamin and riboflavin in foods to be separated and quantitated by HPLC simultaneously.

Microbiological analyses depend on the extent of growth of a thiamin-dependent organism such as *Lactobacillus*(*L.*) *fermentum* and *L. viridescens. L. fermentum* is susceptible to matrix effects such as carbohydrates, fats, and some minerals in a growth medium [58], but *L. viridescens* is more specific for thiamin and not susceptible to matrix effects. Microbiological assay is still used in food analysis, but AOAC International [11] does not provide an official method for thiamin determination using microorganisms.

HPLC methods have been recently developed to allow the rapid, sensitive, and specific analysis of thiamin and its phosphorylated forms in drugs and biological materials. Thiamin is measured itself with absorbance detection, usually at 245–254 nm, or with fluorescence detection systems after conversion to thiochrome. Although absorbance detection has sensitivity for high-thiamin-containing foods, it may not be appropriate for foods containing small amount of thiamin. Fluorescence detection is much more sensitive than absorbance detection. Therefore, HPLC with fluorescence detection has been widely used for the determination of thiamin in foods including animal-derived foods. Thiamin itself does not fluoresce, so thiamin should be converted to thiochrome using reagents for alkaline oxidation either by post-column or pre-column derivatization. The maximum fluorescence of thiochrome is excitation λ 365–375 nm and emission λ 425–435 nm. Recently, Lebiedziñska et al. [7] reported the simultaneous determination of thiamin, vitamins B_6 and B_{12} in several types of foods using HPLC method with electrochemical and UV detection. The mobile phase pH should be kept above 8, because the fluorescence intensity of thiochrome is pH-dependent and reaches a steady state at a pH above 8 [59]. Milligrams of thiamin are frequently used for expressing the content of thiamin in foods.

10.3.2 Riboflavin (Vitamin B_2)

Riboflavin, known as vitamin B_2, is a water-soluble vitamin naturally found in foods. Riboflavin acts as an integral component of two coenzymes: flavin adenine dinucleotide (FAD) and flavin mononucleotide (FMN). Riboflavin occurs naturally in foods as free riboflavin and as the protein-bound coenzymes, FAD and FMN. Several edible animal by-products including heart, kidneys, liver, and pancreas are excellent sources of riboflavin. Riboflavin is stable to heat, acidic conditions, and oxidation if light is excluded. Riboflavin is destroyed by exposure to UV and visible light within the range of 420–560 nm. The rate of destruction is accelerated by increasing temperature and pH. Thus, riboflavin is generally stable during heat processing and normal cooking of foods if protected from light.

Several methods have been proposed for the determination of riboflavin in foods, usually involving the conversion of FAD and FMN to free riboflavin. The fluorometric methods of AOAC Official Methods 970.65 and 981.15 [11], microbiological assays [60], and HPLC methodologies using fluorescence detection [55,61–63] are used for measuring total riboflavin in foods. HPLC can separate individual free riboflavin, FAD, and FMN in foods.

To measure total riboflavin contents as free riboflavin in foods using these methods, acid hydrolysis with autoclaving is used to release the riboflavin from association with proteins and to convert FAD and FMN to free riboflavin. However, to complete the conversion of FMN to free riboflavin, enzyme hydrolysis is required with diastatic enzymes after acid hydrolysis [64]. Combined extractions for thiamin and riboflavin assays have been usually used for food analysis [6,52,55].

AOAC Official Method 940.33 [11] is an approved microbiological method only for riboflavin in vitamin preparations. However, microbiological assay applying *L. rhamnosis* (formerly *L. casei*) has been used to determine total riboflavin content in foods. Because lactic acid bacteria utilize riboflavin and FMN, but not FAD, an acid hydrolysis step is necessary to convert FAD and FMN to free riboflavin, but enzyme hydrolysis for completing the conversion of FMN to riboflavin is not required. *L. rhamnosis* is affected by starch and fatty acids. The matrix effect by starch in the media is eliminated by acid hydrolysis. Fatty acids stimulate or inhibit the growth of *L. rhamnosis*. Hence, for high fat foods, the fat extraction step with petroleum ether or hexane should be conducted before acid hydrolysis.

Reversed-phase HPLC with fluorescence detection is frequently utilized for chromatography of riboflavin assays in foods. Extraction procedures for total riboflavin analyses include acid and enzyme hydrolysis. Chromatography is capable of separating FMN and free riboflavin, and the concentrations of FMN and free riboflavin by HPLC are summed to obtain total riboflavin concentration, so, in this case, enzyme hydrolysis can be skipped. To quantify individual free riboflavin, FAD, and FMN in foods, Viñas et al. [63] used an extraction method utilizing acetonitrile without acid hydrolysis. Solid-phase cleanup procedures are often used prior to injection to remove some of the interfering materials. UV detection with reversed-phase HPLC has been used for riboflavin analyses in foods at 254 nm [54]; however, fluorescence detection (excitation λ: 440–500 nm; emission λ: 520–530 nm) is more sensitive and specific for riboflavin quantitation than UV detection [6,54,55]. Riboflavin content in foods is commonly expressed as mg of riboflavin.

10.3.3 Niacin (Vitamin B_3)

Niacin is one of the water-soluble B-vitamins known as vitamin B_3. The term niacin is the generic descriptor for nicotinic acid and nicotinamide, which are essential for formation of the coenzymes, nicotinamide adenine dinucleotide (NAD), and nicotinamide adenine dinucleotide phosphate (NADP) in the body. Niacin can be biosynthesized from the amino acid, tryptophan. Most animal organs contain large amounts of niacin. Especially, the liver is an excellent source containing over 50% of niacin DV (Table 15.2). Nicotinic acid is found mainly in plant foods, but animal foods contain nicotinamide that is bioavailable. In uncooked foods, niacin is present as NAD and NADP, but these nucleotides may be hydrolyzed to nicotinamide by cooking [65]. Niacin is not affected by thermal processing, light, oxygen, and pH. It is stable during processing, storage, and cooking of foods. Thus, acid or alkali hydrolysis can be used for extraction of niacin from food samples.

Niacin in foods can be determined by the colorimetric methods of AOAC Official Methods 961.14 and 975.41 [11] using the König reaction in which nicotinic acid and nicotinamide react with cyanogen bromide and the aromatic amine, sulfanilic acid. Microbiological assays [66], including AOAC Official Method 985.34 [11], and HPLC methodologies [67–70] are also currently used for determining niacin in foods.

Because niacin in animal-based foods is present in free forms (nicotinic acid and nicotinamide) and bound forms (NAD and NADP), hydrolysis procedures are required. Acid hydrolysis with HCl or H_2SO_4 or alkaline hydrolysis with NaOH or $Ca(OH)_2$ are used as the initial step in niacin extraction procedures. Acid hydrolysis liberates nicotinamide from bound forms and hydrolyzes it to nicotinic acid; however, nicotinic acid, mainly distributed in cereal products and biologically unavailable niacin, is not completely liberated from bound forms by acid hydrolysis. Alkaline hydrolysis liberates most bound forms and provides a measure of total niacin in cereal products [71].

Microbiological assay is used for the determination of total niacin using *L. plantarum*, which responds to nicotinic acid, nicotinamide, nicotinuric acid, and NAD. However, this method does not account for tryptophan. To determine total niacin concentration, AOAC Official Methods 944.13 and 985.34 [11] using *L. plantarum* are provided for vitamin preparations and for ready-to-feed milk-based infant formulas, respectively. Solve et al. [72] developed an automated microplate method with *L. plantarum*, which reduced time expenditure and materials compared to conventional microbiological procedures.

HPLC determination of niacin has generally been carried out with ion-pairing or RPC with UV detection. Either acid or alkali hydrolysis is used to liberate free niacin from bound forms. Water or methanol is used to measure only free forms of nicotinic acid and nicotinamide in foods by HPLC [70,73]. Cleanup procedures like cartridge extractions and column switching are usually performed to improve selectivity and sensitivity by eliminating interfering materials before HPLC. Most studies have used UV absorbance detection of nicotinic acid and nicotinamide at 254 or 264 nm. Fluorescence detection (excitation λ: 322 nm; emission λ: 380 nm) may be used to increase specificity and sensitivity of HPLC. Niacin is not naturally fluorescent, but fluorescent derivatives

can be formed using cyanogen bromide and ρ-aminophenol [74]. A post-column UV irradiation in the presence of hydrogen peroxide and copper (II) ions also induces fluorescence [66,75]. Lombardi-Boccia et al. [6] determined niacin, thiamin, and riboflavin together by using reversed-phase HPLC with a photodiode array detector after acid and enzyme hydrolysis.

Niacin content in foods is commonly expressed as mg of niacin equivalents (NE). Sixty milligrams of dietary tryptophan is considered equivalent to 1 mg of niacin [76]. Thus, 1 mg NE is equal to 1 mg of niacin or 60 mg of dietary tryptophan.

10.3.4 Vitamin B_6

Vitamin B_6 is a water-soluble vitamin. Vitamin B_6 consists of derivatives of 3-hydroxy-2-methylpyridine, i.e., pyridoxal (PL), pyridoxine (PN), pyridoxamine (PM), and their respective 5′-phosphates (PLP, PNP, and PMP). PLP is a metabolically active B_6 vitamer. Vitamin B_6 in free and bound forms is found in a wide variety of foods including edible animal by-products. Some of animal organs are a good to rich sources of vitamin B_6. PLP, bound to the apoenzyme by a Schiff base in animal tissues, is the major form of vitamin B_6 in animal tissue, which is bioavailable. PMP is also found in edible animal by-products. Hence, PL, PLP, PM, and PMP are determined in edible animal by-products as a result of interconversion of aldehyde and amine forms during processing and storage. However, PN and PNP, found in plants, are not detected in animal foods. Vitamin B_6 is unstable to light, both visible and UV. The vitamin is stable in acidic conditions if protected from light. The stability of vitamin B_6 toward heat treatment, processing, and storage depends upon the pH of the media. Losses of the vitamin increase as pH increases. PN is more stable to heat than PL and PM.

Several different methodologies have been developed to analyze vitamin B_6 in foods. These include animal growth, microbiological, enzymatic, fluorometric, GC, and HPLC assays [77]. Currently, microbiological assays and HPLC methods are often used in the vitamin B_6 determination in foods. AOAC International provides both microbiological (Official Methods 961.15 and 985.32) and HPLC (Official Method 2004.07) methods to measure total vitamin B_6 in ready-to-feed milk-based infant formula and reconstituted infant formula, respectively [11]. Also, several HPLC methodologies have been reported for determining total vitamin B_6 as well as individual B_6 derivatives in foods [7,78–80].

Before being analyzed by microbiological, chromatographic, and other methods, food samples are usually extracted by acid hydrolysis with autoclaving in HCl or H_2SO_4 to dissociate vitamin B_6 from proteins. The phosphate esters of PNP, PLP, and PMP are also hydrolyzed by this procedure. In the AOAC microbiological methods [11] for measuring total vitamin B_6, sample foods are autoclaved with 0.055 N HCl for 5 h at 121°C. However, the AOAC liquid chromatographic method for total vitamin B_6 uses enzymatic hydrolysis using acid phosphatase followed by a reaction with glyoxylic acid in the presence of a Fe^{2+} catalyst to transform PM into PL [11]. For separation of individual B_6 vitamers, metaphosphoric acid, perchloric acid, trichloroacetic acid, and/or sulfosalicylic acid are used as deproteinating agents. Phosphorylated forms in foods are preserved by using these agents.

The total vitamin B_6 composition in foods is usually estimated microbiologically using a turbidimetric assay. *Saccharomyces uvarum* (formerly *S. carlsbergenesis*) is the commonly used microorganism, which is also used in the AOAC method. Acid hydrolysis is necessary for determining total vitamin B_6 because the microorganism utilizes only nonphosphorylated B_6 vitamers. *S. uvarum* responds unequally to PL, PM, and PN. The growth response of *S. uvarum* to PL relative to that to PN is practically equal, but the response of the microorganism to PM is frequently 60%–80% of that to PL and PN [81]. The problem of differential response of *S. uvarum* can be overcome by separating PL, PM, and PN chromatographically and analyzing each form of the vitamin. Cation-exchange chromatography on Dowex AG 50 W-X8 resin resolves the vitamers in the acid extractant and allows their individual quantitation [82,83]. *Kloechera brevis* (formerly *K. apiculata*) may be used for vitamin B_6 assay. However, the results in responses of the organism to PL, PM, and PN are not consistent [77], and *K. brevis* has not seen wide usage in food analysis.

Ion-exchange and reversed-phase HPLC with fluorescence detection has been used for quantitative determination of vitamin B_6 in foods. HPLC has the ability to separate and quantitate PL, PM, PN, and their 5′-phosphate esters, and also vitamin B_6 metabolites such as 4-pyridoxic acid. HPLC provides high resolution and high sensitivity to the B_6 vitamers. To determine total vitamin B_6 by quantitating PL, PM, and PN, hydrolysis of the phosphate esters is usually completed with a commercial phosphatase or treatment with H_2SO_4. For preservation of the phosphorylated vitamers and metabolites, deproteinizing agents are used. Because of the native fluorescence of PL, PM, PN, and their 5′-phosphorylated derivatives and a relatively low UV detection sensitivity, most of the HPLC methods have used fluorescence detection. The intensity of fluorescence among the B_6 vitamers is pH dependent. The B_6 vitamers are suited to ion-exchange because of their pH-dependent ionic nature. In reversed-phase HPLC, sample components are separated according to their relative affinity for a nonpolar bounded stationary phase and a polar mobile phase [84]. HPLC with fluorescence detection has been recommended for quantitative determination of vitamin B_6 in foods because individual vitamers can be determined [85,86], but microbiological assays are still used in total vitamin B_6 measurements in foods. Milligrams of vitamin B_6 are generally used for expressing the content of vitamin B_6 in foods.

10.3.5 Folate

Folate is a generic term for a water-soluble vitamin and includes naturally occurring food folates and folic acid found in dietary supplements and used in food fortification. Folate can vary in structure by reduction of the pteridine moiety to dihydrofolic acid and tetrahydrofolic acid (THF). Folate exists predominantly as polyglutamyl forms of THF, which are biologically active folate coenzymes in the body. Edible animal by-products provide small amounts of folate, except kidneys and liver. Major folate vitamers in animal tissues are polyglutamyl forms of THF, 5-methyl-THF, and 10-formyl-THF [87]. The composition of 5-formyl-THF in animal tissues is low, but heating can increase it by isomerization of 10-formyl-THF [88]. Folate is sensitive to heat, acids, oxidation, and light. Folate losses by food processing and storage are variable by food matrices, oxygen availability, heating times, and forms of folate in foods. Folate is quite stable in dry products if protected from light and oxygen, but folate losses are large in water. Reducing agents such as ascorbic acid increases folate retention, while metals like Fe^{2+} increase folate losses. Folic acid is generally more stable than naturally occurring folates.

Methodologies for the determination of folates in foods include microbiological assay [89] including AOAC Official Method 2004.05 for total folates in cereals and cereal foods [11], HPLC with UV, fluorescence, or electrochemical detection [90–96], and HPLC or GC with mass spectrometric (MS) methods [97,98] using stable isotopes. Microbiological assay for total folate determination is still the most widely used procedure. HPLC methods allow measurement of each form of folates.

The traditional food folate extraction method includes heat treatment to release folate from its binding proteins and folate conjugase treatment to hydrolyze polyglutamyl folate to di- or monoglutamyl folate. Insufficient enzymatic deconjugation may result in underestimation of folate when measured by either microbiological or HPLC methods using single-enzyme digestion. Several studies have reported that treatment of food homogenates with α-amylase, protease, and folate conjugase (trienzyme extraction) enhances the yield of measurable folate in folate assays [94–96]. The use of α-amylase and protease allows for a more complete extraction of folate trapped in carbohydrates or proteins in foods. This trienzyme extraction method has become widely used in the extraction of folate from food samples. Antioxidants, such as ascorbic acid, 2-mercaptoethanol, and dithiothreitol, should be added to prevent the destruction of labile folates during heat treatment.

For routine food analysis purposes, microbiological assay with *L. rhamnosus* (formerly *L. casei*) after extraction with folate conjugase is used for the determination of folate. *L. rhamnosus* has greater capacity for response to the γ-glutamyl folate polymers compared to the other assay

organisms. Although *L. rhamnosus* is the commonly used and accepted organism for folate analysis in foods, its ability to respond on an equimolar basis to metabolically active folates is controversial [99]. Chicken pancreas conjugase is used to hydrolyze polyglutamates to diglutamyl and monoglutamyl folates, which are used by *L. rhamnosus*. AOAC Official Method 2004.05 [11] is the microbiological method using *L. rhamnosus* after extracting samples by the trienzyme procedure. The method can determine turbidity semiautomatically by using 96-well microtiter plates and autoplate readers [100].

The major advantage of HPLC analysis is the ability to quantify the specific folate forms. Current HPLC systems for separating folates use either ion-pair or RPC with UV, fluorometric, or electrochemical detection. Trienzyme extraction procedures are commonly used for HPLC analysis of food folates. Because HPLC systems for determining folate are able to detect only monoglutamates, human or rat plasma conjugase, not chicken pancreas conjugase, is used to deconjugate polyglutamyl folates to monoglutamyl folates. To remove interfering substances in extracts, purification is recommended with affinity chromatography using immobilized folate-binding protein or solid-phase extraction using silica-based strong anion-exchange cartridges. Due to its sensitivity and selectivity, fluorescence detection is most commonly used, particularly for reduced folate forms. UV detection is useful in detecting the folic acid found in fortified foods, but not for naturally occurring folates due to a lack of sensitivity. UV spectra and fluorescence excitation and emission spectra for the different forms of folates have been published by Ball [101]. Electrochemical detection has sensitivity for 5-methyl-THF; however, it has not been widely used in food analysis. Mass spectrometer with an HPLC system using stable isotope-labeled analyte standards has been used for folate detection to improve sensitivity and selectivity.

Folate content of foods is expressed in either mg or μg of naturally occurring folate and fortified folic acid in the foods or dietary folate equivalents (DFE). Micrograms of DFE are calculated based on μg of food folate plus fortified folic acid multiplied by the factor 1.7 [102].

10.3.6 Vitamin B_{12}

Vitamin B_{12} is a water-soluble vitamin and a family of compounds called cobalamins, which contain cyanocobalamin, hydroxocobalamin, and the two coenzyme forms 5′-deoxyadenosylcobalamin (adenosylcobalamin) and methylcobalamin. Cyanocobalamin and hydroxocobalamin are the forms of vitamin B_{12} used in most dietary supplements, and are converted to adenosylcobalamin and methylcobalamin in the body. Vitamin B_{12} found in nature appears to be from synthesis by bacteria and other microorganisms growing in soil and water. Edible animal by-products are rich sources of vitamin B_{12}, with heart, kidneys, livers, pancreas at the top of the list. The most prevalent forms of vitamin B_{12} in the foods are adenosylcobalamin, hydroxocobalamin, and metylcobalamin. Vitamin B_{12} is generally stable if protected from light. Cobalamins are considered to be stable to thermal processing, but large losses of the vitamin occur by leaching into the cooking water. Cyanocobalamin is the most stable form of vitamin B_{12}. Strong alkaline and acid conditions, intense visible light, and oxidizing agents inactivate the vitamin.

The determination of total vitamin B_{12} may be performed by microbiological assays, including AOAC Official Methods 952.30 and 986.23 [11], radioisotope dilution methods [103,104], and HPLC methods [105,106]. Microbiological assay is most widely used for the determination of total vitamin B_{12} in foods. Radioassay kits for clinical samples are not useful for analysis of food samples. Radioisotope dilution methods lack selectivity as the intrinsic factor used for the assay could also bind other cobalamins or analogues [107]. Currently, these methods are not routinely used for vitamin B_{12} analysis of foods. The HPLC method lacks the sensitivity to measure vitamin B_{12} in non-fortified food products.

The extraction procedures of the AOAC microbiological methods [11] are usually utilized for determining total vitamin B_{12} content in foods. Extraction procedures liberate cobalamins from protein and convert the labile, naturally occurring forms to a single, stable form, which are

cyanocobalamin or sulfitocobalamin [108]. The extraction is completed by homogenizing the sample in the extraction solution, autoclaving the sample at 121°C for 10 min. To protect cobalamins in samples, metabisulfite or ascorbic acid is added to the extracting solutions.

L. delbrueckii subsp. *lactis* (*L. leichmannii*) is frequently used for the determination of vitamin B_{12} in foods. *L. delbrueckii* has a similar response to nitritocobalamin, hydroxocobalamin, dicyanocobalamin, and sulfitocobalamin. However, adenosylcobalamin produces a greater response, and methylcobalamin, a lesser growth response. If the sample extracts are exposed to light before analysis, adenosylcobalamin and methylcobalamin are completely converted to hydroxocobalamin, so that vitamin B_{12} activity can be measured accurately [109]. *L. delbrueckii* responds to deoxyribonucleosides. Treatment of the sample with alkali and heat destroys the vitamin cobalamins, leaving the deoxyribonucleosides intact; thus, the activity attributable to deoxyribonucleosides can be determined. Dilution of deoxyriboside concentrations to less than 1 μg/mL of assay solution can also eliminate the effect [110]. The vitamin B_{12} concentrations in foods are expressed in mg or μg of vitamin B_{12}.

10.3.7 Pantothenic Acid

Pantothenic acid, also known as vitamin B_5, occurs primarily bound as part of coenzyme A and acyl carrier proteins. Pantothenic acid is found in plant- and animal-derived foods because of its diverse metabolic functions as a structural component of coenzyme A. Organs contain a considerable amount of pantothenic acid. Pantothenic acid is stable to atmospheric oxygen and light, whereas large losses of the vitamin can occur in the blanching and boiling of foods. The stability of pantothenic acid is highly pH dependent. The vitamin is stable in slightly acidic solutions at pH 5–7.

Microbiological assay [111] is most commonly used for determining pantothenic acid in foods. Microbiological assay has been accepted by AOAC (Official Methods 945.74 and 992.07) [11] for quantification of pantothenic acid in vitamin preparations and milk-based infant formula, respectively. Other methodologies for the determination of the vitamin in foods include radiometric microbiological assay [112], radioimmunoassay [113,114], enzyme-linked immunosorbent assay [115] optical biosensor inhibition immunoassay [116], capillary electrophoresis [117], GC-MS using stable isotope dilution [118], and HPLC methods [119–121]. Methods for the pantothenic acid determination in foods vary widely in approach, because the methodologies for pantothenic acid determination in food products remain limited by their low sensitivity and poor selectivity. And, the applications of the analytic methods have several drawbacks including being time-consuming, use of radioisotopes and scintillation counting, and sample derivatization procedures.

The microbiological assay is time-consuming and lacks specificity [122], but determination of pantothenic acid in foods has most frequently been accomplished by this type of assay. The commonly used microorganism is *L. plantarum*. The organism does not respond to phosphopantetheine or intact forms of coenzyme A. Due to instability of the vitamin in acid and alkaline conditions, enzyme hydrolysis should be utilized to obtain free pantothenic acid and pantetheine. Intestinal phosphatase to cleave the phosphate linkage and avian liver peptidase to break the linkage between mercaptoethylamine and pantothenic acid are used in the enzyme hydrolysis, which is also utilized in the AOAC microbiological method for milk-based infant formulas [11]. Fatty acids in foods stimulate the growth of *L. plantarum*; therefore, a fat extraction step may be necessary before enzyme hydrolysis in fat-containing foods [111]. The pantothenic acid content of foods is commonly expressed as mg of pantothenic acid.

10.3.8 Biotin

Biotin is a water-soluble B-vitamin, which contains sulfur. The biotin molecule contains three asymmetric carbon atoms, and therefore eight different isomers are possible. Of these isomers, only the dextrorotatory (+) *d*-biotin possesses biotin activity as a coenzyme. Biotin is widely distributed

in many foods, but its concentrations are relatively low compared to that of other water-soluble vitamins. Liver contains considerable amounts of biotin. Most of biotin in animal products is in a protein-bound form. Biotin is generally stable to heat, but is gradually destroyed by UV light. In strong acidic and alkaline solutions, biotin is unstable to heating. The sulfur atom in biotin is susceptible to oxidation with formation of biotin sulfoxide and biotin sulfone during food processing, which leads to loss of biotin activity.

Microbiological assays [123], as well as protein-binding assays [124,125], and a biosensor-based immunoassay [126] have been developed for the determination of biotin content of foods. HPLC methods [127–129] have been also used for quantification of biotin and biocytin in foods. Currently, an AOAC Official Method for biotin determination does not exist [11].

The most widely used method for determination of biotin in foods is a microbiological assay using *Lactobacillus plantarum*. The microorganism, which requires biotin for growth and reproduction, is incubated with diluted sample extracts. The resulting increased turbidity of the extract is measured, and correlated with the biotin content of the sample. The microorganism cannot utilize bound forms of biotin including biocytin, thus acid hydrolysis with autoclaving is required to liberate biotin completely from food samples. Although *L. plantarum* is more specific for biotin-active forms than other biotin-requiring organisms, *L. plantarum* responds to dethiobiotin that spares biotin and can thus overestimate biotin content [130].

HPLC can separate free biotin, biotin sulfoxides, biotin sulfones, and other biotin analogs. Extractions with acid have commonly been used for foods to ensure liberation of bound biotin forms. The biotin molecule does not have enough UV absorbance and native fluorescence; thus UV and fluorescence detection are not useful. However, avidin can be labeled with a fluorescent marker and the complex used as a post-column derivatizing agent. The fluorescence of the labeled protein is enhanced on binding of its specific ligands, biotin, and biocytin. HPLC methods with avidin-binding detection, formation of fluorescent derivatives, or usage of other detection systems, including MS and electrochemical detection, are ways to increase the sensitivity of the detection after HPLC separation. Analytical results are expressed in μg of biotin.

10.3.9 Vitamin C

Vitamin C is a water-soluble vitamin and occurs in two forms, the reduced ascorbic acid and the oxidized dehydroascorbic acid. Ascorbic acid is reversibly oxidized to dehydroascorbic acid. Further oxidations convert dehydroascorbic acid to the inactive and irreversible compound diketoglutamic acid. There are two enantiomeric pairs, L- and D-ascorbic acid and L- and D-isoascorbic acid. L-Ascorbic acid and D-isoascorbic acid (known as D-araboascorbic acid and erythobic acid) have the biological activity of vitamin C. D-Isoascorbic acid has 1/20 the activity of L-ascorbic acid [131]. L-Ascorbic acid and dehydroascorbic acid are naturally occurring forms of vitamin C, but D-isoascorbic acid is not found in foods. Organs are good to rich sources of vitamin C. Vitamin C is very susceptible to oxidation during the processing, storage, and cooking of foods, especially under alkaline conditions. Maximal stability occurs between pH 4 and 6; however, degradation rates are influenced by oxygen availability, the presence of antioxidants, thermal processing conditions, transition metal catalysis, oxidizing lipid effects, and the presence of ascorbic acid oxidase [132].

Methodologies for determination of total vitamin C in foods include the titrimetric method using oxidation-reduction indicators [11], the fluorometric method including derivatization procedures [11], enzymatic methods [133], and electrochemical procedures [134]. Total vitamin C content is also usually determined by HPLC using UV, fluorescence, or electrochemical detection [135,136]. Only HPLC procedures simultaneously separate L-ascorbic acid, dehydroascorbic acid, and D-isoascorbic acid in foods [137–140]. The titrimetric method employed in AOAC Official Methods 967.21 and 985.33 [11] uses 2,6-dichloroindophenol in measuring ascorbic acid, not dehydroascorbic acid. This method cannot distinguish between L-ascorbic acid and D-isoascorbic acid; thus, the titrimetric method cannot be used for processed food products containing D-isoascorbic acid.

The fluorometric method of AOAC Official Methods 967.22 and 984.26 [11] determines total vitamin C content in vitamin preparations and foods, respectively, by derivatization of dehydroascorbic acid using the *o*-phenylenediamine condensation reaction.

Because vitamin C is destroyed easily, extraction procedures should be conducted to stabilize the vitamin. The choice of extracting solution is dependent on the sample matrix and determination method, but the solutions should maintain an acidic environment, chelate metals, inactivate ascorbic acid oxidase, and precipitate proteins and starches [132]. The extracting solution usually is 3%–6% metaphosphoric acid dissolved in 8% glacial acetic acid or ethylenediaminetetraacetic acid (EDTA). Metaphosphoric acid prevents metal catalysis and activation of ascorbic acid oxidase, and precipitates proteins. EDTA also chelates metals, and acetic acid precipitates starches in extractants.

HPLC methodologies are widely used for determining ascorbic acid and its degradation products in foods. RPC with and without ion suppression, and ion-pair RPC with C_{18} columns, and ion-exchange chromatography are currently employed for the analysis of vitamin C. UV, electrochemical, and fluorescence detection are commonly used for quantitation of L-ascorbic acid, dehydroascorbic acid, and D-isoascorbic acid in foods. UV detection is not suitable for low-vitamin C-content foods due to poor sensitivity of dehydroascorbic acid. Dehydroascorbic acid is electrochemically inactive; therefore, to measure total vitamin C contents, it is reduced to L-ascorbic acid before electrochemical detection. Fluorescence detection is accomplished after *o*-phenylenediamine derivatization using pre- or post-columns. Both electrochemical and fluorescence detection have excellent selectivity and sensitivity to vitamin C analysis by HPLC. The vitamin C contents of foods are commonly expressed as mg of vitamin C.

10.4 SUMMARY

Vitamins are classified as to their solubility in fat organic solvents (fat-soluble vitamins) or water (water-soluble vitamins). The solubility properties are related to the distribution of vitamins in foods as well as the analytical methods employed. Fat-soluble vitamins A, D, and E are contained in edible animal by-products in varying amounts. Most of organs are good to excellent dietary sources of most of the B-vitamins and vitamin C. Vitamins are generally susceptible to oxidation, heat, pH, moisture, light, degradative enzymes, and metal trace elements. Thus, processing, storage, preparation, and cooking methods can affect the concentrations of vitamins in foods.

To liberate vitamins bound in lipid or protein fractions, food samples may need to be hydrolyzed using acids, alkalines, and/or enzymes, or extracted directly with solvents without hydrolysis. The extract solutions may require some forms of cleanup before the vitamins are measured to remove interfering substances and to improve the sensitivity and selectivity of the analytical methods. Antioxidants such as BHT, pyrogallol, or ascorbic acid, are frequently added in extraction solvents to prevent oxidation and conversion of vitamins. Most of the vitamins are liable to light, and, therefore, food samples must be protected from light during the entire analysis.

Various methodologies, including colorimetric, fluorometric, titrimetric, and spectrophotometric methods, have been developed and used for determining the vitamins in foods. However, microbiological and HPLC methods are most frequently used in estimating the vitamins in foods, because these methods have sufficient sensitivity and selectivity to quantitate low concentrations of naturally occurring vitamins. Microbiological assay can be applied to all the B-vitamins. Lactic acid bacteria are suitable for determining the B-vitamins turbidimetrically, except for vitamin B_6, which may be determined using yeasts. Fat-soluble vitamins and vitamin C are most commonly determined by HPLC. HPLC methods can also be used for estimating the B-vitamins. HPLC distinguishes between naturally occurring and added (fortified or enriched) vitamins, and also separates the individual forms of vitamins. Some vitamins, having low UV absorbance or fluorescence responses, can be determined after conversion to fluorescent derivatives using pre- or post-column derivatization. However, some edible animal by-products such as the kidneys and liver contain large amounts of fat- and water-soluble vitamins, thus a dilution step may be needed to obtain clear separations.

Biospecific methods for determining some of the water-soluble vitamins include immunoassays and protein-binding assays. Newer techniques continue to be developed for quantitating the concentrations of the various vitamins in all types of foods, including edible animal by-products.

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11 Minerals and Trace Elements

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CONTENTS

11.1 INTRODUCTION

The processing of animals for meat in slaughter houses generates different by-products, including offal or variety meats, the edible internal organs, and external parts of the animal. Offal includes any of various non-muscular parts of the carcasses of beef and veal, mutton and lamb, and pork, which are either consumed directly as food or used in the production of other foods. According to EU regulation 853/2004 [1], offal means fresh meat other than that of the carcass, including viscera and blood.

Blood is the first by-product obtained while processing animals for meat and is one of the most underutilized slaughter house by-products. Since the invention of cooking, offal has been part of the human diet, because cooking renders the indigestible animal parts edible. Different offal meats are representative of traditional foods and dishes, such as foie gras, kidney pie, black pudding, tripe, etc.

Liver of different origins (beef, lamb, pork, or goose) and kidneys are among the offal meats most often found in the diet, and such meats can also include treated stomach, bladder, and intestines.

Derived products such as gelatin obtained by the partial hydrolysis of collagen from bones, hides and skins, tendons, and sinews of animals, are widely used in the food industry.

In nutritional terms, the interest of offal meats lies in their provision of proteins and certain vitamins and minerals. Mineral contents are often higher than in muscle tissue [2] (Table 11.1). Kidney and liver are a good source of essential elements (notably Fe, Cu, Zn, and Se) in the human diet, but

TABLE 11.1
Iron and Zinc Contents (mg/kg) in Raw Meat (Chop or Sirloin) and Liver of Different Animal Species

	Beef		Lamb		Pork		Veal	
	Meat	Liver	Meat	Liver	Meat	Liver	Meat	Liver
Iron	16.4	49.0	17.5	73.7	8.5	233.0	7.8	64.0
Zinc	35.7	40.0	32.1	46.6	20.5	57.6	25.5	120.2

Source: Data from USDA-ARS, USDA National Nutrient Data Base of Standard Reference, Nutrient Data Laboratory, Beltsville, MD, at http://www.nal.usda.gov/fnic/foodcomp/search/

they also easily accumulate heavy metals. Therefore, offal can contain higher levels of toxic metals than many other foodstuffs.

As an example, the contents in minerals of nutritional interest in different forms of offal (brain, heart, liver, and kidneys) from a range of edible animal species (beef, lamb, pork, and veal) are reported in Table 11.2 [2]. In general, the range of mean contents of a given mineral in the same organ from different animal species is narrow, with some exceptions such as calcium in brain; iron in liver; selenium in brain, heart, and kidneys; and copper in liver.

TABLE 11.2
Elements of Nutritional Interest Expressed in mg/kg, Except for Se (µg/kg) of Different Raw Offal (Brain, Heart, Kidney, and Liver)

	Ca	Fe	Mg	P	K	Na	Zn	Cu	Mn	Se
Brain										
Beef	430.0	25.5	130.0	3620.0	2740.0	1260.0	10.2	2.9	0.3	213.0
Lamb	90.0	17.5	120.0	2700.0	2960.0	1120.0	11.7	2.4	0.4	90.0
Pork	100.0	16.0	140.0	2820.0	2580.0	1200.0	12.7	2.4	1.0	159.0
Veal	100.0	21.3	140.0	2740.0	3150.0	1270.0	11.1	2.2	0.4	100.0
Heart										
Beef	70.0	43.1	210.0	2120.0	2870.0	980.0	17.0	4.0	0.3	218.0
Lamb	60.0	46.0	170.0	1750.0	3160.0	890.0	18.7	4.0	0.5	320.0
Pork	50.0	46.8	190.0	1690.0	2940.0	560.0	28.0	4.0	0.6	104.0
Veal	50.0	42.4	180.0	2110.0	2610.0	770.0	14.7	3.4	0.4	333.0
Kidney										
Beef	130.0	46.0	170.0	2570.0	2620.0	1820.0	19.2	4.3	1.4	1410.0
Lamb	130.0	63.8	170.0	2460.0	2770.0	1560.0	22.4	4.5	1.2	1269.0
Pork	90.0	48.9	170.0	2040.0	2290.0	1210.0	27.5	6.2	1.2	1900.0
Veal	110.0	33.6	160.0	2410.0	2720.0	1780.0	19.7	5.0	0.8	800.0
Liver										
Beef	50.0	49.0	180.0	3870.0	3130.0	690.0	40.0	97.6	3.1	397.0
Lamb	70.0	73.7	190.0	3640.0	3130.0	700.0	46.6	69.8	1.8	824.0
Pork	90.0	233.0	180.0	2880.0	2730.0	870.0	57.6	67.7	3.4	527.0
Veal	50.0	64.0	200.0	3790.0	3080.0	770.0	120.2	118.6	2.5	227.0

Source: Data from USDA-ARS, USDA National Nutrient Data Base of Standard Reference, Nutrient Data Laboratory, Beltsville, MD, at http://www.nal.usda.gov/fnic/foodcomp/search/

TABLE 11.3
Maximum Residue Limits (mg/kg Wet Weight) for Copper and Zinc in Bovine Liver and Kidney in Various Countries

	Liver	Kidney	Country
Copper	100	100	United States
	100	100	Australia
	150	150	Canada
	60	60	Slovac Republic
Zinc	500	500	United States
	500	500	Canada
	150	150	Australia
	100	100	Canada
	80	80	Slovac Republic
	100	100	Russia

Source: Data collected by Nriagu, J. et al., *Ecot. Environ. Saf.*, 72, 564, 2009.

Mineral element contents of offal are determined with the following aims:

- To know the essential element content (Cu, Fe, Zn, and Se) in order to estimate offal adequacy as a dietary source or as a required step for estimating total mineral intake
- To check compliance with the maximum residue limits established for some offal meats in various countries and by different organizations [3] (Table 11.3)
- To study interrelations between essential (Cu, Zn) and toxic elements, mainly (Cd, Pb, and As)

Studies on the contents of elements of nutritional interest, such as copper and zinc in offal, are relatively scarce in comparison with those measuring heavy metals.

Zinc contents of different offal meats are reported in the study of zinc levels in foods from southeastern Europe. A relatively large number of different offal meats were analyzed, though the number of samples of each type of product was limited [4].

The interest of zinc in human nutrition and the importance of an adequate dietary supply to avoid deficiencies has led to compilation of the zinc contents of selected raw foods, originating from different continents (Europe, Asia, Africa, or America), carried out by Scherz and Kirchhoff [5]. A remarkable consistency in zinc content in raw foods, especially in some vegetable raw materials and edible animal offal meats, was found. The zinc contents, expressed as mg Zn/kg of edible fresh product, were significantly higher in pig liver (43.4–90.0) and pig kidney (22.0–30.5) than in beef liver (36.3–45.5) and beef kidney (16.0–21.0).

Copper and zinc concentrations in the liver, kidney, and muscle (meat) from 438 calves (6–10 months old) and 56 cows (2–16 years old) slaughtered in Galicia (Spain) in 1996 were determined. The arithmetic mean fresh weight concentrations found in calf liver, kidney, and muscle, respectively, were 64.6, 4.91, and 0.677 mg/kg for copper and 47.7, 14.4, and 47.8 mg/kg for zinc. In cows, these respective concentrations were 60.3, 3.67, and 1.26 mg/kg (copper) and 59.8, 20.0, and 52.7 mg/kg (zinc) [6].

Liver and especially kidney copper concentrations were higher in calves than in cows. The comparison with copper contents reported for animals from different countries has shown liver copper levels in Galician animals to be approximately twice those found in cattle elsewhere. These levels

exceed the acceptable maximum concentrations established in some countries, with the exception of Canada [7], where liver copper concentrations in calves were some 4 ± 5 times greater than in cattle from most other countries and 2 ± 3 times higher than in Galician calves. Liver copper concentrations in adult cattle were also elevated in Canada and in the United Kingdom compared with most other countries. In the case of zinc, it was noted that in cattle, liver and muscle zinc contents are similar and two- to threefold higher than in kidney. Copper and zinc concentrations in the livers from a number of Galician cattle exceeded the regulatory limits adopted by some countries, though the frequency with which this occurred depended upon which acceptable maximum concentration was used—regulatory values differing markedly between countries [6].

On the other hand, the consumption of these livers is not considered to constitute a risk to consumers. In fact, the consumption of offal may be beneficial, particularly in areas where copper intake is low or even deficient. In Galicia, the contribution of cattle products to copper and zinc intake has been estimated to be 0.12 and 2.54 mg/person/day, respectively. Liver accounted for most (67%) of the dietary copper intake derived from cattle [8].

In a later study by the same group [9], nutritionally essential elements (Ca, Co, Cr, Cu, Fe, Mn, Mo, Ni, Se, Zn) were evaluated in the tissues (liver, kidney, and muscle) of 120 cattle from Spain, with the aim of detecting significant associations between essential metals. Trace essential metals were found to be within the adequate ranges. Very strong correlations were found between Ca, Cu, Fe, Mn, Mo, and Zn in the kidney. In liver, Co was moderately correlated to most of the essential metals. The knowledge of these correlations may be essential to understand the kinetic interactions of metals and their implications in trace metal metabolism.

In a recently published study [3], copper, selenium, and zinc (among other elements) have been determined in bovine kidneys and livers of 100 animals from various parts in Jamaica. The aim of the study was to evaluate the influence of essential trace elements (Zn, Cu, and Se) on the accumulation of toxic elements (Cd, Pb, and As) in the kidney and liver samples. Contents in liver and kidney, expressed as mg/kg wet weight, were in the following ranges: Cu (< detection limit—99.8), Se (0.058–2.71), Zn (5.28–73.6); and Cu (< detection limit—21.9), Se (< detection limit—3.58), Zn (3.17–45.2), respectively.

These data suggest that some animals may suffer from copper and zinc deficiency. In this study, dietary data on consumption rates for kidney and liver were 2.2 and 5.2 g/day, respectively, which is reflected in the intake of Cu and Zn of 0.06 and 0.18 mg/day, respectively, which is well below the recommended daily allowance of these elements.

11.2 SAMPLE TREATMENT

11.2.1 Organic Matter Destruction-Mineralization

When measuring trace elements in a variety of meats/offal, a prior sample pretreatment stage is mandatory. Wet or dry decomposition procedures can be successfully applied. The choice of procedure depends on the element to be determined and the analytical technique applied, as well as on the fact of whether multiple-element analysis is involved or not.

11.2.1.1 Wet Ashing

Wet digestion with concentrated acids is the most common sample pretreatment for element determination in a variety of meats/offal.

Different volume ratios of acid mixtures (HNO_3/H_2SO_4, $HNO_3/HClO_4$, and $HNO_3/HClO_4/H_2SO_4$) were tested for the digestion of reference material (bovine liver 1577a), for selenium determination by spectrofluorometry and hydride generation atomic absorption spectrometry (HG-AAS).

In the case of spectrofluorometry, the mixture of HNO_3–H_2SO_4 was effective in decomposing resistant organic material, but the presence of sulfuric acid caused the formation of crystallized

2,3-diaminonaphthalene (DAN), which interfered with the fluorescence signal. Filtering or addition of $HClO_4$ to the HNO_3–H_2SO_4 mixture failed to prevent formation of the DAN crystals. The digestion with HNO_3–$HClO_4$ procedure was the most efficient option, and did not cause any interference. In the case of HG-AAS, the presence of nitrate interfered with the formation of hydride and caused suppression of the absorption signal. The presence of perchloric acid in the HNO_3–$HClO_4$ and HNO_3–$HClO_4$–H_2SO_4 mixtures yielded the best recoveries, increased the oxidizing power, and shortened the digestion time [10].

Microwave energy has allowed a reduction of digestion time, and different acid digestion procedures have been developed using concentrated acid. This type of procedures allow a complete destruction of the solid samples matrix, so the digest can be easily analyzed using conventional sample introduction systems in different techniques for mineral determination. Microwave digestion methods have become popular because they are more reproducible, more accurate, and less time-consuming than conventional digestions on hot plates in open beakers [11].

A partial vapor-phase acid digestion procedure is proposed using a focused microwave oven operating at atmospheric pressure for Fe and Co determination in biological samples (including bovine liver SRM 1577b) by electrothermal atomic absorption spectrometry (ETAAS). Time digestion effect (microwave-heating program between 6 and 10 min at 115°C), addition of water or H_2O_2 to the sample for the vapor-phase, and comparison with a sample preparation using a closed vessel microwave approach (with HNO_3 and H_2O_2) were tested. Better digestion was obtained using a longer acid-vapor exposure time (10 min instead of 6 min). Co and Fe extraction were more efficient when water was added, which promoted sample wetting and minimized microwave energy transfer to the sample—thereby avoiding charring. The efficiency of the metal extraction was apparently the same with either water or H_2O_2, though with the latter the extraction time was lowered from 60 to 45 min, which yielded a clearer resulting solution and could potentially be used in colorimetric methods. The application of vapor-phase digestion associated with the single-vessel technique gave low blank values because of fewer pretreatment steps and less sample handling [12].

New sample pretreatment methods such as acid leaching assisted by microwave or ultrasonic bath have been developed. The procedure involves the solubilization of minerals in the leaching solvent (an acid and/or oxidant agent), without sample matrix decomposition. This type of procedure minimizes the main limitations of the wet digestion methods. The parameters associated with ultrasonic solid–liquid extraction (ultrasonic bath or ultrasonic probe, particle size, acid concentration, sonication time and sonication amplitude, and analyte–matrix binding) for elemental analysis have been reviewed [13].

The determination of Zn by flow injection-flame atomic absorption spectrophotometric (FI-FAAS) in lamb kidney, rabbit liver, and reference material (BCR-186 pig kidney), after pretreatment by continuous ultrasound-assisted extraction (CUES), was optimized and compared with a conventional off-line sample digestion method. Nitric acid concentration, hydrochloric acid concentration, sonication time, leaching temperature, flow rate of the CUES, and leaching solution volume were studied.

A nitric acid or hydrochloric acid concentration of 0.75 M as leaching solution proved enough to obtain a quantitative extraction efficiency for Zn of 100.2% and 100.1%, respectively. With regard to sonication time and leaching solution volume, it was found that 0.5 min and 1 mL provided quantitative recoveries of 100.6% and 100.3%. For leaching temperature and flow rate of the CUES, the optimum values were 20°C and 3.5 mL/min, because they simplified the manifold. The sample particle size (smaller than 30 µm and between 30 and 100 µm) did not affect the extraction process, due to the high energy supplied by the ultrasound (frequency of 40 kHz), which increased contact between the sample and the leaching solution.

The results obtained by both sample treatments (CUES vs. off-line digestion) did not reveal significantly different values for the zinc concentrations. Recovery, precision and LOD (0.6 µg/g) yielded satisfactory results [14].

Although wet digestion is the organic material destruction technique most widely used with a variety of meats/offal, few studies involving foods of this kind have compared and validated different procedures for sample preparation in this manner.

Two microwave digestion systems (open-focused and closed-pressurized) were tested for the mineralization of bovine liver (NIST SRM 1577a) as dissolution steps prior to Bi, Cd, Co, Cs, Cu, Fe, Hg, Mn, Mo, Pb, Rb, Sb, Sn, Sr, Tl, and Zn determination by inductively coupled plasma mass spectrometry (ICP-MS). Digestion parameters (mass of sample, digestion mixture, and power/time steps) were optimized. Digestion with the open-focused microwave system requires larger volumes of nitric acid and hydrogen peroxide, and the detection limits are higher than the system with the closed-pressurized digester [11].

A partial microwave-assisted wet digestion (HNO_3 + H_2O_2) using a baby-bottle sterilized with a domestic microwave oven was compared with a total microwave-assisted wet digestion using a commercial microwave digestion system. These procedures were applied to Ca, Cu, Fe, Mg, Mn, and Zn determination by inductively coupled plasma absorption emission spectroscopy (ICP-AES) in bovine liver (SRM 1557b). The results obtained reveal that the proposed partial digestion method can be successfully applied to different bovine tissues, providing the same efficiency as complete digestion. Besides, it is an inexpensive alternative for digestion, producing low amounts of residue and lower blanks [15].

A critical review of the state of the art of enzymatic digestion and ultrasonication in sample treatment for total metal determination and speciation by atomic spectrometry was described [16].

11.2.1.2 Dry Ashing

This organic material destruction procedure is scantly used for determining mineral elements in a variety of meats/offal. The official Association of Official Analytical Chemists (AOAC) methods include some ashing procedures for Zn, Cd, and Fe determination in foods, including liver paste (AOAC, 999.11) [17].

11.2.2 Other Sample Pretreatments

A simple alternative to circumvent the digestion of difficult to solubilize samples is slurry sampling, which combines the advantages of direct solid sampling (shortening the sample preparation time, and reducing risk of both sample contamination and analyte losses) and liquid sampling (sampling dispensing by using an autosampler, with calibration carried out with aqueous reference solutions) [18]. A review of the use in the last decade of slurry sampling and ETAAS, including animal tissues, was carried out by Cal-Prieto et al. [19]

Pereira-Filho and Arruda [20] reported the development of a mechanized procedure for on-line slurry food sample digestion and ETAAS determination. The method allows the digestion of simultaneous complex matrixes such as reference materials (bovine liver) by using three PTFE coils inserted into the microwave oven cavity. The off-line spectrophotometric determination of cobalt and iron was performed by using a spectrophotometric monosegmented system.

A solid sampling ETAAS has been applied to Cu and Zn determination in bovine liver. Several pyrolysis and atomization temperatures, with or without permanent chemical modifiers (W + Rh or Ir), were tested. Considering the good performance presented by Cu in the absence of a chemical modifier, this condition was chosen for the subsequent studies. In the case of Zn, better performance was found for W + Rh.

With a particle diameter below 90 μm, which was obtained by cryogenic mill grinding during 6 min, sample homogeneity is guaranteed. In general, bovine liver samples subjected to cryogenic pretreatment (drying and grinding) showed good homogeneity, even for small sample masses [21].

11.3 DETERMINATION METHODS

11.3.1 Reference Materials

Certified reference materials are widely used to verify the reliability of the obtained analytical data and demonstrate the credibility of the analytical results. In relation to edible animal by-products, bovine, beef or pork liver, pig kidney, animal blood, and bone meal are the available reference materials used as appropriate standards for application for quality control in minerals analysis (see Table 11.4).

In 1972, SRM 1577 bovine liver was the first CRM, representing a benchmark for trace element contents in mammalian tissues. It included 16 elements with certified mass fraction values and 13 elements with noncertified values. In 1982, it was replaced by SRM 1577a, followed by SRM 1577b in 1991. In the latest material SRM 1577b, 24 elements were assigned certified mass fraction values, and an additional 5 elements were assigned noncertified values, based on the determinations by 29 analysts of the National Institute of Standards and Technology (NIST) and two collaborating laboratories. At present, a new bovine liver SRM 1577c is being characterized [22].

11.3.2 Official Methods [17]

There are few official methods of the AOAC for the determination of minerals in variety meats/offal. The FAAS method for copper in liver has been described by the AOAC (985.40), following liver tissue mineralization (overnight at 60°C) with HNO_3.

TABLE 11.4
Minerals and Trace Elements Determination in Variety Meats/Offal Reference Materials

		Elements	
Organism	**Reference Material**	**Certified Values**	**Information Values**
International Atomic Energy Agency (IAEA) (Austria)	A-13 animal blood	Br, Ca, Cu, Fe, K, Na, Rb, S, Se, Zn[a]	Mg, Ni, P, Pb
Institute for Reference Materials and Measurements (IRMM) (Belgium)	BCR-186 pig kidney	Mn, Fe, Cd, Hg, Pb, Cu, Se, As, Zn	
	BCR-185R bovine liver	As, Cd, Cu, Mn, Pb, Se, Zn	Cr, Hg
National Institute of Standards & Technology (NIST) (USA)	1486 bone meal	Ca, Mg, P, Fe, Pb, K, Sr, Zn	Al, As, Cd, Cu, Fe, Mn, Se, Si, Na, C
	SRM 1577b bovine liver	Ag, Ca, Cd, Cl, Cu, Fe, K, Mg, Mn, Mo, Na, P, Pb, Rb, S, Se, Sr, Zn	Al, As, Br, Co, Hg, Sb, V
National Research Centre for Certified Reference Materials (China)	NCS ZC71001 beef liver	Ca, Cl, Co, Cu, Fe, K, Mg, Mn, Mo, Na, P, Rb, Se, Sr	Al, Ba, Br, Cd, F, Hg, Rb, Pb, S, Ti
National Research Council (Canada)	LUTS-1 nondefatted lobster hepatopancreas	Ag, As, Cd, Ca, Cr, Co, Cu, Fe, Pb, Mg, Mn, Ni, K, Se, Sr, Zn	
	TORT-2 lobster hepatopancreas	As, Cd, Cr, Co, Cu, Fe, Pb, Mn, Hg, Mb, Ni, Se, Sr, V, Zn	Sn
	DOLT-4 dogfish liver	Ag, As, Cd, Cu, Fe, Pb, Hg, Ni, Se, Zn	

[a] Recommended values.

The total phosphorus in gelatin can be determined by a gravimetric method with quinolinium molybdophosphate. The sample is mixed with $MgO\text{-}Mg(NO_3)_2$, evaporated under an IR lamp, ashed for 1 h at 500°C, and dissolved in HCl heating for 30 min on a steam bath.

The determination of Zn, Cu, and Fe in foods by FAAS after dry ashing (AOAC 999.11) or microwave digestion ($HNO_3 + H_2O_2$) (AOAC 999.10) has been proposed by the AOAC. The results of the interlaboratory study in several matrixes including liver paste support the acceptance of this method for application in variety meats. The analyte range for Zn, Cu, and Fe has been established as ≥0.7, 0.2, and 4 mg/kg to dry ashing and ≥4, 0.2, 7 mg/kg to wet ashing.

11.3.3 Electroanalysis

Voltammetry, together with appropriate pretreatment of the sample (digestion methods that are suitable for analysis by atomic spectrometry in which the digest is atomized but may not be suitable for voltammetric analysis), has been shown to be a valid analytical procedure that is certainly applicable to simultaneous metal determinations in multicomponent complex matrixes.

The determination of some minerals (mainly Cu, Zn) in offal (mainly liver), based on voltammetric techniques such as stripping voltammetry (SV) or differential pulse polarography (DPP), is simple, rapid, and requires relatively inexpensive instrumentation—with good sensitivity (capable of determining elements accurately at trace to ultra-trace levels) and the possibility of multielement determination. Electroanalytical techniques provide an alternative to the use of atomic spectroscopic methods for heavy metal determinations and are free of interferences detected by AAS procedures. The standard addition method must be used for quantification [23].

An automatic-continuous method for the simultaneous determination of Cu and Pb based on flow injection analysis (FIA) and SV was proposed by Izquierdo et al. [23]. The method was applied to three different reference materials (bovine liver (NIST SRM 1577a and BCR No. 285) and pig kidney (BCR No. 186a)) and fresh bovine liver, following wet digestion with nitric and sulfuric acids. The method affords determination of the analytes at ng/mL level with good precision, though the Cu content obtained in bovine liver (SRM 1577) and pig kidney (BCR No. 186a) does not coincide with the certified value.

When potentiometric stripping analysis (PSA) is used as electrochemical method for analysis, total organic matter destruction is unnecessary. This technique is an interesting alternative when other electrochemical methods (such as anodic stripping or DPP) fail. In PSA, the potential change of the working electrode is measured as a function of time, and the determination is not based on current measurements; this eliminates measurements errors caused by the liquid resistance between the electrodes and the potential drop over the electrical double layer around the working electrode. Such no-current measurement reduces the background signal and provides the possibility of analyzing samples with a high content of organic redox compounds, without these interfering with the metal determination. The process can be carried out, from the lyophilized sample, in approximately 3–4 h [24]. The application of these techniques is shown in Table 11.5.

11.3.4 Neutron Activation Analysis

Neutron activation analysis (NAA) is a powerful analytical method for the determination of minerals in foods. Different variants of NAA can be employed. Radiochemical neutron activation analysis (RNAA) is used in the case where the induced radionuclides of trace elements are masked by matrix activity. The resulting radioactive sample is chemically decomposed and the radionuclides are isolated by chemical separations. Before separation, the samples have to be chemically decomposed. Usually, samples are digested using various acids or are burned in a oxygen atmosphere. RNAA has the advantage of high sensitivities to many elements, and the data obtained are accurate. As disadvantages, it is rather time-consuming, and the radiochemical procedures can often be very complicated. Instrumental neutron activation analysis (INAA) does not require decomposition of

TABLE 11.5
Minerals and Trace Elements Determination in Variety Meats/Offal by Electroanalytical Methods

Element	Food	Sample Treatment	Technique	Reference
Pb	Bovine liver (1577a and BCR-285)[a], pig kidney (BCR-186)[a], fresh bovine liver	HNO_3–H_2SO_4	FIA-ASV	[23]
Cu, Zn	Bovine liver (1577a and BCR-285)[a]	Dry ashing	DPASV	[44]
Cu, Zn	Liver	HNO_3–$HClO_4$ (microwave radiation	DPASV	[45]
Cu	Fish liver tissues	(1) Sodium dodecylsulfate and sodium deoxycholic acid + sonication; (2) low temperature ashing	PSA	[24]

[a] Standard reference material.

the radioactive sample. The method is not destructive and does not involve complicate handling. All problems related to decomposition of the sample and separation (dissolution, contamination, and losses) are avoided [25].

NAA is currently one of the analytical techniques used in the certification process of minerals from standard reference material (SRM). In new bovine liver SRM, the contributions of INAA to Ca, Cl, Co, Cr, Cs, Cu, Fe, K, Mg, Mn, Mo, Na, Rb, Se, and Zn certification content and of radiochemical procedures (RNAA) to Ag, As, Cd, Cu, Mo, Sb, and Se certification content have been established [22].

Selenium is an element found in food at low concentrations. Highly sensitive techniques are, therefore, required to determine Se. NAA is considered to be the technique of choice, due to its inherent sensitivity, selectivity, and freedom from reagent blank [26].

INAA using both short- and long-term irradiation and RNAA, after wet ashing of samples in a microwave digestion unit, was used for selenium assay in foods from Libya, including as quality control the NIST SRM 1577b (bovine liver). For selenium, better counting statistics and more than one order of magnitude lower detection limits (down to 2 µg/kg) were achieved using long irradiation with radiochemical separation [26].

Selenium distribution in porcine tissue samples (heart, liver, spleen, lung, kidney, cerebrum, cerebellum, and others parts of the brain) from a mercury-polluted area and from a nonmercury exposed area in China was determined by INAA. SRM NIST-bovine liver 1577a and IAEA horse kidney H-8 were used for analytical quality control [27].

A pseudo-cyclic instrumental NAA method in conjunction with anti-coincidence gamma-ray spectrometry (PC-INAA-AC) has been developed for the determination of ppb levels of Se, and applied to foods including organ meats and bovine liver (SRM 1577b). The Se detection limits for this reference material using both conventional and anti-coincidence counting modes are 13 and 9.1 ng, respectively. The precision and detection limits are significantly improved by the proposed method (PC-INAA-AC), and the total experimental time is reduced. The PC-INAA-AC method can be applied to routine analysis of foods for Se without the need to dissolve the samples or to use tedious chemical treatments [28]. The application of these techniques is shown in Table 11.6.

11.3.5 X-Ray Fluorescence

X-ray fluorescence (XRF) can provide simultaneous multielement analyses of foods. It is rapid compared to the multielement atomic spectrometry techniques, and is nondestructive and matrix independent, in the same way as NAA; thus, measurement can be carried out directly on solid samples.

TABLE 11.6
Minerals and Trace Elements Determination in Standard Reference Materials by Neutron Activation Analysis

Element	Food	Method	Reference
Se	Bovine liver 1577b	PC-INAA-AC thermal neutron flux 5×10^{11} n cm^{-2} s^{-1}, irradiation time 30 s, decay time 10–50 s, counting time 20–60 s, HPGe p-type coaxial detector	[28]
Ca, Cl, Co, Cr, Cs, Cu, Fe, K, Mg, Mn, Mo, Na, Rb, Se, and Zn	Bovine liver 1577c	INAA thermal neutron flux 1×10^{14} n cm^{-2} s^{-1}, gamma-ray detector	[22]
Ag, As, Cd, Cu, Mo, Sb, and Se		RNAA thermal neutron flux 1×10^{14} n cm^{-2} s^{-1}, gamma-ray detector	
Se	Bovine liver 1577b	Thermal neutron flux 8×10^{13} n cm^{-2} s^{-1}, fast neutron flux 3×10^{13} n cm^{-2} s^{-1}, HPGE detectors INAA irradiation time 10 s, decay time 20 s, counting time 20 s, long-term (Short-term irradiation time 10 s, decay time 20 s, counting time 20 s, long-term irradiation time 2 h, decay time 33–40 day, counting time 2 h) RNAA decay time 12 day, counting time 1 h	[26]
Hg		INNA long-term irradiation time 2 h, decay time 33–40 day, counting time 2 h RNAA irradiation time 20 h, decay time 7 day, counting time 2 h	
Mg, Cl, Mn, Se, Br, Zn, Co, Fe, Rb, Cd, and Cu	Bovine liver 1577	INAA thermal neutron flux 4×10^{12} n cm^{-2} s^{-1}, irradiation time: 5 min (Mg, Cl, Mn, K) 10 h (Se, Br, Zn, Co, Fe, Rb, Cd, Cu)	[46]
Na	Bovine liver 1577	INNA thermal neutron flux $.10^{16}$ to 10^{18} n m^{-2} s^{-1}	[47]
Se	Bovine liver 1577	CINAA: (irradiation time 20 s, decay time 10 s, counting time 20 s) number cycles 4. Thermal flux 5×10^{11} n cm^{-2} s^{-1}; detector Ge	[48]
I		EINAA: (irradiation time 30 min, decay time 1–6 min, counting time 30 min); detector Ge (Li)	
Se	Bovine liver 1577a, animal blood IAEA-A-13	CINAA: (irradiation time 20 s, decay time 10 s, counting time 20 s) number cycles 4. Thermal flux 5×10^{11} n cm^{-2} s^{-1}; detector Ge (Li)	[49]

Although sample matrix effects can be significant in XRF, they are relatively constant for foods, due to their predominant contents of carbon, oxygen, nitrogen, and hydrogen. An advancement for quantitative XRF analysis without standards is the use of channel electron multiplied arrays (CEMAS) quantitation, similar to NAA in that it does not require standards of similar physical and chemical form as the samples, and it relies on fundamental parameters of x-ray physics for quantitation of the x-ray intensities. This approach eliminates the need for calibration standards of composition similar to the samples, and allows the use of SRMs only for monitoring accuracy and precision [29,30].

The main limitations of the XRF methods are the reduced number of elements to which the method is applicable—only elements having an atomic number between 22 (titanium) and 55 (cesium)—and the need to work under special conditions or with low sensitivity, when light elements such as magnesium are to be measured. Other drawbacks of the XRF methods are high cost, important matrix effects when the salt contents are high, and the fact that the radiation intensity is not a lineal function of the element or element contents [31].

The CEMAS XRF method for multielement analysis of foods involving the determination of Mn, Fe, Cu, and Zn in eight standards (including bovine liver SRM 1577a) and in a variety of

TABLE 11.7
Minerals and Trace Elements Determination in Variety Meats/Offal by X-Ray Fluorescence Methods

Elements	Food	Technique	Reference
Cu, Fe, Mn, and Zn	Bovine liver (1577)[a]	XRF and CEMAS quantitation	[29]
Ca, Br, Cl, Fe, K, Mn, P, Zn, Rb, S, and Sr	Bovine liver (1577)[a]	Lyophilized sample/XRF and CEMAS quantitation	[30]
Ca, Mn, Fe, Ni, Cu, Zn, As, Br, Rb, and Sr	Beef and chicken liver and chicken heart	Water washed/freeze-dried and ground/PIXE	[50]
K, Ca, Mn, Fe, Co, Ni, Cu, Zn, As, Se, Br, Rb, Sr, Hg, and Pb	Fish liver	Freezer and ground/XRF	[51]

[a] Standard reference material.

food materials (without including variety of meats) compared with independent determinations by FAAS, following ashing, has been characterized and validated [29]. The detection limits varied most for Mn, due to the variation in matrix composition of the standards; as a result, Mn in this sample was below the detection limit (1.4 μg/g). In a later study, the analysis was extended to the determination of P, S, Cl, K, Ca, Mn, Fe, Zn, Br, Rb, and Sr in the same SRM, and the method was validated by comparison with FAAS, following ashing. Good agreement was obtained for both techniques, with correlation coefficients from XRF vs. FAAS plots for Mn, Fe, and Zn of 0.94, 0.97, and 0.97, respectively [30]. The application of these techniques is shown in Table 11.7.

11.3.6 Atomic Spectrometry

Atomic spectroscopic techniques are the best choice for elemental analysis, because of their widespread availability and ease of use. The extensive annual literature reviews in the section entitled *Atomic Spectrometry Update-Clinical and Biological Materials, Food and Beverages* of the *Journal of Analytical Atomic Spectroscopy*, reflects the major role that atomic spectroscopy has played in the development of the currents databases on minerals in foods. Comprehensive reviews of literature reports on methods focused on the progress for single and multielement analysis, sampling and sample preparation, reference materials, developments in analytical methodology and instrumentation, and applications in foods, including a variety of meats/offal/edible animal products.

Comparison of spectrofluorometry and HG-AAS for selenium determination in foodstuffs (including bovine liver 1577a) was evaluated by Tinggi et al. [10].

The levels of concentration at which other trace elements such as iron, copper, zinc, manganese, and chromium interfered with hybrid generation of selenium were also investigated. No suppression of the absorption signals was observed at the 15 μg/g level, but at 20 μg/g, the dark green precipitation of copper in the form of chlorocomplexation was observed, and this was found to cause marked suppression of the absorption signal.

The accuracy of the methods was similar for both methods. However, spectrofluorometry was much more sensitive than HG-AAS (detection limit 0.001 vs. 0.033 μg/g). However, it was found to be tedious and time-consuming, and not ideal for the routine analysis of a large number of samples [10].

FAAS and ICP-AES methods were compared for mineral determination in foods including bovine liver 1577a. After wet ashing (nitric acid 80°C overnight), Ca, Cu, Cr, Fe, Mg, Mn, and Zn were determined by FAAS, and Ca, Co, Cu, Cr, Fe, Mg, Mn, Ni, P, V, and Zn were determined simultaneously using ICP-AES. Detection limits for both techniques were in the ppb range, precision was similar, and both provided accurate elemental food composition data [32].

A collaborative study was conducted for evaluating Pd and Cd determination by FAAS, and Zn, Cu, and Fe determination by ETAAS following microwave digestion (nitric acid and hydrogen peroxide) under pressure in a closed vessel. This was done in foods that included bovine and pig liver. Precision and detection limits for all elements were given [33].

López Alonso et al. [6] determined copper and zinc in liver, kidney, and muscle from calves and cows by FAAS, following wet digestion with nitric acid (120°C for 1 h) and 30% hydrogen peroxide. The interest of this work lies in the strict analytical quality control program used during the study. The detection limit (15.3 µg/L for Cu and 19.4 µg/L for Zn in acid digest of blank), precision (<10.7%), and analytical recoveries from a certified reference material (CRM) (pig kidney CRM 186, BCR) and spiked recoveries (100% for both minerals, and similar to those from the CRM) were determined. In addition, published data on copper and zinc concentrations in liver and kidney for animals from different countries were compared, and regulatory limits for these minerals in both matrixes in various countries (Australia, Canada, Slovac Republic) were indicated.

The suitability of quadrupole ICP-MS for Al, As, Cd, Cr, Cu, Fe, Mn, Mo, Ni, Pb, Se, Sn, V, and Zn analysis of foods (including bovine liver 1577a), following microwave closed vessel digestion of sample, has been evaluated. The authors indicated the need for correction of matrix-induced interferences and of the spectral interferences caused by the C, Cl, and Ca content of digests. The method proposed is rapid (8 samples per hour) and reliable for the multielement analysis of food samples [34].

Concentrations of Cd, Pb, and Zn (determined by ICP-MS) in fish blood, carcass, and liver as possible biomonitoring parameters of aquatic contamination have been proposed by Brumbaugh et al. [35]. Cd and Pb concentrations in fish blood were found to be a better indicator than liver or carcass, and there were practical advantages for sampling blood in comparison with other tissues. Zn determination in blood was no better or worse than in carcass or liver, but none of these tissues seemed particularly useful [35].

The use of a preconcentration step with a SiO_2-C_{18} solid-phase extraction (SPE) column and a complexing agent such as diethyldithiophosphate (DDTP) to improve the detection limit and avoid matrix effects of the ETAAS technique was evaluated for the determination of selenium in some reference materials (animal blood IAEA A-13, bovine liver BCR 185, pig kidney CRM 186). Different quantities of SiO_2-C_{18}, for minicolumn filling, were tested. Best results were achieved by using 30 mg of the sorbent material, and to secure reasonable precision in the procedure, not less than 200 µL of ethanol should be used for elution of the DDTP-selenium complex adsorbed in the minicolumn. The use of chemical modifiers such as Rh, Ni, Ir, and Pd in order to minimize the depression of selenium absorbance in the presence of DDTP, was tested. Clearly, the addition of Rh proved to be the best choice, in either the chloride or the nitrate form, whereas Pd depressed selenium absorbance even more. No memory effects were detected with any of the metallic modifiers evaluated.

It was observed that complexation and absorption of the DDTP-selenium complex can be performed in 0.2%–2% v/v nitric acid or at even higher concentrations, though it is convenient to keep the acid concentration low (e.g., 0.2% v/v), in order to preserve the column. When the optimized preconcentration conditions were applied to CRM, some matrix effects were clearly present also with DDTP-SiO_2-C_{18}, but they were overcome when appropriate dilution of the sample digest was carried out [36].

A thermospray flame furnace atomic absorption spectrometer (TS-FF-AAS) was employed for Co determination in biological materials, including bovine and pig kidney and reference material (bovine liver 1557b). Hydrochloric acid is used for cobalt extraction, following derivatization with ammonium pyrrolidine dithiocarbamate and a cloud point extraction step with Triton X-114. This method has been compared with FAAS. The proposed method is 670-fold more sensitive than that obtained by conventional FAAS. As advantages, mention should be made of low cost, and fast and effective performance, because no sample digestion is required [37]. The application of these techniques is shown in Table 11.8.

TABLE 11.8
Minerals and Trace Elements Determination in Variety Meats/Offal by Atomic Spectrometric Methods

Element	Food	Sample Treatment	Technique	References
Ca, Cu, Fe, Mg, Mn, K, Na, Zn	Bovine liver (1577a)[a]	Microwave digestion at ambient temperature ($HNO_3 + H_2O_2$)	FAAS	[52]
Mn, Cu, Zn, Fe	Liver and kidneys of duck, geese, turkey, chicken, hen, rabbit, sheep	Dry ashing	FAAS	[53]
Cu, Zn	Bovine liver and kidney, pig kidney (CRM-186)[a]	Wet digestion ($HNO_3 + H_2O_2$) at 120°C	FAAS	[6,54]
Cu, Zn, Mn, Fe, Co	Liver and kidney of cattle	Dry ashing (450°C)	FAAS	[55]
Zn, Cu, Fe	Liver paste	Dry ashing (450°C). Addition of HCl and evaporation to dryness. Residue dissolved in HNO_3	FAAS	[56]
Zn, Cu, Fe	Bovine and pig liver	Microwave acid-assisted digestion ($HNO_3 + H_2O_2$)	FAAS	[33]
Cu, Fe, Mn, Zn	Liver, kidney and blood of cattle, pig kidney (CRM-186)[a]	Wet digestion ($HNO_3 + H_2O_2$)	FAAS	[57]
Cu and Zn	Liver and kidney of sheep and cattle	—	AAS	[58]
Co, Cu, Fe, Mn, Ni, Zn	Liver and kidney of bovine (bull, steer, cow, heifer, calf), ovine (sheep and lambs), porcine (market hogs, boar/stag, sow), poultry (chicken, turkey, and duck)	Dry ashing (500°C–550°C). Dissolution of ashes with HCl	AAS	[59]
Ca, Cu, Fe, Mg, Mn, Zn	Chicken heart, liver, kidney, gizzard and spleen	Wet digestion ($HNO_3 + H_2SO_4 + HClO_4$) at 80°C. After cooling, demineralized water is added, heating to 150°C	AAS	[60]
Zn, Cu, Mn, Ni, Se, Co, Al	Pig liver and kidney and bovine liver (1577 and 1577a)[a]	Dry ashing: 450°C Se: Dry ashing: 450°C (matrix modifiers: $MgNO_3 + MgO$)	Zn, Cu, Mn: FAAS Se: HG-AAS Ni, Co, Al: ETAAS	[61]
Cu, Mb, Fe, Co, Mn, Zn,	Liver, kidney, heart, spleen, lung, cerebrum, cerebellum, pancreas of camels	—	FAAS Se: HG-AAS	[62]
Cu, Se, Zn	Bovine liver and kidney Bovine liver[a] (1577a)	Wet digestion ($HNO_3 + HClO_4 + H_2SO_4$), 200°C	Cu, Zn: FAAS Se: HG-AAS	[63]
Cd, Cu, Se, Zn	Liver and kidney of cattle, swine, poultry, horses, calves, and sheep	Automated wet digestion ($HNO_3 + HClO_4 + H_2SO_4$)	Cu, Zn: FAAS Se: HG-AAS	[7]

(*continued*)

TABLE 11.8 (continued)
Minerals and Trace Elements Determination in Variety Meats/Offal by Atomic Spectrometric Methods

Element	Food	Sample Treatment	Technique	References
Cd, Cu, Zn, Se	Liver and kidney of cattle and bovine liver (1577)[a]	Cd, Cu, Zn: wet digestion (HNO_3), 80°C Se: wet digestion (HNO_3 + $HClO_4$ + H_2SO_4)	Cu, Zn: FAAS Se: HG-AAS	[64]
Zn, Cu, Fe, Ni	Liver paste	Dry ashing (450°C). Addition of HCl and evaporation to dryness. Residue dissolved in HNO_3	Zn, Cu, Fe: FAAS Ni: HG-AAS	[65]
Zn	Pig kidney (BCR-186)[a], lamb kidney, rabbit liver	CUES (HNO_3 or HCl) incorporated to an on-line FI-FAAS Wet digestion (HNO_3)	FI-FAAS	[14]
Se	Bovine liver (1577a) [a]	Wet digestion (HNO_3 + $HClO_4$)	HG-AAS	[10]
Se	Rabbit tongue and kidney, chicken liver and heart, lamb lung, pork kidney, liver, lung, brain, and heart	Wet digestion (HNO_3)	HG-AAS	[66]
Se	Porcine heart, liver, spleen, lung, kidney, cerebrum, cerebellum	Wet digestion (HNO_3 + $HClO_4$), addition of HCl (to reduce Se (VI) to Se (IV)). Addition of $NaBH_4$-acid system to generate H_2Se	HG-ETAAS	[27]
Cu, Zn	Bovine liver (1577a)[a]	Microwave oven drying and grinding	Solid sampling-ETAAS	[21]
Se	Bovine liver (BCR-185)[a], animal blood (IAEA-A-13)[a], pig kidney (CRM-186)[a]	Microwave acid-assisted digestion (HNO_3) and preconcentration using a SiO_2-C_{18} minicolumn and DDTP	ETAAS	[36]
Fe, Co	Bovine liver (1577b)[a]	Vapor-phase acid (HNO_3) digestion using a focused microwave oven	ETAAS	[12]
Co	Bovine liver (1577a)[a]	Acid extraction (HCl)	TS-FF-AAS	[37]
Ca, Cu, Fe, Mg, Mn, Zn	Bovine liver (1577a)[a]	Wet ashing: HNO_3, 80°C; then H_2O_2 at 100°C until digest were clear and HCl	FAAS	[32]
Ca, Co, Cu, Fe, Mg, Mn, P, Zn			ICP-AES	
Al, Co, Cu, Fe, Mn, Mo, Ni, Se, Sn, V, Zn	Bovine liver (1577b)[a]	Microwave acid-assisted digestion (HNO_3 + H_2O_2)	ICP-MS	[34]
Zn	Blood and liver of common carp, channel- and flathead catfish, largemouth- and spotted bass, and white crappie	Wet digestion (HNO_3 + H_2O_2), 110°C	ICP-MS	[35]
Bi, Co, Cs, Cu, Fe, Mn, Mo, Rb, Sb, Sn, Sr, Tl, Zn	Bovine liver (1577a)[a]	Microwave acid-assisted digestion (HNO_3 + H_2O_2)	ICP-MS	[11]

Cu, Se, Zn	Bovine kidney and liver Bovine liver (1577b)[a]	Wet digestion ($HNO_3 + H_2O_2$), 70°C	ICP-MS	[3]
Fe, Na, Al, K, Mg, Ca, Zn	Liver, kidney, spleen, and heart of sheep	—	ICP-MS	[67]
Ag, Al, As, Ca, Cd, Co, Cr, Cu, Fe, K, Li, Mg, Mn, Mo, Ni, Pb, Se, Sn, V, Zn	Dogfish (DOLT-2)[a]	Microwave acid-assisted digestion (HNO_3 + HCl), at 180°C	ICP-MS	[68]
Ni, Cu	Bovine liver and kidney, pig kidney (CRM-186)[a]	Microwave acid-assisted digestion ($HNO_3 + H_2O_2$)	Ni: ICP-MS Cu: ICP-AES	[69]
Co, Cr, Ni, Cu, Fe, Mn, Mo, Se, Zn	Liver and kidney of cattle, pig kidney (CRM-186)[a]	Wet digestion ($HNO_3 + H_2O_2$)	ICP-AES	[70]
Co, Cr, Ni			ICP-MS	
Fe, Se	Bovine liver (CRM-185R)[a]	Microwave acid-assisted digestion (HNO_3)	CCT-ICP-MS	[71]
Mn, Cu, Mb, Zn, Fe, Se	Bovine liver (1577b)[a]	Microwave acid-assisted digestion ($HNO_3 + H_2O_2$)	Mn, Cu, Mb, Zn: ICP-MS Se, Fe: ICP-DRC-MS	[72]
Se	Liver, kidney, and serum of hen		ICP-AES	[73]
Na, K, Mg, Ca, P, Cu, Zn, Fe, Mn	Brain, lungs, heart, spleen, liver, kidney, pancreas, digestive tract, and offals of deer and bovine liver (1577b)[a]	Wet ashing (HNO_3), 480°C	ICP-AES	[74]
Ca, Cu, Fe, Mg, Mn, Zn	Bovine liver (1577b)[a]	Partial microwave acid-assisted digestion ($HNO_3 + H_2O_2$)	ICP-AES	[15]
Cu	Bovine liver	Microwave acid-assisted digestion ($HNO_3 + H_2O_2$)	ICP-AES	[75]
Ca, Co, Cr, Cu, Fe, Mn, Mo, Ni, Se, Zn	Liver and kidney of cattle, pig kidney (CRM-186)[a]	Wet digestion ($HNO_3 + H_2O_2$)	ICP-AES	[9]
Sb, Se, Sn	Lobster hepatopancreas (LUTS-1)[a], dogfish liver (DOLT-2)[a]	Slurry with HCl and thiourea and ultrasonic sonication	SS-HG-MIP-AES	[18]
Bi, Ge, Sb, Se, Sn, Fe, Mg, Mn, Zn	Lobster hepatopancreas (LUTS-1)[a], dogfish liver (DOLT-2)[a]	Slurry with HCl and thiourea and ultrasonic sonication	SS-CVG/ NEB-MIP-AES	[76]

[a] Standard reference material.

11.3.7 Speciation

Elemental speciation in foods shows that metals can be present in the ionic form and/or complexed to various binding proteins. A considerable amount of the proteins in mammalian tissues are metal binding proteins. Especially in relation to enzymes, metals are essential for life. In the post-genome era, the challenges and needs of research also comprise characterization of the metallome, i.e., the entire set of trace element species in the organism [38]. Elemental speciation in biological samples provides crucial evidence for determination of the bioavailability, toxicity, and environmental behavior of elements.

A review of speciation analysis by hyphenated techniques has been published. Separation mechanisms including size-exclusion, anion- and cation-exchange, and reverse-phase (HPLC), and flat bed and capillary zone electrophoresis, are discussed. The advantages and the limitations of various element-selective (e.g., AAS, ICP-AES, ICP-MS) and molecule-specific (electrospray mass spectrometry MS/MS) detection techniques are commented. The application to mineral speciation in animal tissues has been compiled [39].

Size-exclusion chromatography (SEC) as a first step to isolate the methallothionein-like protein fraction in mussel hepatopancreas, followed by anion-exchange chromatography coupled with ICP-TOF-MS, has been applied to the specific detection of 16 metals in aquarium mussel samples living in seawater spiked with 16 metals. This method makes it possible to determine many metals in a single chromatographic run. However, owing to the lack of sensitivity of the ICP-TOF-MS system, biological sample from uncontaminated areas require the use of more sensitive detection methods such as quadrupole or double-focusing ICP-MS detectors [40].

SEC with a double-focusing ICP-MS device has been successfully applied to Cu, Zn, Mn, Fe, Cd, S, P, Mo, Co, Ca, and Mg distribution in bovine liver 1577a. A magnetic sector mass spectrometer eliminates polyatomic ion interferences for Fe, S, and P, and provides high sensitivity. Sample preconcentration is not needed. This method generates information on the approximate molecular weight of proteins containing particular elements, without using a standard sample of the same protein. This is one of the advantages of SEC compared to RP-HPLC or ion-exchange chromatography [41]. SEC and RP chromatography coupled either on-line to ICP-MS for species quantification via their trace element content or off-line to electrospray ionization (ESI-MS) for species identification via their molecular weight were applied to fresh porcine liver in order to investigate metallothioneins and SOD, Fe, Cu, Zn, and Mn species. Freezing porcine liver for several weeks did not significantly affect the stability of the quantified species. However, lyophilization is not recommendable [38].

A method has been developed for the simultaneous determination of selenomethionine and selenocysteine in offal (heart, liver, and kidney). The technique allows the conversion of all protein-bound selenium, to either carbamidomethylated selenocysteine or to carbamidomethylated selenomethionine, with subsequent quantification by HPLC-ICP-MS [42].

SEC and/or anion-exchange chromatography coupled to ICP-MS was used to investigate the speciation of Se, As, Cu, Cd, and Zn in tissue extract (including gonad and liver) from largemouth bass (*Micropterus salmoides*) collected from habitats with different levels of contamination. In liver and gonad, Cu, Cd, and Zn are associated with metallothionein, but Se was not associated with this protein. Only in gonad did Se appear to be associated with a high molecular weight protein [43].

11.4 CONCLUSIONS

Unfortunately, there is little information on the mineral and trace elements in a variety of meats/offal—the existing data being fundamentally related to these minerals in fresh liver and kidney and reference materials. In mineral element analysis of variety of meats/offal, wet digestion is the technique most often used in sample preparation. The most widely used techniques for mineral

determination in these foods are atomic absorption, ICP-AES, and ICP-MS—these being the most reliable options for multielemental analyses.

Elemental speciation in these foods provides crucial evidence for defining the bioavailability, toxicity, and environmental behavior of mineral and trace elements. The hyphenated techniques are the most commonly used options for this purpose.

ABBREVIATIONS

AAS	Atomic absorption spectrometry
AOAC	Association of Official Analytical Chemists
ASV	Anodic stripping voltammetry
CCT-ICP-MS	Collision cell technology inductively coupled plasma-mass spectrometry
CEMAS	Channel electron multiplied arrays
CINAA	Cyclic instrumental neutron activation analysis
CUES	Continuous ultrasound-assisted extraction system
DAN	2,3-Diaminonaphthalene
DDTP	Diethyldithiophosphate
DPASV	Differential pulse anodic stripping voltammetry
DPP	Differential pulse polarography
EINAA	Epithermal instrumental neutron activation analysis
ESI-MS	Electrospray ionization
ETAAS	Electrothermal atomic absorption spectrometry
FAAS	Flame atomic absorption spectroscopy
FI	Flow injection
FIA	Flow injection analysis
HG-AAS	Hydride generation atomic absorption spectrometry
HG-AFS	Hydride generation atomic fluorescence spectrometry
HPLC	High performance liquid chromatography
ICP-AES	Inductively coupled plasma absorption emission spectroscopy
ICP-DRC-MS	Dynamic reaction cell inductively coupled plasma-mass spectrometry
ICP-MS	Inductively coupled plasma-mass spectrometry
ICP-TOF-MS	Inductively coupled plasma time-of-flight mass spectrometry
INAA	Instrumental neutron activation analysis
MS	Mass spectrometry
MSIS	Dual-mode sample introduction system
NAA	Neutron activation analysis
PC-INAA-AC	Pseudo-cyclic neutron activation and anti-coincidence gamma-ray spectrometry
PIXE	Proton-induced x-ray emission
PSA	Potentiometric stripping analysis
PTFE	Polytetrafluoroethylene
RNAA	Radiochemical neutron activation analysis
SEC	Size-exclusion chromatography
SEC-ICP-MS	Size-exclusion chromatography inductively coupled plasma-mass spectrometry
SEC-ICP-TOF-MS	Size-exclusion chromatography inductively coupled plasma time-of-flight mass spectrometry
SPE	Solid-phase extraction
SS-CVG/NEB-MIP-AES	Slurry sampling chemical vapor generation pneumatic nebulization microwave induced plasma optical emission spectrometry

SS-HG-MIP-AES	Slurry sampling hydride generation microwave induced plasma optical emission spectrometry
SV	Stripping voltammetry
TS-FF-AAS	Thermospray flame furnace atomic absorption spectrometry
XRF	X-ray fluorescence

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Part IV

Safety

12 Spoilage Detection

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CONTENTS

12.1 INTRODUCTION

Once an animal is slaughtered and the carcass is eviscerated, washed, and split into halves, a complex series of processes related to changing the muscle into edible meat start. In addition, a considerable amount of material is obtained as by-products.

Although consumption of meat by-products widely varies among countries and regions, it is necessary to control the quality of this food and its wholesomeness and sanitation. It is a task that requires a deep knowledge of chemical changes occurring during the product lifespan, related to microflora selection due to the particular microecology of a given by-product and to chemical changes such as lipid oxidation and proteolysis.

The most frequently used edible by-products are blood, liver, heart, kidney, tongue, stomach, and lungs, although other authors [1] report that variety meat is normally limited to liver, heart, tongue, kidney, sweetbreads, brain, and tripe. The specific by-product—organ or tissue—considered edible by a given human population group depends on facts such as tradition, religion, and culture.

12.2 FACTORS AFFECTING MEAT BY-PRODUCT SPOILAGE

Spoilage of meat and meat by-products implies deterioration following two mechanisms: chemical, mainly due to the presence of endogenous enzymes that remain active after slaughtering, and *rigor mortis*; in some cases, like cathepsins and calpains in the striated muscle, the enzymes are activated

due to *postmortem* conditions. A wide variety of enzymes involved in the various biochemical pathways remain active after slaughtering, acting upon the tissues and promoting decomposition due to lipolysis and proteolysis in organs such as stomach, intestines, and brain. Enzymes in meat by-products were discussed in previous chapters.

The other spoilage mechanism is due to the presence of colonizing microorganisms. As any other food, the spoilage—chemical or microbial—of an edible meat by-product depends on a given set of conditions allowing deterioration to proceed at a maximum rate. Microbial spoilage depends on two main factors: the dominant microfloras growing and colonizing the product and the produced microbial metabolites. Both are a result of the available substrate and the specific microecology supporting the microbial growth.

Spoilage depends on intrinsic and extrinsic parameters affecting the biochemistry of the most abundant or most active strain [2]. Conversely, as with the striated muscle in which the chemical composition is roughly the same for all muscles in the carcass, by-products have a wide variety of compositions, microstructures, and environmental parameters. Due to this diversity, edible viscera are colonized by different microflora and, in consequence, the spoilage mechanism may vary.

12.2.1 Intrinsic Factors

The main intrinsic factors determining the time for microbial proliferation in meat and meat products are substrate availability and initial microbial counts. Shelf life, to a large extent, is determined by the initial microbial load, although certain microorganisms have a higher relationship to changes in quality characteristics than others due to the presence of specific enzymes altering the substrate. High total viable counts considerably shorten the shelf life [3]. As meat is a highly nutritious food for humans as well as for microorganisms, all nutrients for microbial proliferation are present. However, glucose is the main nutrient determining the type and rate of microbial growth [4].

Microorganisms involved in meat spoilage mainly originate in the rumen, intestines, skin, ganglions, and feathers. In addition, handling and contact with the workers' clothing, floors, walls, and instruments are also sources of contamination [5]. Several toxins could also generate due to in vivo malfunction of certain organs such as liver, spleen, or pancreas and accumulate in the viscera. Due to the availability of compounds easily metabolized by indigenous microbial flora meat by-products decompose faster than skeletal muscle. By-products can also contain pathogens without showing signs of deterioration [2]. Finally, pH in the living striated muscle is near 7.0 and drops to 5.5–5.6 within 24 h after slaughtering; high final pH values result when the animals are exhausted during transportation [6]. In this type of meat, spoilage bacteria multiply and shorten the shelf life [7]. In a similar fashion, in most meat by-products pH remains higher after slaughtering (Table 12.1), allowing microbial spoilage to take place.

12.2.2 Extrinsic Factors

Those factors involved in meat by-product spoilage are similar to extrinsic factors affecting any protein-rich food, namely, oxygen availability, water activity, pH, temperature, presence of bacteriostatic compounds, and initial microbial load. Aerobic bacteria have an absolute requirement for oxygen; therefore availability of this element determines their growth. The most important spoilage bacteria (*Pseudomonas* spp.) are strict aerobes. Anaerobic bacteria grow within the tissues as they need absence of oxygen. Facultative anaerobes grow better in the presence of oxygen. Redox potential, related to oxygen concentration in the gas environment is relatively low (–50 mV) although it increases in the surface; strict or facultative aerobes are the most frequently found microorganisms [8]. Water activity is also a factor controlling food spoilage; bacteria stop growing at $a_w = 0.90$, whereas yeast and moulds can grow up to $a_w = 0.80–0.88$ [3]; an exception is the halophiles that can grow at salt concentrations as high as 200 g/L and pH close to 11, $a_w = 0.75$; these organisms include denitrifiers, sulfate reducers, and methanogens [9] and therefore produce

TABLE 12.1
Average pH of Some Tissues of Various Species

Organ	Average pH	References
Blood	7.3–7.4	[16]
Brain	7.02	[24]
	6.8 (abnormal)	
Heart	7.0–6.8 (*in vivo*)	[59]
	5.7 (*postmortem*)	
Intestine		
Large	8.0–8.3	[14]
Small	7.5–8.0	[14]
Kidney	6.8	[23]
Liver	6.99 (*in vivo*)	[36,56]
	6.3 (*postmortem*)	
Lung	6.69	[58]
Pancreas	8.1–8.5	[60]
Rumen	6.2	[14]
Spleen	6.8–7.0	[16]
Stomach (non-ruminant)	1.5–3.0	[16]
Thyme	7.28–7.32	[57]
Tongue	8.1 (*in vivo*)	[16]
	6.0 (*postmortem*)	
Uterus (heifers)	7.1–7.2	[14]

specific spoilage-related compounds. Several meat by-products are traditionally preserved by salting; therefore, halophiles may grow in this environment.

Bacteria relevant to meat, meat products, and other foods are divided into three groups according to the temperature range within which they can grow: mesophiles (10°C–45°C), psychrophiles (0°C–28°C), and psychrotrophs (10°C–45°C). Mesophiles do not grow below 10°C, but psychrotrophs, of which *Pseudomonas* are the most important in meat and meat-like foods, can grow even at 0°C. The nearer to 0°C, the slower the growth rate and the longer the shelf life. Many mesophiles cause spoilage, but since meat is generally kept under refrigerated temperatures, most spoilage is due to psychrophiles [5]. By-products are frequently exposed to careless handling as they are considered of less commercial value than meat; failure in the refrigeration, storage, and transport is frequently the cause of losses due to spoilage [10].

As mentioned before, pH is a determinant factor for by-product spoilage. On high pH (>6.0) spoilage proceeds at lower microbial loads than in normal pH (<5.8) [11]; with the exception of the stomach, pH in most viscera is close to or above neutrality.

12.3 SPOILAGE AND BY-PRODUCT COMPOSITION

After evisceration and by-product separation, the initial microbial load depends on the counts previously incorporated during slaughtering and evisceration, and selected due to the availability of nutrients in the substrate [12]. Any protein-rich food is easily contaminated by bacteria, and meat by-products have high protein content (Table 12.2). The liver, tail, ears and feet of cattle have a protein level close to that of lean meat tissue, although high collagen content is present in most smooth muscles [1]. The lowest protein level is found in the brain and the highest in the liver [13]. Meat by-products are particularly rich in proline, hydroxyproline, and glycine, mainly in lungs, stomach,

TABLE 12.2
Composition (%) of Selected Animal By-Products (Range Considering Beef, Pork, and Lamb Averages)

	Protein (g/100 g)	Fat (g/100 g)	Saturated Fat (g/100 g)	Long-Chain Unsaturated Fat (mg/100 g)
Brain	10.3–12.7	7.6–8.6	46.1–48.4	51.6–53.9
Heart	16.8–28.5	2.7–3.6	40.9–46.0	54.0–59.1
Kidney	15.4–25.4	2.6–6.8	43.8–56.5	43.5–56.2
Liver	20.0–23.7	3.7–7.8	37.3–38.3	61.7–62.7
Pancreas	14.7–28.5	4.0–19.9		
Tongue[a]	15.3–22.2	14.6		
Blood[b]	17.0–17.4			
Spleen			46.8–50.6	49.4–53.2
Tripe	12.3–13.1	20–23	0.9–3.2	14–23

Sources: Ockerman, H.W. and Hansen, C.L., Edible meat by-products, in *Animal By-Product Processing*, Ockerman, H.W. and Hansen, C.L., Eds., Ellis Horwood, Chichester, U.K., 1988; Liu, D.C., Better utilization of by-products from the meat industry, Extension Bulletin, Food and Fertilizer Technology Center for the Asian and Pacific Region, Taipei, Taiwan, 2001; Randall, D. et al., *Eckert Animal Physiology: Mechanisms and Adaptation*, W.H. Freeman, London, U.K., 2002; Hill, R.W. et al., *Animal Physiology*, Sinauer Associates, Sunderland, MA, 2004.

[a] Beef.

[b] Beef and pork.

and intestines, and have low levels of tryptophan and tyrosine. Blood has high protein content (17.0 g/100 g), with a good amino acid balance [14].

Several organs contain more polyunsaturated fatty acids than lean meat; brain, heart, kidney, liver, and lungs have the lowest level of monounsaturated fatty acids and the highest level of polyunsaturated fatty acids (Table 12.2). There is three to five times more cholesterol (260–410 mg/100 g) in organs than in lean meat, brain being the one containing the highest cholesterol (1352–2195 mg/100 g) and phospholipids levels [13,15,16].

The smooth muscles usually contain larger amounts of vitamins than the striated muscle in the same animal species. Organs are particularly rich in riboflavin (kidney and liver); niacin, B12, B6, and folic (liver and kidney) and ascorbic acid (liver). Kidneys, especially from lamb, as well as lung and spleen from pork contain high iron and vitamin levels; copper is high in livers of large species and phosphorus and potassium in sweetbreads. Sodium content is lower than the one found in striated muscle, with the exception of brain, kidney, lungs, and spleen [13,15,16].

With the exception of the non-ruminant stomach, the smooth muscle pH is close to neutrality (Table 12.1). This fact allows a rapid colonization of a wide number of pathogens and spoilage microorganisms. pH decrease in liver has been used by some authors [17,18] as a spoilage indicator due to lactic acid bacteria contamination.

12.3.1 Blood

As it is a significant part of the animal body weight (2.4%–8.0%), blood is a valuable by-product usually recovered from pigs, cattle, and lambs. During industrial processing, blood is separated into plasma (60%–80%) and red cells (20%–40%). The plasma consists mainly of 7%–8% protein and 91% water. The red cells consist of 34%–38% protein and 62% water [15]. On rare occasions, blood is consumed directly; in general it is utilized as a raw material for a wide variety of ethnic

products. However, as these processed foods usually undergo other preservation treatments, such as salt addition, pasteurization or vacuum packaging, the product has an extended shelf life due to a combination of factors [19,20]. The use of blood in meat processing colors the final product [13], whereas the plasma provides excellent functional properties (water retention, emulsifying capacity, cohesion) without notably modifying the product's original pigmentation. Due to its content in nutrients (water, amino acids, proteins, carbohydrates, lipids, hormones, vitamins, electrolytes, dissolved gases, and cellular wastes), the main spoilage mechanisms in blood are via microbial metabolism. Proteins are easily denatured during blood concentration and drying, making them easily spoiled by proteolysis, decarboxylation or microbial growth [15,21]. The two main spoilage bacteria in vacuum-packaged blood sausages are *Weissella viridescens* and *Leuconostoc mesenteroides* [19].

12.3.2 Liver

Liver is the most widely consumed meat by-product, either directly or processed. Livers from lambs, veal calves, and young cattle are preferred for the table because they have a lighter flavor and texture [1,15]. It is rich in protein (20%–24%), fat (5.6%; 60%–62% unsaturated and 15% saturated fatty acids), carbohydrates (2.73%), vitamins, mainly B12 and folic acid, and minerals (Na, K, Ca, Mg, Fe, Cu) [17,22]. Due to its composition, it easily undergoes lipid oxidation and microbial contamination, glycogen being the main substrate for liver microbial spoilage. Its pH is around 6.99 *in vivo* and 6.3 *postmortem* (Table 12.1).

12.3.3 Heart

Heart is commonly used as a table meat or as an ingredient in processed meats. The cardiac muscle is rich in mitochondria, as it undergoes oxidative phosphorylation; myoglobin is the pigment responsible for oxygen storing [16]. Most of its energy proceeds from fat, although carbohydrates also provide energy resources [14]. It contains 18% protein, 4.5% fat (1.7% saturated, 116 mg/100 g cholesterol, 75 mg/100 g polyunsaturated), and it is not especially rich in vitamins [13]. Its pH ranges from 7.0 to 6.8 (*in vivo*), but declines to 5.7 *postmortem* (Table 12.1). Due to its similarity in chemical composition and pH with the striated muscle, it is assumed that it can be colonized by microflora closely related to carcass meat.

12.3.4 Kidney

Kidneys are widely accepted as foods in most of the world. They are removed from the fatty capsules that hold them in place; the urethra and blood vessels need to be trimmed before the kidneys are prepared for processing [15]. The approximate composition of kidneys are: 17.5% protein, 2% fat; (0.7% saturated), 75 mg polyunsaturated fatty acids/100 g, and 330 mg cholesterol/100 g; it is high in vitamins, specially in folic acid [13]. Kidney pH is around 6.8; due to this high pH, colonization by pathogens is likely to occur [23].

12.3.5 Brain

The brain, nervous system, and spinal cord are usually prepared direct for the table rather than processed. The membranes (the pia mater and arachnoid meninges) are peeled from the brain before cooking [13]. Brains, nervous systems and spinal cords are a source of cholesterol which is the raw material for the synthesis of vitamin D3. At pH around neutrality [24] and because of its rich chemical composition, it is easily decomposed by bacterial as well as chemical mechanisms. It is such a rich substrate for microbial growth that, together with heart and liver, brain extracts are the most commonly used selective culture media.

12.3.6 Rumen, Stomach, and Intestines

The ruminant stomachs from cattle and lamb have four compartments: rumen, reticulum, abomasum, and an omnivore stomach. The rumen and reticulum are those most often used as food [25]. They are generally processed at the place of collection by washing, scalding, and bleaching, and processed or sewn to form a casing. Pig stomachs are composed mainly of smooth muscle and collagenous connective tissue [14]; they are cleaned and scalded to remove the mucosa lining, and also consumed directly or used as a casing for sausages. Due to the possibility of microbial colonization, intestines must be thoroughly cleaned and stored at temperatures lower that 3°C.

Animal intestines are used as food after being boiled in some countries. These are also used in pet food or for meat meal, tallow, or fertilizer. However, the most important use of the intestines is as sausage casings [15]. Animal intestines, when removed from the carcass, are highly contaminated with microbes and very fragile. They must be cleaned immediately after the slaughter of the animal. The fat is separated from the intestines, and the feces stripped out; the inner mucosa membrane is separated from the casing, all strings and blood are removed. Intestines must be emptied and cleaned and passed through a manure stripper to squeeze out the intestinal contents.

12.3.7 Sweetbread

Sweetbreads are divided into the thymus gland or throat sweetbread, and the pancreas or stomach sweetbread, the most prized because of its larger size. Both sweetbreads come from young animals, lambs, and calves; pork sweetbreads are generally discarded. The thyme glands are rich in connective tissue that forms a fibrous capsule penetrating into the gland and dividing it into lobules; it is also rich in fat. Both components, connective tissue and fat, increases with the age of the animals [16], this being the reason why only sweetbreads from young animals are consumed.

Throat sweetbread is commonly used for further processing such as pâté, sausages, or terrines [16]; whereas stomach sweetbread is used for direct consumption. Therefore, microbial contamination occurring during slaughtering and evisceration is considerably reduced in throat sweetbread due to further heat treatment. This does not occur in pancreas, where initial microbial loads could increase during storage, and if not properly processed, before serving. In addition, the thymus glands from lambs and calves are blanched to firm the tissue, and peeled from the capsule before they are cooked. *In vivo* pH for both organs is above neutrality (7.28–7.32 for thyme and 8.1–8.5 for pancreas) (Table 12.1).

12.3.8 Other Edible Meat By-Products

Depending on tradition, food availability, and tastes, a wide variety of meat by-products are considered as suitable for human consumption. Most of them share the characteristics of rapid spoilage due to their enzymatic content, pH close to neutrality, and the presence of compounds easily utilized by the contaminating microflora. Edible udder is obtained only from bovines; it is mainly composed of connective tissue, and generally used for direct consumption in certain ethnic dishes. It is not suitable as raw material due to its poor physical functionality (emulsifying capacity, water retention, etc.) [13]. Spleens are widely consumed in processed foods in the United Kingdom and the United States; *in vivo* pH is 6.8–7.0, making spleen a suitable environment for pathogen proliferation, mainly *Staphylococcus* that may also be found *postmortem*. Nonpregnant pig uteruses are the only ones consumed as human food; generally they contain high microbial loads [1]. Pig, calf, and lamb lungs are mainly used to make stuffing and some types of sausages and processed meats [15]. Tongues include small portions of the trachea, larynx, hyoid bones, associated muscle, and salivary glands; the protective covering membrane is removed before storage [13]. Tongue undergoes *postmortem* proteolysis and, as a consequence, *rigor mortis*; pH declines from 8.1 *in vivo* to 6.0 *postmortem*. Therefore, microbial colonization is similar to that found in raw striated muscle.

12.4 MICROBIAL-RELATED SPOILAGE

The native microflora in raw meat is mainly composed by yeasts, bacilli, micrococcus, staphylococci, corinebacteria, *Brochothrix thermosphacta*, *Moraxella*, *Acinetobacter*, *Carnobacterium* spp., *Enterobacteriaceae*, *Lactobacillus* spp., *Leuconostoc* spp., *Salmonella* spp., *Pseudomonas* spp., *Shewanella putrefaciens*, and *Listeria* spp. [27]. Microorganisms mainly involved in meat by-products spoilage are *Pseudomonas* ssp., *B. thermosphacta*, *Enterobacteriaceae*, and lactic acid bacteria [28]. Microbial contamination of viscera is higher than in other meats due to either the food origin or the poor hygienic and chilling conditions [10]. *Enterobacteriacea* predominate in poor refrigeration conditions (above 10°C), off-odor occurs when the population is above 10^7 cfu/g; average microbial counts in offal from various animal species are 1.3–4.6 were 1.4–4.1 log cfu/g before and after chilling, respectively.

However, as microorganisms first utilize low molecular weight nutrients such as glucose, glucose-6-phosphate, ribose, glycerol, amino acids, and lactate [3,4,12,29,30] those foods with high content of these compounds will be readily altered. This is the case of liver, rich in glycogen, and blood. Glucose is the only substantial component of meat that supports its growth [31]; under anaerobic conditions, its spoilage potential is very low, producing lactic acid and small amounts of volatiles, so the result is a slight off-odor [31–33]. In an aerobic complex medium such as meat by-products, it produces highly odoriferous compounds such as acetoin, acetic, isobutyric, isovaleric acids, and their aldehydes and alcohols [31,33]. In addition, there is a wide range of facultative anaerobes that preferentially utilize glucose; although some utilize glucose-6-phosphate, their metabolism produces catabolic repression on amino acid degradation [34].

Pseudomonads and lactic acid bacteria are the main components of the spoilage flora. Although *Pseudomonas* spp. are the main organisms responsible for putrid odors, the volatiles produced appear only when the metabolized substrate changes to amino acids, producing malodor, esters, and acid [12,32]. However, when *B. thermosphacta* counts are higher than *Pseudomonas* sp., large amounts of end products are detected. Some *Pseudomonas* strains can utilize compounds produced by *B. thermosphacta* such as diacetyl, acetoin, propylene, and butylene glycols, as carbon sources [26,29].

When buffalo liver was aerobically stored in refrigerated conditions, pH decreased from 6.43 to 5.96 in 6 days [17,35] but no off-odors were detected during the initial storage time, just until the microbial load reached 10^7 cfu/g when off-odor was evident. These authors confirmed that microorganisms preferentially utilized glucose.

Liver microbial spoilage is due to several microbial types; at 5°C the microflora is formed by Gram-positive cocci, chromogens and non-chromogens, sporeformers, presumptive coliforms, and Gram-negative rods. When this population reach levels 10^7 cfu/g, after 7–10 days, the food is organoleptically unacceptable, although Gram-negative bacteria do not exceed 10^6 cfu/g [36]. If liver is stored at 10°C, a variable distribution of microbial populations takes place; whereas deep tissue flora is dominated by anaerobic and facultative organisms (*Lactobacillus*, *Enterobacter*, *Aeromonas*); surface flora is mainly composed of strictly aerobic organisms (*Pseudomonas, Acinetobacter*); facultative anaerobic *Enterobacter* is predominant in the drip. Even when spoilage has reached an advanced stage, no spoilage odors are evident, although initial pH (6.4) decreases as lactic acid accumulates, concluding that pH could be a spoilage indicator [37] were pH 6.15 indicates incipient spoilage [18].

A decrease in liver pH from 6.3 to 5.9 results in souring due to the growth of lactic acid bacteria [36]. These microorganisms generally do not produce off-flavors or odors; their spoilage effect is due mainly to souring, unless sulfide-producing strains are present [38]. Lactic acid bacteria only become dominant after other spoilage microorganisms such as *B. thermosphacta* or *Enterobacteriaceae* are detected [3].

Rumen microbiology is an extraordinary consortium of a wide variety of microbial species [25]; therefore, special care must be taken to reduce the microbial load if this organ is intended for human

consumption. Intestines must be removed from the carcass, and immediately washed and scalded [39]. Whereas stomach pH is between 1.5 and 3, and increases *postmortem* due to spoilage-related products, rumen pH is around 5.4–6.9.

Bacteria growing on adipose tissue at high pH (>7.0) utilize glucose first, but when exhausted, colonies attack amino acids, producing malodorous substances which are detectable as spoilage odors when the cell density is about 10^6 cfu/cm^2. Even if growth ceases due to substrate limitation these odoriferous compounds remain [40].

12.5 SPOILAGE ANALYSIS METHODS

Intrinsic and extrinsic parameters that encourage or prevent decomposition of any food material are practically the same as those for selecting a given microbial population. Based on the available substrate, microbial load, and environmental parameters, a given microbial group is likely to grow and proliferate. These growth models are reported in the literature as predictive microbiology, and computer models are developed accordingly [12,41,42]. A viable microbial population sets a limit to shelf life. Fast and accurate detection of spoilage, even before evident signs appear, is necessary to prevent losses during production, distribution, and storage of foods. Various authors report the advantages of analyzing the chemical compounds related to spoilage, mainly of microbial origin [43,44].

Alternatively, chemical compounds present in a given food can be indicative of deterioration as there is a correlation between substrate consumption and production of quantifiable metabolites by specific microorganisms. This method has been referred to as chemometrics. A review on predictive and chemometrics methods was previously reported [5].

12.5.1 pH

Several authors have demonstrated that pH can be a quick and reliable indicator of incipient spoilage, mainly in liver. Devatkal and Mendiratta [17] found a significant pH decrease from 6.43 to 5.96 during buffalo liver storage in air at 4°C. Liver spoilage organisms preferentially utilize simple carbohydrates, such as glucose, and do not produce offensive odor during the initial storage period. The high glucose content of liver allows the spoilage bacteria to form visible colonies on the exposed surface before any off-odor develops. These findings suggest that odor parameter may not be useful for the assessment of shelf life of liver, because an off-odor appeared when microbial counts were 10^7 cfu/g and the livers had been previously rejected for overall appearance and the presence of surface colonies. Therefore, based on the results on shelf life evaluation, it was concluded that buffalo liver was acceptable microbiologically and organoleptically up to the third day of storage at 4°C. Hernández-Herrero et al. [18] demonstrated that all microbial groups (except micrococci) showed differences in their growth rates between 0°C and 3°C. When aerobic plate counts at 37°C reached 10^5–10^6 and 10^6–10^7 cfu/g at 20°C, and pseudomonad counts reached 10^6–10^7 cfu/g, surface colonies were observed in liver samples. The authors concluded that pH below 6.15 may be considered as indicative of beef liver spoilage.

12.5.2 Chemometrics

It is based on detection of specific metabolites produced by a given spoilage-related microorganism. To be used as an efficient spoilage analysis method, the correlation between volatiles production and microbial population must be high, and metabolites must be quantifiable. Depending on the main metabolite produced, or the responsible evidence of spoilage, chemical analysis is designed. Consumption of specific substrates has been also proposed as a measure of spoilage microorganisms growth. Among the metabolites used as spoilage indicators of meat and meat-related foods are biogenic amines and volatile compounds.

Biogenic amines are produced by bacterial decarboxylases; the most abundant biogenic amines in meat by-products are: cadaverine (pentamethylene diamine), putrescine (1,4-diaminobutane), spermidine [*N*-(3-aminopropyl) butane-1,4-diamine], histamine [2-(3H-imidazol-4-yl) ethanamine], tryptamine [3-(2-aminoethyl) indole], agmantine (β-phenyl-ethylamine), ornithin (2,5-diaminovaleric acid), tyramine (4-hydroxy-phenethylamine), and spermine [*N*,*N*′-bis(3-aminopropyl)butane-1,4-diamine]. The main decarboxylase-producing microorganisms involved in meat by-product spoilage are *Enterobacteriaceae, Bacillaceae,* species of *Lactobacillus, Pedicococcus,* and *Streptococcus* [45]. Although biogenic amines can be analyzed by specific culture media or by enzymatic methods, the use of high pressure chromatography is the usual technique due to its reproducibility and time for analysis [46]. Analysis of specific biogenic amines has been developed using ion mobility spectrometry (IMS)—separating and detecting electrically charged particles sorted according to the speed they travel through an electric field [47]. Putrescine, cadaverine, and histamine can be also analyzed using a polymeric sensor and colorimetry [48]. Cadaverine and putrescine have been analyzed by chemiluminescence-flow injection in a photomultiplier detector, initiated by hydrogen peroxide produced by enzymatic oxidation of biogenic amines [49], and by biosensors of Ag/AgCl and platinum electrodes coupled to immobilized oxidases [50].

Microbial spoilage populations generally produce particular metabolites; most of them are highly volatile or can derivatize to increase volatility. Analyzing these products can be an index of by-product spoilage [4,29]. Volatile analysis is also carried out by instrumental methods such as GC, GC/MS, and capillary gas liquid chromatography/mass spectrometry. Fourier transformed infrared spectroscopy (FT-IR) is a non-invasive technique to quantitatively analyze microbial spoilage that provides "fingerprints" of metabolites produced by spoilage microorganisms. Interpretation of FT-IR spectra was carried out by statistical analysis techniques, estimating the bacterial loads, and considering the extent of proteolysis [51].

12.5.3 Microbial Populations and Predictive Microbiology

In a similar way as with striated muscle, by-product spoilage determination by microbial population analysis makes use of several techniques, depending on a particular product. As the contaminant microflora is initiated at the abattoir and proliferates throughout transportation and storage, the analysis of a particular population must use selective culture media. Indicator microbial populations are obtained by differential culture media and specific culture techniques. However, conversely, as with striated muscles, different by-products may present different indicative microorganisms.

Expressing, in terms of a mathematical model, the effect of intrinsic and extrinsic parameters on a given microbial population allows forecasting of the product shelf life. This method is known as predictive microbiology. The intensity of these parameters must be also considered as a function of limiting factors [51]. Once the microbial criteria in response to time are established (population loads, consumed substrate or indirect responses such as absorbance or turbidity, or mathematical models including microbial parameters such as growth rate, lag time, etc.), calculations are carried out by the use of computer models [52]. The most commonly used model in predictive microbiology is the growth curves, such as the Gompertz model [42], thermal processing parameters (*D*, *Z* and *F* values), overall process calculations [53], response surface methodology [41], among other models.

Molecular techniques are also useful to detect spoilage. DNA-probes allow fingerprinting of spoilage-related microorganisms at subspecies level. *Leuconostoc* spp., *Clostridium* spp., *Pseudomonas* spp., *Alcaligens* spp. [54,55] have been accurately detected by the use of 16S rDNA-directed primers. Although these techniques are expensive, they are highly valuable for spoilage strain identification.

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13 Microbial Foodborne Pathogens

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CONTENTS

13.1 INTRODUCTION

During the slaughter of animals, several by-products of particular economic interest are produced. The majority of these products are edible and they can be used for nutritional purposes, while others are not suitable for direct human consumption and undergo further processing in order to be used at later processing stages by the food or chemical industry.

Edible animal by-products should be handled with extreme caution so as to ensure the microbiological safety of food supply. If this does not prove to be the case, then the possible mishandling of these products could result in severe health-related problems (i.e., foodborne infections and intoxications) or even death (Table 13.1). The presence of microorganisms, such as pathogenic bacteria, originates from the natural microbiota of the raw material and those organisms are introduced in the course of slaughter, processing, storage, and distribution. The types of microorganisms will be determined by the properties of the product, the storage environment, the attributes of the organisms themselves, and the effect of processing [3]. As a consequence, certain food safety issues regarding the consumption of the above products come before the footlights. For instance, ubiquitous psychrotrophic pathogens such as *Listeria monocytogenes* and *Yersinia enterocolitica* are of great concern because they are able to grow well at refrigeration temperatures (2°C-4°C), making the control of these two foodborne pathogens even more difficult [4]. Moreover, certain animal intestines are very high in coliform population and need to be washed and cooked thoroughly to be safe for eating. These examples demonstrate the importance of effective microbiological testing schemes needed to assure the safety of by-products. The materials and methods necessary to perform those schemes are discussed in the following paragraphs. For more detailed information on protocols and culture media and for all the microbiological methods described, there are reference works that should be consulted [5–8].

TABLE 13.1
Listeriosis Outbreaks during the Past 20 Years Implicating Several Edible By-Products of Animal Origin

Year	Implicated Food	Country	Cases	Deaths
1987–1989	Pâté and spread meat products	England	355	94
1990	Pâté and spread meat products	Australia	11	6
1992	Pork jelly	France	279	63
1993	Pâté	France	38	11
1999	Pâté	United States	11	?
1999–2000	Pork jelly	United States	26	7
2000	Pork jelly	France	32	Unknown
2005	Blood sausage and blood pudding	United States	Unknown	Unknown
2005	Pork blood sausage	United States	Unknown	Unknown

Sources: Food Safety and Inspection Service, FSIS recalls, Food Safety and Inspection Service, Washington, DC, 2010. http://www.fsis.usda.gov/Fsis_Recalls/Recall_Case_Archive/index.asp. Accessed November 28, 2010; FDA/Center for Food Safety and Applied Nutrition—USDA/Food Safety and Inspection Service, 2003. *Listeria monocytogenes Risk Assessment: II*, Hazard Identification, http://www.fda.gov/food/ScienceResearch/ResearchAreas/RiskAssessmentSafetyAssessment/ucm183981.htm

Roughly speaking, there are two categories of analytical tools used for the evaluation of microbiological safety and quality in by-products. The first category comprises culture-dependent methods that nowadays are considered classic and which basically study the growth of the microorganism(s) under examination in a Petri dish or a test tube that contains the appropriate growth medium. The other category comprises culture-independent methods that are more advanced, quick, and reliable.

13.2 CULTURE-DEPENDENT METHODS

Cultural or traditional methods are widely used for determining the number of microbes present in a food sample, simply by letting them grow in predetermined solid or liquid growth media under controlled laboratory conditions. Cultural methods are simple, inexpensive, and easy to use, but, on the other hand, they are time-consuming and demand a great deal of effort, not to mention the possibility of obtaining misleading or even false results due to technical failures and/or improper sample treatment.

The implementation of these methods generally consists of three steps. It is of vital importance to define the sample that will be analyzed. For that reason, the sample withdrawn for microbiological analysis should be representative and randomly selected from different areas of the food to assure detection of the target microorganism if this is not uniformly distributed in the food, which very often is the case for solid foods [9]. Practical aspects of sampling and analysis, together with information on the statistical basis of sampling plans, are provided by Jarvis [10].

The second step, following the proper sampling of the product, is the selection of the appropriate medium for the desired microorganism or group of organisms. This selection will depend not only on what group of organisms is being studied, but also on the overall purpose of the study. There are three basic types of laboratory media used to subculture the microorganisms present in a food sample: (a) elective, (b) selective, and (c) differential or diagnostic media [3,11]. Elective media are designed to support the growth of the target microorganism by containing special agents (e.g., microelements) that encourage the more rapid growth of one species or group of microorganisms, without nonetheless inhibiting the growth of the accompanying microbiota. The latter is achieved by the use of the selective media, which contain inhibitory agents, such as inorganic salts,

triphenylmethane dyes, surface-active agents, and antibiotics. These compounds inhibit the growth of the nontarget organisms as well as, in some cases, the growth of the microorganism under study but in lesser degree. Attention is needed, therefore, if cells of the target organism have been subjected to sublethal injury, because they may not be able to grow on the medium without a resuscitation step to allow them to repair. Finally, differential or diagnostic media contain reagents that allow the differentiation of microorganisms, by providing a visual response to a particular reaction taking place because of bacterial metabolism (e.g., chromogenic media), making it possible to recognize individual species or groups due to the presence of a specific metabolic pathway or even a single enzyme. In the case of chromogenic media, the presence of indicators (such as neutral red, phenol red, eosin, or methylene blue) leads to the production of a specific color when these agents react with the forming colonies, ultimately changing the color of the media. As a conclusion, there is a wide variety of media readily available to the microbiologist (Table 13.2), many of which in practice combine selective reagents, elective components, and diagnostic features to ensure the proper identification of the target microorganism. An interesting example is the Baird-Parker agar used for the presumptive isolation of *Staphylococcus aureus* in foods. This medium contains lithium chloride and tellurite (i.e., selective agents) to inhibit the growth of accompanying microorganisms, whereas pyruvate and glycine (i.e., elective agents) selectively stimulate the growth of staphylococci. The diagnostic features shown by most strains of *St. aureus* are provided by the addition of egg yolk (i.e., the differential agent), which is responsible for the formation of characteristic opaque zones

TABLE 13.2
Selection of Laboratory Solid Media Commonly Used to Subculture Microbial Foodborne Pathogens

Solid Growth Medium	Type	Target Microorganism(s)	Main Reference
Agar *Listeria* Ottaviani and Agosti (ALOA)	Selective/differential (Chromogenic)	*Listeria* spp./*L. monocytogenes*	[12]
Baird-Parker agar	Elective/selective/differential	Staphylococci/*Staphylococcus* spp./*St. aureus*	[13]
Brilliant green agar	Selective/differential	Salmonellae/*Salmonella* spp. (except *S. typhi*)	[14]
Campylobacter selective agars	Selective/differential	*Campylobacter* spp./*C. jejuni*	[15]
Cereus selective agar (or MYP Agar)	Selective/differential	*Bacillus cereus*	[16]
MacConkey agar with sorbitol, cefixime and tellurite (CT-SMAC)	Selective/differential	*Escherichia coli* O157:H7	[17]
Oxford agar	Selective/differential	*Listeria* spp.	[18]
PALCAM agar	Selective/differential	*Listeria* spp.	[19]
RAPID'L. mono Agar	Selective/differential (chromogenic)	*Listeria* spp./*L. monocytogenes*	[20]
Salmonella Shigella Agar with sodium deoxycholate and calcium chloride (SSDC)	Selective/differential	*Yersinia enterocolitica*/*Y. enterocolitica* O:3	[21]
Tryptone bile X-glucuronide (TBX) agar	Selective/differential (chromogenic)	*Escherichia coli*	[22]
Tryptose sulfite cycloserine (TSC) agar egg-yolk-free	Selective/differential	*Clostridium perfringens*	[23]
Xylose lysine desoxycholate (XLD) agar	Selective/differential	Enteric pathogens/*Shigella* spp. (primarily) and *Salmonella* spp.	[24]
Xylose lysine tergitol-4 (XLT-4) agar	Selective/differential	Enteric pathogens/Non-typhi *Salmonella* spp.	[25]

and rings as a result of lipolysis and proteolysis due to lecithinase activity, surrounded by a halo of clearing due to proteolytic activity.

Finally, the third step is the enumeration of microorganisms. The enumeration methods mainly used are the plate count and most probable number. The plate count method is used more often, while the most probable number is used for certain microorganisms, such as coliforms [26] and *Escherichia coli* [27].

13.2.1 Plate Count

Plate count is the most popular cultural enumeration method, also known as total plate count, total viable count providing an estimation of population as a number of colony-forming units (cfu). This method is used for the enumeration of viable microorganisms, which are able to form visible colonies after plating on various media and then incubating at selected temperatures, depending on the microorganism under study. It is widely accepted that reasonably accurate results are obtained when plates counted contain 30–300 colonies. However, due to the fact that by-product samples may contain hundreds of thousands or even millions of microbes, it is often necessary to dilute a sample before enumeration to obtain plates with number of colonies in between the above mentioned range. This procedure is named the 10-fold dilution series and is considered one of the fundamentals in food microbiology.

The diluent used for 10-fold dilutions must be correctly prepared in terms of buffer capacity and osmotic pressure, so as not to cause any damage, such as an osmotic shock, to the microorganisms. In any other case, the microbial cells may be stressed influencing the final result. Consequently, the use of sterile distilled water is inappropriate and so saline peptone water or a maximum recovery diluent, as it is better known, is commonly used. This diluent contains 0.1% peptone and 0.85% sodium chloride.

The plate count method consists of two techniques; the pour plate and the spread plate technique. In the pour plate technique, an adequate volume of sample (1 mL) from the appropriate dilution is pipetted directly into a sterile Petri dish and mixed with an appropriate volume of molten agar. Even if the molten agar is carefully tempered at 40°C-45°C, the upcoming thermal shock may result in the inability of some bacteria (especially psychrotrophs) to form a visible colony. This problem is avoided, though, in the spread plate technique, where a smaller sample volume (0.1 mL) is spread on the surface of solidified agar plates, ensuring also growth under aerobic conditions.

It is common experience that the apparent numbers of cfu increase as we move towards higher decimal dilutions and use them to calculate the microbial population in a food sample. This may reflect the breaking up of clumps by the action of pipetting, and/or reduced competition on less-crowded plates [3]. A recent critical review of measurement uncertainty in the enumeration of microorganisms in foods is given by Corry et al. [28].

The confidence in the results obtained by this method could be increased simply by plating a number of replicate samples from each dilution. In other words, instead of just using the counts of a single dilution for the calculation of the microbial population in the original sample, given by Equation 13.1,

$$N = \frac{\overline{u}}{V \times d} \tag{13.1}$$

where

N = cfu g^{-1}
$\overline{u}$ is the mean count per plate
V is the volume of the sample plated
d is the dilution factor

It is better to use the counts from several sequential dilutions and estimate a weighted mean (Equation 13.2.),

$$N_w = \frac{C_1 + C_2}{n_1 + n_2/10} V d_1 \tag{13.2}$$

where

N_w is the weighted mean
C_1 is the total count on n_1 replicates at dilution d_1
C_2 is the total count on n_2 replicates of the next dilution

13.2.2 Most Probable Number

The most probable number (MPN) method is normally used for the enumeration of relatively low numbers of viable microorganisms. The concept of this method correlates with the fact that samples of the same size, coming from the same product, are expected to have more or less the same number of microbes. So, despite any slight differences on the microbial population of the samples acquired, the mean is the most probable number of microbes on the original food.

The method is usually based on inoculating replicate tubes (usually three, sometimes four or five) of an appropriate liquid growth medium (broth) with three different sample sizes or 10-fold dilutions of the by-product to be studied. After incubating the tubes for a sufficient period of time at a specific temperature, according to the microorganism or group of organisms under examination, the tubes showing turbidity (growth) are considered positive, otherwise they are taken as negative. The number of viable cells in a food sample is assessed through the comparison of positive tubes with probability tables based on statistical analysis and processing [6,9]. In that way, MPN is obtained by referring to results such as that shown in Table 13.3.

TABLE 13.3
Selection of MPN Values Using Three Replicate Tubes[a]

Number of Positive Tubes	MPN	95% Confidence Limits
0 0 0	<0.30	
1 0 0	0.36	0.02–1.7
2 0 0	0.92	0.15–3.5
2 1 0	1.5	0.4–3.8
3 0 0	2.3	0.5–9.4
3 1 0	4.3	0.9–18.1
3 1 1	7.5	1.7–19.9
3 2 0	9.3	1.8–36
3 2 1	15	3.0–38
3 3 0	24	4.0–99
3 3 1	46	9.0–198
3 3 2	110	20.0–400
3 3 3	>110	

Source: Adams, M.R. and Moss, M.O. *Food Microbiology*, 3rd edn. RSC Publishing, Cambridge, U.K., 2008.

[a] Based on 3 × 1 g + 3 × 0.1 g + 3 × 0.01 g samples (expressed as organisms per 1 g, i.e., cfu g^{-1}).

13.2.3 Detection Methods

Microorganisms present in processed edible by-products of animal origin are mostly able to form visible colonies on plate count agars. This is due to the fact that cells of these organisms remain fully intact after processing, exhibiting no signs of damage. However, apart from those microorganisms being killed, a number of microbial cells are injured or stressed during processing, suffering damages that lead to a temporary change in physiology and subsequently ending up in a loss of culturability.

Injured cells may show an extended lag phase, restricted temperature range for growth, and increased sensitivity to selective agents, salt, acidity, and oxidative stress. In some cases, it has been possible to identify the site of cellular injury leading to the observed phenotype [29]. Although those cells may remain undetected on selective agars, they are still viable and are commonly referred to as viable but nonculturable (VBNC) cells. Normally, the use of nonselective media provides ideal growth conditions for the cultivation of these cells. In most cases, the inducing stress is starvation, but VBNC cells have also been reported in response to biocide treatment, osmotic stress, exposure to sunlight, oxidative stress, aerosolization, and survival in soil [30–32]. Sublethal injury and its repair have been reviewed in numerous articles and book volumes [32–38].

Detection methods have been developed to determine the presence or absence of a specific pathogen and to estimate its number. These cultural methods include the additional steps of enrichment or pre-enrichment prior to inoculation on/into a selective medium, allowing resuscitation of pathogens to take place, which leads to their recovery by giving them the opportunity and necessary time to repair damages.

The VBNC state should not be underestimated and needs to be taken into account when preservation treatments or environmental stresses are applied. Under conditions that favor their growth, injured pathogenic cells might undergo resuscitation either in the food or following ingestion, and pose a serious threat to public health through the manifestation of food poisoning outbreaks. As mentioned above, there are many factors influencing the resuscitation of injured cells, such as the composition and characteristics of the medium and environmental parameters [39].

13.3 CULTURE-INDEPENDENT METHODS

13.3.1 DNA-Based Detection

Advancements in the field of molecular biology allowed the development of more sophisticated techniques for the detection of foodborne pathogens. Among others, the DNA molecule has been widely used for this purpose. DNA-based detection relies on the existence of sequences specific to the bacterial pathogen at the desired level of detection (i.e., genus/species/strain). It has been generally accepted that compared to classical microbiological techniques, these methods are faster and offer improved selectivity, sensitivity, specificity, and reliability. Moreover, polymerase chain reaction (PCR) evolution along with advancements in the field of molecular beacons enabled the quantitative detection of a specific microbial population within a natural microbial consortium.

Employing DNA-based detection of foodborne pathogens leads the researcher to face certain issues:

1. Low numbers of the microorganisms whose detection is the objective. Furthermore, the detection should be performed in the presence of a natural dominant microbiota that is present in much higher population.
2. An optimal DNA extraction method should be applied to avoid interference with the detection limit and, on the other hand, to remove all compounds that may impose some kind of inhibiting action to the detection method.

3. Specificity, and thus reliability, of the detection. In a detection protocol, the PCR method itself may also become the limiting factor, in terms of template DNA concentration. It is generally accepted that detectable DNA through PCR is achieved when the microbial population is at least 10^3 cfu mL^{-1}, provided that the DNA extraction method is optimal. No PCR technique can ensure detection below that limit.
4. The detection step itself, i.e., any post-PCR techniques that are used to detect the amplified DNA target.

Many researchers have offered a wide range of alternatives to face the above mentioned challenges, and the most effective interventions are discussed in the following paragraphs.

Pathogenic microbiota can be regarded as a secondary one that may accompany a dominant microbial consortium that varies according to the product characteristics and storage conditions. The method applied should enable the effective detection of the target microorganism(s), to a population as low as one cell per 25 g of product. Given the inability of PCR-based techniques to ensure detection below a population of 10^3 cfu mL^{-1}, selective propagation or concentration of the target microorganism is a prerequisite. The former is the intervention most frequently selected, due to simplicity and reliability, especially when the target is only one pathogen. When the simultaneous detection of more than one pathogen is the case, then the correct selection of the enrichment broth and incubation conditions are crucial to obtain an equal propagation of the target microorganisms, while at the same time inhibit any other microbiota present. The simplest simultaneous enrichment has been successfully applied by Murphy et al. [40], Lee et al. [41], and Perelle et al. [42] for the simultaneous propagation of *E. coli* O26, O111, and O157 in *Salmonella typhimurium*, *S. enteritidis*, and thermophilic campylobacters, respectively. The former was performed with tryptic soy (TS) broth supplemented with 20 g L^{-1} novobiocin and incubation at 42°C for 16 h, whereas the latter with Bolton broth and incubation at 42°C for 20 h. Salmonellae were enriched with buffered peptone water. In a study conducted by Kawasaki et al. [43], the simultaneous enrichment of *L. monocytogenes*, *S. enteritidis*, and *E. coli* O157:H7 was attempted. It was concluded that TS broth was inadequate for the efficient growth of *L. monocytogenes* that only reached 2.9 and 5.3 log cfu mL^{-1} after 24 and 48 h of incubation at 35°C, respectively, while natural biota (termed as aerobic bacteria) as well as *S. enteritidis* and *E. coli* O157:H7 managed to reach 8.0 log cfu mL^{-1} after 24 h incubation at 35°C. Thus, enrichment with TS broth would render *L. monocytogenes* detection quite difficult. On the other hand, enrichment with broth named No. 17 enabled growth and consequent *L. monocytogenes* detection. Similarly, Li et al. [44] successfully used brain–heart infusion broth and incubation at 37°C for 24 h for the simultaneous propagation of *E. coli* O157:H7, *S. typhimurium*, and *Shigella flexneri*. However, due to the inherent difficulty of such an approach, it is not common practice. On the other hand, the most commonly applied approach consists of parallel enrichments, each for every target microorganism. This was the case in the study by Jofre et al. [45], in which the simultaneous detection of *Salmonella* sp. and *L. monocytogenes* was performed by parallel enrichments with buffered peptone water and half Fraser broth, respectively. Similarly, parallel enrichments with buffered peptone water and EC broth, both supplemented with novobiocin, were used for the detection of *Salmonella* sp. and *E. coli* O157:H7, respectively [46].

Alternatively, when the propagation of the target population is not feasible, this population can be concentrated by a number of techniques. Immunomagnetic separation (IMS) has been successfully applied to separate microbial cells from biological samples. Magnetic beads are coated with specific antibodies that will bind to antigens present on the surface of cells. Then the bead-target complexes are separated from the food matrix with a magnet, thus obtaining concentration and purification from the food sample. This technique has been successfully applied, among others, for the detection and separation of *S. typhimurium* in raw meats [47], *Campylobacter jejuni* in spiked chicken wash samples in an assay time of 8 h without sample enrichment [48], *E. coli* O157:H7, *Salmonella*, and *Shigella* present in ground beef [49], salmonellae in poultry [50], and ground beef [51], as well as *L. monocytogenes* in turkey meat [52].

Another promising technique for the separation and concentration of foodborne pathogens is buoyant density gradient centrifugation (BDC). According to this, a density gradient is created by layering solutions of varying densities with the dense end either at the bottom (sedimentation) or the top (flotation) of a centrifuge tube. Then, the sample is added and the constituents are separated according to their density. Both sedimentation as well as flotation BDC have been successfully applied in meat products. The former allowed detection limits for *S. flexneri* [53], *E. coli* O157 [54], and *Y. enterocolitica* [55,56], of 10^2 to 10^3 cfu mL^{-1}, despite the only partial recovery of bacteria (25%). The latter, which has been successfully combined with real-time quantitative PCR (RTi-qPCR), showed that cell concentrations of at least 4.2×10^3 *Y. enterocolitica* cfu mL^{-1} of pork juice [57] and as low as 8.6×10^2 *C. jejuni* cfu mL^{-1} of chicken rinse [58] could be positively identified without culture enrichment. However, for future applications, technical pitfalls that may occur in a routine setup along with the characteristics of food constituents should be taken into consideration for an effective application [59].

Regarding DNA extraction, the method employed should be suitable in terms of effectiveness, as quantified by both DNA recovery and removal of inhibitors, and safety, in terms of absence of reagents such as phenol and chloroform. A wide range of protocols are currently available, each of them offering specific advantages and suffering from specific drawbacks. The selection of the appropriate method should take into consideration the nature of the target microorganisms, e.g., Gram reaction, as well as the food matrix from which the isolation is attempted, in terms of composition and need for purification steps to remove inhibiting compounds. The simplest techniques are boiling and alkali lysis. In the former, cells are suspended in a Tris–EDTA buffer and boiled in a water bath. Cell debris is removed by centrifugation and the DNA is delivered in the supernatant. Alkali lysis involves mixing of the cells with an alkali solution (mostly 3% KOH), vigorous mixing, and centrifugation. Both techniques are very effective for Gram-negative bacteria. However, in most of the cases, PCR inhibitors may be present and the utilization of a method involving precipitation steps is necessary. Guanidine isothiocyanate (GuSCN) is very often utilized and is very effective for both Gram- positive and negative bacteria. According to this technique, treatment of the cells with GuSCN and vigorous mixing ensures cell lysis. Then, the supernatant is treated with isopropanol to ensure removal of PCR inhibitors. This technique has been successfully combined with IMS for effective separation of the target cells, as well as with prior lysozyme treatment, which is very effective, particularly when dealing with Gram-positive bacteria [43,60]. However, utilization of IMS is costly and involves cross-contamination risks [61]. Another effective and relatively fast protocol, especially for Gram-positive bacteria, involves lysis with either lysozyme or sonication in a buffer containing EDTA and, consecutively, treatment with EDTA containing Tris–HCl solution, SDS, potassium acetate, isopropanol, and ethanol precipitation, ensuring relatively high DNA yield in terms of both quantity and purity as well as a complete removal of inhibitors. Similarly, a quite often encountered protocol, especially for Gram-negative bacteria, involves the mixing of the cells with Chelex 100 and incubation at elevated temperatures. After chilling on ice and centrifugation, DNA is accessible in the supernatant fluid. In case of the presence of inhibitors, a dilution of the template DNA (1:50) with a TE buffer is very often used. Finally, a wide variety of standardized kits are currently commercially available.

Once DNA has been successfully extracted, it should be amplified to obtain a readable signal, according to the detection method. The most commonly applied family of techniques is based on specific amplification. According to this, primers that anneal specifically to the desired level of identification are used. Thus, the specificity of polymerization is ensured. Then, a detection method is applied to verify amplification. When the target consists of merely one microorganism, then a pair of primers designed to anneal specifically to it, are incorporated. This approach has been successfully applied for the detection of an extended list of microorganisms, in nearly all food matrices. It is, however, more practical to detect as many pathogens as possible with a single reaction. Multiplex PCR, i.e., the polymerase chain reaction in which more than one set of primers is used aiming at more than one target microorganism, has been successfully applied for that reason. In Table 13.4, the oligonucleotides used as primers along with the target gene and the

TABLE 13.4
Oligonucleotides (5′–3′) Used as Primers, Target Gene and Amplicon Size in Pathogen Detection from Meat and Meat Products

Target Microorganism	Primers (5′–3′)	Target Gene	PCR Size (bp)	Reference
Salmonella enterica	SAL-F: AATTATCGCCACGTTCGGGCAA SAL-R: TCGCACCGTCAAAGGAACC	*invA*	278	[62]
Listeria monocytogenes	LIS-F: TCATCGACGGCAACCTCGG LIS-R: TGAGCAACGTATCCTCCAGAGT	*prfA*	217	
E. coli O157:H7	ESC-F: GGCGGATAAGACTTCGGCTA ESC-R: CGTTTTGGCACTATTTGCCC	*eaeA*	151	
E. coli O26	O26-F: GCGCTGCAATTGCTTATGTA O26-R: TTTCCCCGCAATTTATTCAG	*wzx*	147	[40]
E. coli O111	O111-F: TAGAGAAATTATCAAGTTAGTTCC O111-R: ATAGTTATGAACATCTTGTTTAGC	*rfb*	320	
E. coli O157	O157-F: CGGACATCCATGTGATATGG O157-R: TTGCCTATGTACAGCTAATCC	*rfb*	242	
Helicobacter pullorum	HELIP2: CCAAGGGCTATGACGGGTGTATCC R: GTGGAGTACAAGACCCGGGAA	16 S rRNA	1107	[63]
Campylobacter jejuni	CAMPCJL1: ACTCCTTTTCTTAGGGAAGAATTC R: GTGGAGTACAAGACCCGGGAA	16 S rRNA	946	
Arcobacter butzleri	ARCOB1: TGTAGGCGGATTGATAAGTTTGAA R: GTGGAGTACAAGACCCGGGAA	16 S rRNA	822	
Salmonella spp.	TS-11: GTCACGGAAGAAGAGAAATCCGTACG TS-5: GGGAGTCCAGGTTGACGGAAAATTT	NA	375	[43]
Listeria monocytogenes	LM1: CGGAGGTTCCGCAAAAGATG LM2: CCTCCAGAGTGATCGATGTT	NA	234	
E. coli O157:H7	VS8: GGCGGATTAGACTTCGGCTA VS9: CGTTTTGGCACTATTTGCCC	NA	120	
C. jejuni	C1: CAAATAAAGTTAGAGGTAGAATGT C4: GGATAAGCACTAGCTAGCTGAT	NA	159	[64]
E. coli O157:H7	UidAa: GCGAAAACTGTGGAATTGGG UidAb: TGATGCTCCATAACTTCCTG	NA	252	
S. typhimurium	S29: CAGTATCAGGGCAAAAACGGC S30: TTCAAAGTTCTGCGCTTTGTT	NA	360	
L. monocytogenes	FP: AGCTCTTAGCTCCATGAGTT RP: ACATTGTAGCTAAGGCGACT	NA	450	
S. enterica	ST11: AGCCAACCATTGCTAAATTGGCGCA ST15: GGTAGAAATTCCCAGCGGGTACTG	Random	429	[65]
S. enterica	S18: ACCGCTAACGCTCGCCTGTAT S19: AGAGGTGGACGGGTTGCTGCCGTT	*ompC*	159	
S. enterica	Sal3: TATCGCCACGTTCGGGCAA Sal4: TCGCACCGTCAAAGGAACC	*invA*	275	
E. coli O157:H7	AE22: ATTACCATCCACACAGACGGT AE20-2: ACAGCGTGGTTGGATCAACCT	*eaeA*	397	[46]
E. coli O157:H7	MK1: TTTACGATAGACTTCTCGAC MK2: CACATATAAATTATTTCGCTC	*stx1/stx2*	228/225	

(*continued*)

TABLE 13.4 (continued)
Oligonucleotides (5′–3′) Used as Primers, Target Gene and Amplicon Size in Pathogen Detection from Meat and Meat Products

Target Microorganism	Primers (5′–3′)	Target Gene	PCR Size (bp)	Reference
Salmonella spp.	MFS1F: ACGATGTGGTTTATTCTGGA MFS1R: CTTCACGTCACCATACATAT	Plasmid	166	
Salmonella spp.	INVAF: CGGTGGTTTTAAGCGTACTCTT INVAR: CGAATATGCTCCACAAGGTTA	*invA*	796	
Salmonella spp.	139F: GTGAAATTATCGCCACGTTCGGGCAA 141R: TCATCGCACCGTCAAAGGAACC	*invA*	284	[45]
Listeria monocytogenes	Lip1F: GATACAGAAACATCGGTTGGC Lip2R: GTGTAACTTGATGCCATCAGG Lip3R: TGACCGCAAATAGAGCCAAG	*prfA*	274 (Lip1–Lip2) 215 (Lip1–Lip3)	
E. coli O157:H7	PT-2: GCGAAAACTGTGGAATTGGG PT-3: TGATGCTCCATCACTTCCTG	*uidA*	252	[44]
Salmonella serotype *typhimurium*	ST-11: AGCCAACCATTGCTAAATTGGCGCA ST-15: GGTAGAAATTCCCAGCGGGTACTG	NA	429	
Shigella flexneri	ipaH-1: GTTCCTTGACCGCCTTTCCGATACCGTC ipaH-2: GCCGGTCAGCCACCCTCTGAGAGTAC	*ipaH*	620	
S. enteritidis	SF: CCTTT CTCCATCGTC CTGA A SR: TGGTG TTATC TGCCT GACC	*fimI*	85	[66]
L. monocytogenes	LF: TCCGC AAAAG ATGAAGTTC LR: ACTCCTGGTG TTTCT CGATT	*hly*	98	
ETEC	GGCGACAGATTATACCGTGC CGGTCTCTATATTCCCTGTT	*lt*	450	[67]
ETEC	ATTTTTCTTTCTGTATTGTCTT CACCCGGTACAAGCAGGATT	*st*	190	
EPEC	AATGGTGCTTGCGCTTGCTGC GCCGCTTTATCCAACCTGGTA	*bfpA*	324	
EPEC	GACCCGGCACAAGCATAAGC CCACCTGCAGCAACAAGAGG	*eaeA*	384	
EHEC	CTGGATTTAATGTCGCATAGTG AGAACGCCCACTGAGATCATC	*stx1*	150	
EHEC	GGCACTGTCTGAAACTGCTCC TCGCCAGTTATCTGACATTCTG	*stx2*	255	
EIEC	GGTATGATGATGATGAGTCCA GGAGGCCAACAATTATTTCC	*ial*	650	
EAEC	CACAGGCAACTGAAATAAGTCTGG ATTCCCATGATGTCAAGCACTTC	*aafII*	378	

Note: ETEC, enterotoxigenic *E. coli*; EPEC, enteropathogenic *E. coli*; EHEC, enterohaemorrhagic *E. coli*; EIEC, enteroinvasive *E. coli*; EAEC, enteroaggregative *E. coli*; NA, not available.

amplicon size in pathogen detection through multiple PCR discipline in meat and meat products are shown. The inherent drawbacks of the PCR itself as well as the detection method applied, i.e., agarose gel electrophoresis, permit the simultaneous detection of many microorganisms. Thus, multiplex PCR is not applied for the simultaneous detection of more than four microorganisms. The specificity and concomitantly the reliability of detection are ensured by the selection of the appropriate primers. It has been exhibited that the detection limit of multiplex PCR in pure

culture samples of *E. coli* O157:H7, *L. monocytogenes*, *S. typhimurium*, and *C. jejuni* was not lower than 10^4 cfu/PCR reaction [68]. This technique, although currently widely used, does not allow the quantification of pathogen presence. This is solved by application of quantitative PCR. Real-time PCR for the specific and quantitative detection of pathogens in foods generally, and in meat products specifically, is increasingly being used as it combines rapidity and reliability, the avoidance of cross-contamination risk compared to conventional PCR as well as, at least in some cases, the possibility for automation. As in the case of conventional PCR, a large amount of data concerning detection of single bacterial pathogens currently exists [57,69–73]. Similarly, the most challenging field has been the simultaneous quantitative detection of more pathogens in one reaction. In Table 13.5, the oligonucleotides and probes used for the simultaneous and quantitative detection of bacterial pathogens in meat and meat products, are exhibited. It has been reported that the detection efficiencies of multiplex quantitative PCR to pure cultures were 10^2 cfu mL^{-1} for *E. coli* O157:H7, 10^3 cfu mL^{-1} for *S. typhimurium*, and 10^1 cfu mL^{-1} for *S. flexneri* [49]. However, both sensitivity and efficiency decreased when the same assay was applied to ground

TABLE 13.5
Oligonucleotides (5′–3′) and Probes Used for Simultaneous and Quantitative Detection of Bacterial Pathogens in Meat and Meat Products

Target Microorganism	Oligonucleotide	Sequence	Target Gene	Reference
Campylobacter spp. (*C. jejuni, C. lari, C. coli*)	OT1559	CTGCTTAACACAAGTTGAGTAGG	16S rRNA	[42]
	18-1	TTCCTTAGGTACCGTCAGAA	16S rRNA	
	LC-CPY-3'Fluo	GGAGGCAGCAGTAGGGAATAT	16S rRNA	
	LC-CPY-5'Red640	CGCAATGGGGGAAACCC	16S rRNA	
	LC-150-5'Red705	CTATCCTTGAGCCGTAGGCCACTATC		
Salmonella enteritidis	Prot6e-5 (forward)	ATATCGTCGTTGCTGCTTCC		[74]
	Prot6e-6 (reverse)	CATTGTTCCACCGTCACTTTG		
	Prot6e probe	FAM-AGGCGCTCATCGGTCCTGCTGT-DQ		
E. coli	*E. coli*-1	TTGACCCACACTTTGCCGTAA	*uidA*	[49]
	E. coli-2	GCGAAAACTGTGGAATTGGG		
	E. coli-p	VIC-TGACCGCATCGAAACGCAGCT-TAMRA		
Salmonella spp.	*Sal*-1	GCTATTTTCGTCCGGCATGA		
	Sal-2	GCGACTATCAGGTTACCGTGGA		
	Salmonella-p	FAM-TAGCCAGCGAGGTGAAAACGACAAAGG-TAMRA		
Shigella spp.	*Shig*-1	CTTGACCGCCTTTCCGATA	*ipaH*	
	Shig-2	AGCGAAAGACTGCTGTCGAAG		
	Shigella-p	TET-AACAGGTCGCTGCATGGCTGGAA-TAMRA		
Salmonella typhimurium	SfC-F	TGCAGAAAATTGATGCTGCT	*fliC*	[41]
	SfC-R	TTGCCCAGGTTGGTAATAGC		
	ST-JOE	JOE-ACCTGGGTGCGGTACAGAACCGT-BHQ1a		
Salmonella enteritidis	SsA-F	GGTAAAGGGGCTTCGGTATC	*sefA*	
	SsA-R	TATTGGCTCCCTGAATACGC		
	SE-Cy5	Cy5-TGGTGGTGTAGCCACTGTCCCGT-BHQ1a		

beef due to the presence of various food constituents, such as organic and phenolic compounds and lipids that may have interfered with DNA amplification. Additionally, the sensitivity was markedly affected by the low numbers of the target microorganism in the presence of high numbers of the dominant microbiota and the detection limit was raised to 10^5 cfu g^{-1} for *E. coli* O157:H7, 10^3 cfu g^{-1} for *Salmonella,* and 10^4 cfu g^{-1} for *Shigella*. Application of IMS to separate and concentrate *E. coli* O157:H7 and *Salmonella* from the samples resulted in a relative improvement of the detection limit to 10^3 cfu g^{-1} for both pathogens. Despite the advantages that this technique offers, compared to multiplex PCR, certain drawbacks, remain. The inability of simultaneous detection of many bacterial pathogens has been overcome by the application of DNA microarray technology. Conventional DNA microarrays consist of nucleic acid probes deposited on a planar glass surface usually coated with chemically reactive groups to ensure efficient binding of nucleotidic probes. The presence of target genes is assessed by the labeling of the nucleic acid samples, hybridization onto the array, and washing with different stringency buffers. The remaining signal resulting from specific interactions between probes and target nucleic acids is measured with a confocal microarray scanner [75]. Regarding pathogen detection, cDNA and oligonucleotide microarray technology has already been applied and a single chip multipathogen oligonucleotide microarray was constructed for the simultaneous analysis of foodborne pathogens [76,77]. Moreover, specific 70-mer oligonucleotide probes for eleven foodborne pathogens, including *L. monocytogenes*, *St. aureus*, *S. typhi*, and *S. typhimurium* have been designed and a microarray has been constructed setting a successful step toward rapid and accurate identification of foodborne pathogens [78].

Alternatively, a number of techniques have been successfully applied. Among them, single-strand conformation polymorphism (SSCP) coupled with capillary electrophoresis (CE) has been proved very promising. SSCP relies on the differences of the secondary conformation that single strands of DNA receive. Different conformations can, theoretically, result from the slightest difference in the DNA sequence, i.e., one nucleotide. Moreover, CE is a newer, more feasible alternative to slab-gel SSCP. With CE, electrophoretic separation is performed within arrays of microbore-fused silica capillaries offering higher throughput and sensitivity, greater reproducibility, and full automation [79,80]. With CE, the product of asymmetric PCR was separated on a genetic analyzer enabling the differentiation between strains of *Campylobacter jejuni*, *Clostridium perfringens*, *Bacillus cereus*, *S. typhimurium*, and *E. coli* O157:H7 [81] as well as between strains of *Mycoplasma genitalium*, *Neisseria gonorrhoeae*, *Trichomonas vaginalis*, *C. jejuni*, *Clostridium perfringens*, *E. coli* O157:H7, *Bacillus anthracis*, and *Borrelia burgdorferi* [82].

Polymerase chain reaction denaturing gradient gel electrophoresis (PCR-DGGE) fingerprinting, although only recently introduced into food microbiology, has already proven very efficient, and therefore, has gained its position among the most commonly used culture-independent fingerprinting techniques. According to this technique, PCR-generated DNA fragments of the same size but different sequence can be separated in a denaturing gradient gel on the basis of their denaturation profile. Therefore, total genomic DNA is isolated from the sample and a PCR reaction is set, usually amplifying the 16 S-rRNA gene. Then, polymerized fragments are subjected to an increasing denaturing environment. Since the melting temperature of each fragment is sequence specific, differences in the distance that each fragment runs in a DGGE gel is indicative of differences in the respective sequence. Thus, fragments of the same size but different sequences can be separated by DGGE.

PCR-DGGE has been extensively used in microbial ecology studies, most commonly in combination with culture-dependent techniques in order to provide the viable but not culturable portion of the microbiota [83]. Regarding pathogen detection, although the technique is capable of accurate differentiation [84], inherent disadvantages renders it incompetent to other techniques, mainly due to the rather high detection limit of 10^4 cfu mL^{-1} along with the species dependence that has been repeatedly indicated [85].

13.3.2 Immunological Detection

A series of techniques based on the basic ELISA principle have been established over time. Most of them consist of two discrete and subsequent steps: (1) capture and (2) detection of the target microorganism. Selective culture enrichment is necessary when the required detection limit is around 1 cfu mL^{-1} or g^{-1}.

Immunomagnetic separation and immunocapture are the most often applied capturing techniques. In the first case, small super-paramagnetic beads coated with antibodies against surface antigens of the target cells are used to separate the target microorganisms from the other members of a microbial ecosystem. Then, the target cells can be removed e.g., with a magnetic particle separator and be detected using techniques that will be described later [86].

Immunocapture is performed according to a typical sandwich ELISA scheme, where an antibacterium antibody is bound to a solid support; the bacterium is then "sandwiched" between the primary antibody and a reporter enzyme-labeled antibody conjugate.

The detection step can be performed using various disciplines. The colorimetric detection is the most common one. Alternatively, reflectance measurement, chemiluminescence, electrochemiluminescence, immunoelectrochemical detection, bioluminescence, and time-resolved fluorescence are among the techniques that have been successfully applied.

The typical sandwich-ELISA protocol has been applied by Padhye and Doyle [87] for the detection of *E. coli* O157:H7 in ground beef. Chemiluminescent detection coupled with sandwich immunoassay and enzymatic signal amplification for the detection of *E. coli* O157:H7 inoculated in ground beef has been demonstrated by Gehring et al. [88]. A rather simple and comparatively rapid assay has been reported by Brewster and Mazenko [89] for the detection of *E. coli* O157:H7. According to this study, target cells were labeled with an enzyme–antibody conjugate and captured by filtration of the sample through a 0.2 μm filter. The enzyme-labeled cells were detected by placing the filter on the surface of an electrode that was measuring the current produced by oxidation of the electroactive enzyme product.

Immunomagnetic separation has proven very powerful and compatible with a variety of detection methods. A combination of immunomagnetic separation and electrochemical detection was used to detect *E. coli* O157:H7 in porcine rinse water [90]. Alternatively, *E. coli* O157:H7 has been detected in ground beef using immunomagnetic separation combined with electrochemiluminescence [91,92]. Immunomagnetic separation followed by incubation with an ATP detection reagent has been used to detect *E. coli* O157:H7 in ground beef [93]. Immunomagnetic separation combined with time-resolved fluorescence has been used for the simultaneous detection of *E. coli* O157:H7 and *Salmonella* inoculated into ground beef [94].

13.3.3 Biosensors

The term biosensor is used to describe analytical instruments that incorporate a biological material as a receptor and a physicochemical transducer able to convert into a readable signal, the binding of the target analyte to the receptor. The receptor may be a tissue, a microorganism, or molecules such as enzymes, antibodies, nucleic acids, proteins, aptamers, biomimics, etc. Similarly, the nature of the transducer may be electrochemical, piezoelectric, optical, thermal, magnetic, or micromechanical.

Biosensors have been originally developed to serve the needs of health practitioners and monitoring agencies in the field; in an inexpensive, reliable, and user-friendly manner. Although the majority of current applications are within that original scope, their use has been expanded to food safety and for the monitoring of contaminants [95,96]. The methodologies applied include mostly antibody- and nucleic acid–based assays.

Antibody-based biosensors have been developed for the detection of important foodborne pathogens in various food matrices. The utilization of a quartz crystal microbalance (QCM)-based

biosensor as well as a surface plasmon resonance biosensor resulted in a detection limit of 10^6 cfu mL^{-1}, when *Salmonella* spp. *Pseudomonas aeruginosa*, *Bacillus cereus*, *E. coli* O157:H7, and *Listeria* spp. were the target microorganisms [97–103]. The detection limit was improved to 6×10^3 cells mL^{-1} with an impedance biosensor chip for detection of *E. coli* O157:H7 [104]. Further improvement was achieved by Shah et al. [105] and Muhammad-Tahir and Alocilja [106] with *E. coli* being the target microorganism in both cases. In the former case, the amperometric immunosensor resulted in a detection limit of 40 cfu mL^{-1}, whereas the conductometric biosensor of the latter case exhibited a similar detection limit of 50 cfu mL^{-1} for bacteria. A cyanine 5-labeled antibody was used to develop biosensors able to detect *L. monocytogenes* and *E. coli* O157:H7 [107,108]. In the first case, the biosensor was able to detect 10^1 cfu g^{-1} when contaminated hot dog or bologna was initially subjected to enrichment in buffered *Listeria* enrichment broth.

The development of DNA-based biosensors was based on the specificity of complementary nucleic acid strands hybridization. The utilization of such approaches for the detection of bacterial pathogens in foodstuff has been extended. The detection level of DNA biosensors vary from micro- to femto-grams. The latter has been achieved by Mo et al. [109] with the utilization of a QCM biosensor in combination with PCR amplification of the *lac* gene for the detection of *E. coli* in water and corresponds to a few viable cells in 100 ml of water. RNA-targeting biosensors have been developed to offer the advantage of viable cell detection and thus avoid false-positive signals. The utilization of a simple optical dipstick-type biosensor coupled to nucleic acid sequence-based amplification (NASBA) resulted in the detection of 40 *E. coli* cells mL^{-1} of water [110]. Other biosensors targeting nucleic acids that have been developed include PCR biosensors [111], microcantilever-based cyclic voltammetry biosensor [112], pulsed amperometry- [113], capacitance- [114], absorbance- [115], and MEMS-based biosensors [116].

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14 Mycotoxins and Toxins

Carla Soler, José Miguel Soriano, and Jordi Mañes

CONTENTS

14.1 MYCOTOXINS IN EDIBLE ANIMAL BY-PRODUCTS

14.1.1 Introduction

Mycotoxins, together with antibiotics, are products of the secondary metabolism of molds, with a molecular weight ranging from ca. 200 to 500, that cause undesirable effects, called mycotoxicoses, when animals or humans are exposed to them [1–8]. Several mycotoxins have a content regulation in most countries [9] but edible animal by-products have received less attention. Of ochratoxins, produced by some species of *Aspergillus* and *Penicillium*, OTA (ochratoxin A) is the most important toxin of this family [10–13] and may be found as cited, from 1980 to 2009 in ISI Web of Science, in edible animal-by-products of ruminant and nonruminant animals.

For ruminant animals, OTA is metabolized by rumen microorganisms into a less-toxic metabolite called ochratoxin α (OTAα) (Figure 14.1). The principal difference between OTAα and other ochratoxins is the lack of a phenylalanine group in the chemical structure [14]. Several authors [15–20] observed that ruminating animals, such as sheep and cows, are developing mycotoxicoses less frequently to the toxin than monogastric animals, due to the fact that the rumen flora acts as a first line of defense against mycotoxins. Meat from ruminant animals can be almost excluded from consideration of food risk due to the degrading/converting action of rumen microflora, which drastically reduces transfer to tissues.

For nonruminant animals, swine tends to accumulate OTA, because of a rather long serum half-life of 72–120 h [21]. A minor and indirect route of mycotoxin exposure is via meat in processed and/or cured meats developing surface molds and/or containing contaminated spices. The transfer of mycotoxins into edible tissues of poultry is not considered to be of toxicological significance. Among meat from nonruminant animals, pork is the most susceptible to OTA contamination [22,23]. Indeed, OTA residues can easily appear in pork tissues due to its high incidence in pig feeds and the unfavorable elimination toxicokinetics that lead to a relatively long half-life in edible animal tissues. OTA contamination of pork is a potential concern, particularly in northern European countries and in other parts of the world where climatic conditions lead to OTA contamination of feeds. Italy has a recommended guideline value of 1 ng g^{-1} in pork meat and derived products, and Denmark has enforced limits of 10 and 25 ng g^{-1} in pig kidneys for viscera and entire carcass condemnation, respectively [24]. In Denmark, postmortem inspection programs were indicated to

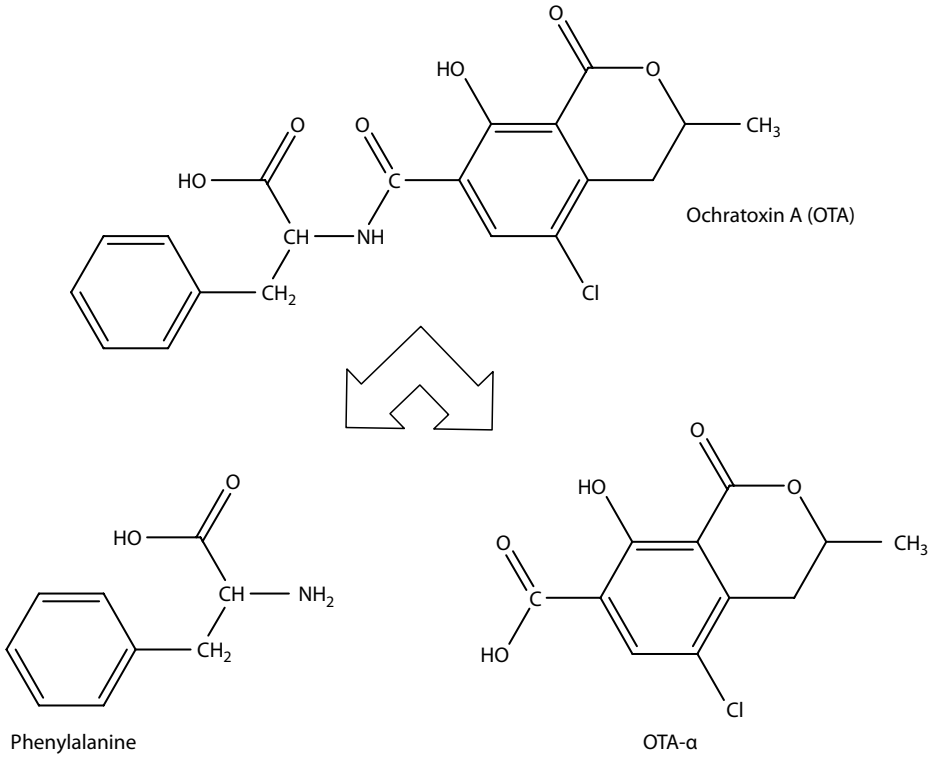

FIGURE 14.1 Chemical structures of OTA and derived products analyzed in edible animal by-products.

be good precautionary practices; it was decided that presence of 10 ppb OTA in pig liver or kidney would result in confiscation of the organ, and that levels exceeding 25 ppb would result in the confiscation of the entire carcass. It can be concluded that pork meat is a route for human exposure to OTA and continuous surveillance is needed, but its contribution is surely much less important than those of other foods [25].

14.1.2 Mycotoxin Analysis

14.1.2.1 Laboratory Precautions

Mycotoxins are extremely toxic chemicals, while crystalline standards are highly electrostatic and can disperse in the work area. As a recommendation, work areas should be decontaminated overnight with sodium hypochlorite at the end of the workday and thoroughly washed to have the surfaces checked for neutrality before starting a new analysis. On the other hand, the handling of standards, either in powder or in concentrated and reference standard working solutions, must be carried out with extreme care under a hood, while protecting the face of the operator with an appropriate mask and the operator's hands with gloves; latex gloves are recommended but not vinyl items. Working solutions should aim for zero operator exposure. Furthermore, the glassware must be scrupulously cleaned by immersion with a powerful oxidant (bleach or sulfochromic mixture). A flask with 10%–20% aqueous solution of sodium hypochlorite must be kept near the bench in case of emergency [26].

14.1.2.2 Sample Preparation, Pre-Concentration, and Cleanup Procedures

According to these products, extraction of OTA is generally performed by means of non-chlorinated solvents, mainly ethyl acetate. However, it poses several problems such as polar interfering substances that are co-extracted to a greater extent. This problem is solved with efficient cleanup of the extract. Solid-phase extraction (SPE) or immunoaffinity (IAC) columns are used for cleanup procedure.

Advantages of SPE are the possibility to regenerate the cartridge for further analysis and the cheaper price than IACs. As an example, Jiménez et al. [27] carried out the purification of patés with C_8 SPE columns and eluted with methanol, previously removed fats of the samples with hexane with the filtered fluid and partition of the two phases by centrifugation, and the aqueous phase was used added in the SPE columns.

The use of IACs helps to considerably increase the reliability of the results owing to its high selectivity, the possibility of analyzing more than one sample simultaneously, reducing the time of analysis, using a small amount of organic solvent, and removing of the matrix quite completely. Several disadvantages working with edible animal by-products could be (1) the acidified solvent extract cannot be directly applied to IAC; to avoid antibody deterioration it requires retroextraction in an aqueous buffer or evaporation to dryness and reconstitution in the buffer, and (2) internal standard cannot be used, which is useful in the following liquid chromatography analysis. However, Monaci et al. [28] developed a fast, simple, and sensitive method to avoid these disadvantages which required a small mass of sample (<2.5 g), a relatively small volume (<15 mL) of a non-chlorinated solvent (ethyl acetate), and the volume of the organic phase was reduced and back-extracted with $NaHCO_3$ pH 8.4. Furthermore, if an emulsion was formed the mix was refrigerated to facilitate separation of the phases.

14.1.2.3 Screening Tests

Three methods of screening tests, have been used to analyze the presence of mycotoxins in edible animal by-products: thin-layer chromatography (TLC), radioimmunoassay (RIA), and enzyme-linked immunoabsorbent assay (ELISA).

For TLC, Golinski et al. [29,30] studied OTA in kidneys and Van Egmond et al. [31] developed a procedure for the analysis of aflatoxins, in concentrations as low as 0.05 µg kg^{-1}, in animal liver. Analytes were extracted with chloroform and phosphoric acid, evaporated after filtration, defatted by liquid–liquid partitioning, and submitted to two-dimensional TLC. Aflatoxins B1 and M1 were confirmed with trifluoroacetic acid (TFA).

For RIA, the first study was developed by Rousseau et al. [32] which applied this procedure in the detection of OTA in porcine kidneys. This sample was extracted with 0.5% phosphoric acid in chloroform. Furthermore, a two-step cleanup was achieved on a Sep-Pak C18 cartridge and Sep-Pak silica cartridge. RIA with monoclonal antibodies coupled to protein A-Sepharose CL-4B was the method of detection of OTA in these samples, at a concentration as low as 0.2 ng g^{-1}. Three years later, Fukal [33] applied and validated a commercial kit for the radioimmunochemical determination of OTA, which has 1 ng g^{-1} as limit of detection, in several samples such as pig kidneys unfit for human consumption being 4.2% positive for this mycotoxin with concentration higher than 5 ng g^{-1}.

For ELISA Clarke et al. [34] developed a procedure which was sensitive, specific, simple, and sufficiently accurate for routine analysis of OTA in swine kidneys. Kidneys were homogenized in acidified ethyl acetate, centrifuged, subsampled, dried, reconstituted with methanol, and directly assayed using an indirect competitive ELISA. The rabbit antisera used in the development of this assay was found to have a high degree of cross-reaction with ochratoxins A and C but not with ochratoxins B, α, 4-OH-OTA, and two structurally similar molecules L-phenylalanine and citrinin, with the values being 100%, 80%, 3.33%, 10%, 1.4%, 0%, and 0.04%, respectively. The lowest reproducible OTA detection limit for ELISA in the spiked swine kidney samples was 7.81 ppb with inter-assay CV of 8.85%. Matrella et al. [35] studied OTA in pared kidneys and muscle samples and confirmed that ELISA method tends to slightly underestimate the mycotoxin concentration with respect to HPLC. According to the suggestion of Iacumin et al. [36], ELISA results must be compared to HPLC measurements using statistical analysis.

14.1.2.4 Quantitative Methods and Detection Systems

Methods used for OTA detection in edible animal by-products are based on liquid chromatography (LC) and the use of octadecyl, as stationary phase, with a saturated and increased percentage of carbon loading (Table 14.1). To date, the use of fluorescence and mass spectrometry detection is mainly applied for OTA in edible animal by-products.

TABLE 14.1
Analysis of Mycotoxins in Edible Animal By-Products

Mycotoxin	Sample	Extraction Technique	Cleanup Technique	Separation	Detection	Recovery (%)	LD (ng g^{-1})	Reference
OTA	Pig liver-derived patés (commercial and home-made)	Shaken with CH_3CN (60%), hexane and water, removed the organic layer	C_8 SPE columns eluted with CH_3OH	Spherisorb ODS-2 (250 × 3.0 mm, 10 µm), 5 mM sodium acetate/ phosphoric acid (pH 2.2)-CH_3OH-CH_3CN from 62:19:19 at 1.5 mL/min	FLD λ_{exc} 225 nm λ_{em} 461 nm	85.7	0.56	[27]
OTA	Pig kidneys, liver and muscle	Homogenization with 1 M phosphoric acid, extraction twice with ethyl acetate	IAC (Ochraprep) eluted with CH_3OH/CH_3COOH (98:2)	Supelcosil LC-18 DB (150 × 4.6 mm, 3 µm), H_2O (1% CH_3COOH)/ CH_3CN (1% CH_3COOH) from 50:50 to 40:60% in 15 min at 0.5 mL/min	FLD λ_{exc} 334 nm λ_{em} 460 nm	70 (liver)–86 (kidney)	0.14 (kidney)–0.15 (muscle)	[28]
OTA	Pig tissues (kidney, liver and muscle)	Homogenization with 1 M phosphoric acid, extraction twice with ethyl acetate	—	Supelcosil LC-18 DB (250 × 2.1 mm, 5 µm), H_2O (1% CH_3COOH)/CH_3CN from 50:50 to 40:60% in 15 min at 0.2 mL/min	ESI-MS^n	86	0.6	[37]

OTA, Ochratoxin A; FLD, fluorescence detection; LC, liquid chromatography; IAC, immunoaffinity column; LD, limit of detection.

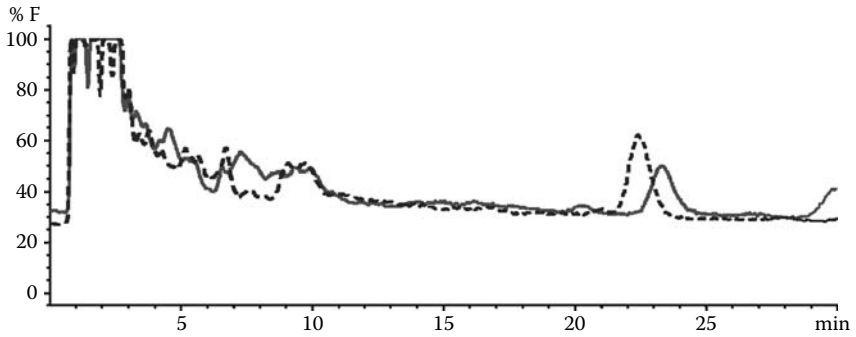

FIGURE 14.2 Confirmation of a positive sample in pig liver-derived patés: (—) chromatogram of a positive sample and (- - -) chromatogram of the same sample after having added OTA and varied the mobile phase. (Reprinted from Jiménez, A.M. et al., *Food Addit. Contam*., 18, 559, 2001. With permission.)

For fluorescence detection (Fld), the excitation and emission wavelengths are 225–334 and 460–461 nm, respectively. According to the great variety of substances present in the reviewed products, Jiménez et al. [27] increased the amount of the aqueous phase in the mobile phase to retard the OTA. These authors achieved a good peak resolution. Confirmation of OTA can be carried out by two procedures. First, derivatization of OTA into its methyl ester and the subsequent determination of this compound is used. However, this method has a problem, as the purity of the reagent employed is frequently uncertain, giving rise to interferences [38]. Furthermore it is not efficient for the confirmation of low levels of this myocotoxin [39–41]. This procedure is not employed for edible animal by-products. However, the hydrolysis of OTA into OTAα by the enzyme carboxypeptidase A, as confirmation of this mycotoxin, was carried out in pig liver-derived patés [27]. A chromatogram of OTA by LC-FLD is shown in Figure 14.2.

For mass spectrometric detection, Losito et al. [37] used the power of electrospray ionization with sequential mass spectrometry (ESI-MS^n) for OTA determination and quantitation in the sub-ppb range, in pig tissues (kidney, liver, and muscle) obtained from local slaughterhouses. Ochratoxin B (OTB) was applied as an internal standard. The choice of internal standard is very important in the analytical procedure. A stable isotope-labeled analyte or analogue (not natural) was used in the matrix. No deuterated analogue of OTA is commercially available but Lindenmeier et al. [42] proposed and recommended a quite complex reaction to synthesize the deuterated analogue of this mycotoxin. The use of deuterated compounds minimize possible interference effects, such as suppression of ESI efficiency, due to co-elution of other matrix components. Losito et al. [37] proposed that the impossibility of synthesized deuterated analogue of mycotoxin can be replaced with the internal standard with an analogue (not natural) in the matrix coupled with a calibration with matrix-matched standards. An improvement in S/N ratio is achieved using consecutive reaction monitoring (CRM) acquisition mode. Figure 14.3 shows the consecutive reaction monitoring (CRM) for OTA and OTB obtained from the extract of a naturally contaminated pig kidney sample.

14.2 BACTERIAL TOXINS IN EDIBLE ANIMAL BY-PRODUCTS

There are now more than 240 well-recognized bacterial toxins. A few of these are entirely responsible for the patterns of clinical illness (e.g., botulinum toxin in botulinum poisoning), but the majority are important components in the spectrum of virulence factors that a pathogenic bacterium uses to cause disease.

A lot of cases have been reported about the presence of bacterial toxins in meat, or meat products. For this reason, a great number of studies have been carried out for the determination of different bacterial toxins in these food commodities [43–45].

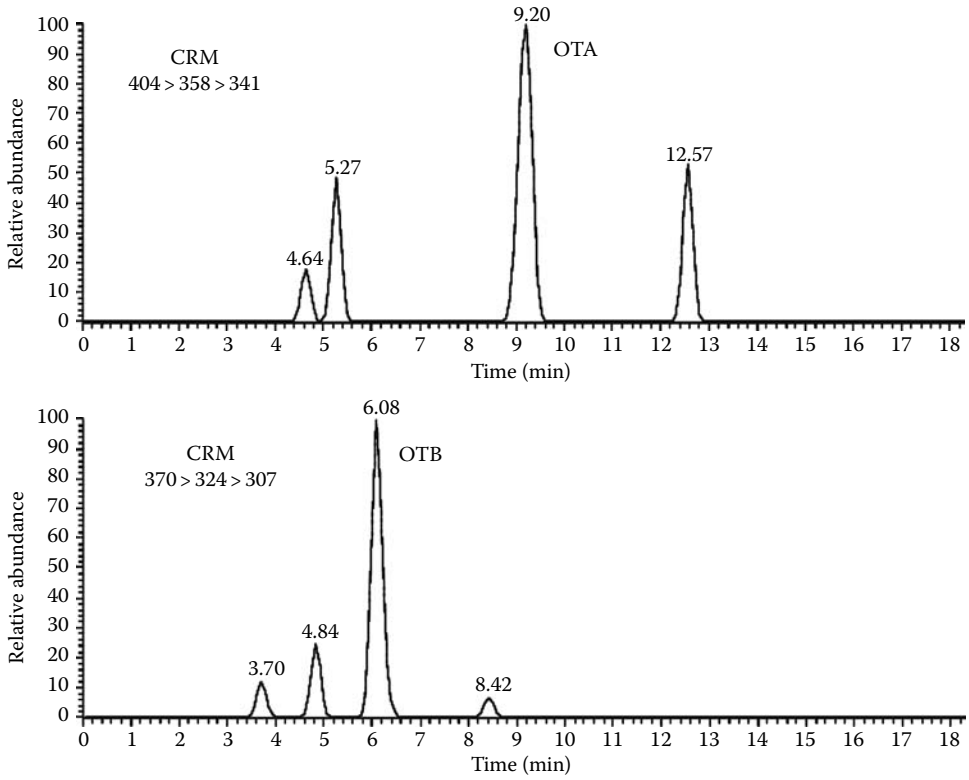

FIGURE 14.3 CRM chromatograms obtained for a pig kidney sample naturally contaminated with OTA spiked with OTB at the 1 ng g^{-1} level. (Reprinted from Losito, I. et al., *Rapid Commun. Mass Spectrom.*, 18, 1965, 2004. With permission.)

However, it is very surprising to realize that there are no studies about the presence of bacterial toxins in edible animal by-products, even though it's true that no occurrence of these toxins are reported in the literature.

The nearest that we can find is a study of Shiga toxin-producing *Escherichia coli* in precooked sausages (morcillas) [46]. "Morcilla" is a mixture of blood of animals approved for consumption, pork fat, ground pigskin, salt, onion, and spices, stuffed into a bovine gut or a synthetic casing. This work demonstrated the high contamination of these products and the necessity to control the bacterial toxins in them.

14.3 CONCLUSIONS

This review has provided an update for the scarce literature about this concern. The inescapable conclusion is that edible animal by-products may be a study subject of the incidence of mycotoxins and bacterial toxins. However, it is also acknowledged that this contamination is of an insignificant degree, and it is not supposed to cause a problem for the consumers' health.

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15 Detection of Bone in Meat

Wolfgang Branscheid and Michael Judas

CONTENTS

15.1 INTRODUCTION

The detection of bone in meat is an important issue in the context of the extraction of meat residues from individual bones or carcasses after deboning. This extracted meat is labeled mechanically recovered, mechanically deboned, or mechanically separated meat (MSM). Due to the production process, an elevated amount of bone particles is to be expected in such meat. Therefore, the content of bone particles is frequently used as a key criterion to identify MSM. A discrimination is required because MSM usually is not equivalent to fresh meat, as reflected in its lower price. Besides other criteria that reduce MSM quality, the elevated bone content is directly responsible for a substantial reduction in sensory quality: consumers are particularly annoyed by grittiness of MSM [1]. But negative gastroenterological side effects that were suspected in past studies could not be confirmed [2]. In contrast, Drake et al. [3] even consider bone as a useful calcium source for nutrition because bone particles are easily soluble in gastric juice. Field [4] points out that calcium additives for food, including baby food, commercially available in the United States, contain bone particles that are sized just as in MSM.

Internationally, there is a consensus that MSM is to be regarded as raw material of decreased quality due to its origin, production, composition, and functional properties, and also due to its limited

consumer acceptance [5]. In numerous countries, this led to legal restrictions on the use of MSM for meat products. But such restrictions can only be effective if the respective raw material is detectable.

Detection methods for MSM take advantage of the high pressure used to separate meat residues from bones. This causes abrasion of bone particles, extrusion of soft tissues (bone marrow, connective tissue), and a modification of muscle structure. Modifications of muscle structure are more difficult to objectify, compared to the various detection methods available for bone. Therefore, for many years, the scientific efforts focused on the bone fraction as a key criterion for MSM (cf. reviews by [6,7]). Only recently, also histomorphological methods to detect modified muscle structure are discussed more intensely [8,9]. But routine quality assurance based on histomorphological assessment of muscle fibers does not appear feasible [10].

15.2 LEGAL BACKGROUND OF BONE DETECTION IN MEAT

Both the United States and the European Union (EU) developed a comprehensive legal framework for the identification of MSM and for the detection of bone in meat (for other legal systems, cf. [11,12]). The two legislations differ distinctly, involving different methods for bone detection.

The system of the United States Department of Agriculture (USDA system) differentiates advanced meat recovery (AMR) and MSM. AMR is a gentle production process that produces meat comparable to hand deboning. The separated bones remain intact enough to keep the total calcium content below 150 mg/100 g (i.e., 1500 ppm). Processed AMR meat needs not to be declared. MSM is analyzed for calcium, protein, and fat contents. In addition, bone fragments are to be described very accurately. Critical values are defined for content of bone and dimensions of bone fragments (Table 15.1). MSM has to be declared as an ingredient of the final product, with a limitation on the amount added (except for poultry MSM, which is not limited).

TABLE 15.1
USDA Definition of Mechanically Separated Meat

	Bone Fragment Characteristics	Analytics of MSM Components
Livestock		
(Veal, pork, lamb, mechanically separated beef is inedible and prohibited for use as human food)	Content of bone fragments <3% of meat wet weight	Calcium ≤0.75% (as a measure of bone solids content of no more than 3%)
	Dimensions of bone fragments:	
	98% of the fragments shall have a maximum size no greater than 0.5 mm in their greatest dimension	Protein ≥ 14%
	No bone fragment larger than 0.85 mm in its greatest dimension	Fat ≤ 30%
Poultry		
	Content of bone solids <1% of meat wet weight	Calcium
	Dimensions of bone fragments:	
	98% of the fragments shall have a maximum size no greater than 1.5 mm in their greatest dimension	≤0.235% from mature chickens or turkeys
	No bone fragment larger than 2.0 mm in its greatest dimension	≤0.175% from other poultry (as a measure of bone solid content of no more than 1%)

Source: Adapted from Code of Federal Regulations *9 CFR 319.5*, http://cfr.vlex.com/vid/319-mechanically-separated-species-19611341 (live stock); Code of Federal Regulations *9 CFR 381.173*, http://cfr.vlex.com/vid/mechanically-separated-kind-poultry-19612333 (poultry).

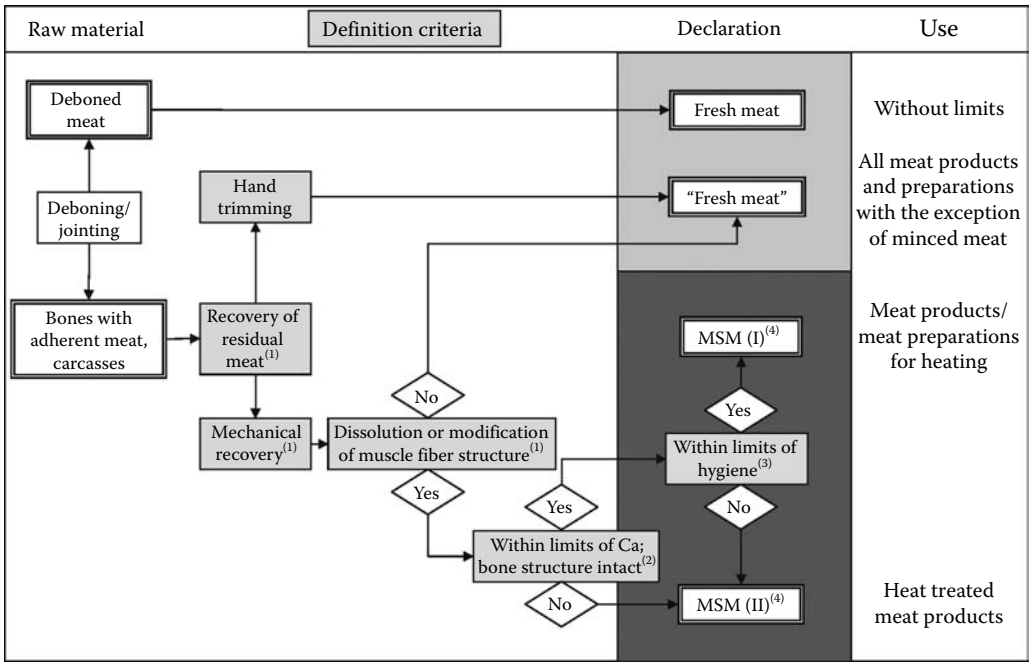


FIGURE 15.1 Definition of mechanically separated meat according to EU regulations. (1) Regulation (EC) Nr. 853/2004, Annex III section V chapter III number 3 and 4. (2) Regulation (EC) Nr. 2074/2005, Annex IV number 1. (3) Regulation (EC) Nr. 2073/2005, Annex I, chapter 1, number 1.7 and chapter 2, number 2.1.7. (4) Directive 2001/101/EC, whereas no. 7. (Adapted from Hildebrandt, G. and Josefowitz, P., *Fleischwirtschaft*, 87, 21, 2007. With permission.)

EU legislation defines properties and use of MSM in a more complex way. Calcium content is a critical criterion, too. Additional MSM criteria are the structure of muscle fibers and microbiological hygiene, but no detailed description of bone fragments. The complex interaction of these criteria is visualized by a decision tree for the identification and delimitation of MSM (Figure 15.1). In this framework, MSM is generally characterized by three properties:

- MSM is produced from meat residues that adhere to bones after deboning.
- These meat residues are mechanically extracted.
- The extraction results in a disintegration of muscle structure, although no details of this disintegration are described.

EU legislation furthermore differentiates such MSM into high-quality MSM (I) and low-quality MSM (II) by the following criteria (cf. Figure 15.1):

- For MSM (I), bone structure has to stay intact and calcium content must not exceed 100 mg/100 g (i.e., 1000 ppm); in addition, MSM (I) material has to comply with the hygienic restrictions for minced meat, especially with regard to salmonellae.
- MSM (II) does not meet the criteria for bone structure or calcium content, or it violates the hygienic restrictions; MSM (II) is the material where elevated amounts of bone particles are to be expected.

MSM (I) and (II) differ in the restrictions on their utilization (Figure 15.1), but, other than in the USDA system, both are declared as "mechanically separated meat" without any differentiation.

The EU system and the U.S. system basically differ in the method of bone detection, since the EU only requires an analytical detection of elevated calcium content, while the USDA system also considers the amount and size of bone particles directly. However, also for European meat quality control, a direct proof of bone particles is of high importance because calcium analysis is but an indirect method of evidence. It is based on the fact that—ceteris paribus—calcium content of meat is a direct function of bone content.

15.3 HISTOLOGICAL BACKGROUND OF BONE PARTICLE DETECTION

All detection methods for bone particles are based on the histological composition of bone tissue (see, e.g., [13] for a comprehensive histology). Bone tissue proper is composed of bone cells (osteocytes) that lie in mineral-free lacunae, and mineralized intercellular substance (tela ossea; Figure 15.2). The tela ossea essentially is an organic matrix with a secondary adsorption of hydroxylapatite (calcium phosphate). 95% of the organic matrix is collagen fibers. The osteocytes access the mineralized matrix by 20–30 extensions each, also allowing for their intercellular contact. The cell extensions run in tiny tubes (canaliculi) that are visible at high magnification. The volume of osteocyte lacunae and their extension tubes is less than 10% of the bone matrix. In adult compact bones, including its marrow, the organic and inorganic substances comprise 35% and 45% of the total volume, respectively (Figure 15.3). The rest, i.e., ca. 20%, is water.

The proportion of mineral tissue (tela ossea) varies between bone types (Table 15.2; cf. [14]). In *compact bones*, it attains as much as 80%–94% of volume. In the compacta fraction, there is space for only rather small, tubular canals (Haversian canals) that form functional units with the so-called osteons of compact bones. Inside the Haversian canals, there is yellow marrow sparse in hematopoietic cells but rich in fat cells. Compact bone forms the major part of the diaphysis of long hollow bones, the corticalis of small bones of the extremities, and the corticalis of flat bones. The compact bone of the diaphysis confines a large cavity that is filled with yellow marrow already at an early age.

Spongious or *trabecular bones* have a considerably lower proportion of bone tissue, namely 25%–30% of volume (Figure 15.2, Table 15.2). Marrow thus comprises 70%–75% of trabecular bone volume. Trabecular bone with red bone marrow is located in the epiphyses of hollow bones, in all short bones of the extremities, and in the bones of the trunk (vertebral bodies, sternum, scapula, ribs, pelvis). In adult and in senescent animals, the proportion of red marrow strongly declines.

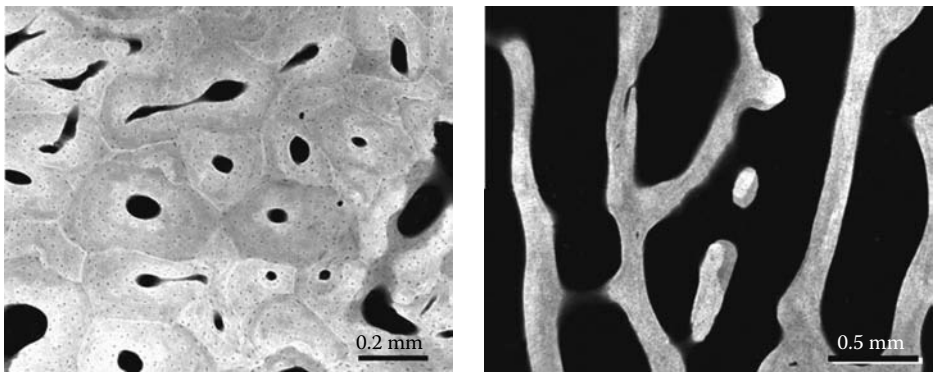


FIGURE 15.2 Microradiographies of compact (A) and trabecular bone (B) from a sesamoid bone of a horse. Only mineralized substance is displayed. (A) Compact bone constitutes the corticalis of the sesamoid bone; between its osteons, there are but small Haversian canals, predominantly filled with yellow marrow. (B) Trabecular bone of the same sesamoid bone confines large cavities with red bone marrow.

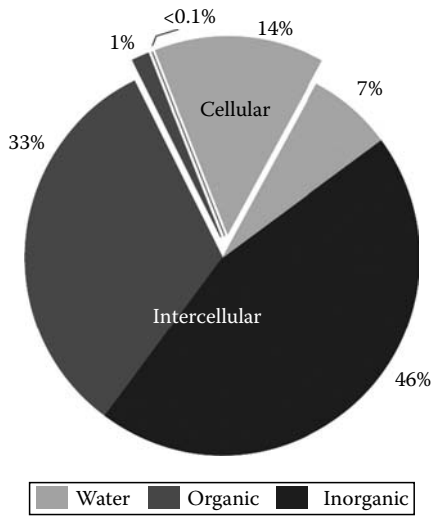


FIGURE 15.3 Composition of compact bone (% vol.). Water, organic, and inorganic compartments are differentiated for cellular components, i.e., bone marrow, vessels, and osteocytes (excentric slices), and for intercellular substance (centric slices). (Original figure from data of Knese, K.-H., *Stützgewebe und Skelettsystem. Handbuch der mikroskopischen Anatomie des Menschen*. 2. Band: Die Gewebe 5. Teil. Berlin/Heidelberg, Springer-Verlag, Germany/New York, 1979.)

TABLE 15.2
Proportion (% Area) of Mineralized Tissue in Different Types of Human Bone

Age Group (Years)	Compact Bone (Mean [Standard Deviation])	Trabecular Bone (Mean [Standard Deviation])
0–1	80 (10)	30 (5)
2–19	92 (4)	27 (7)
20–49	94 (2)	25 (4)

Sources: Adapted from Heuck, F., *Verhandlungen der Deutschen Gesellschaft für Pathologie*, 58, 114, 1974; Heuck, F., Allgemeine Radiologie und Morphologie der Knochenkrankheiten. In *Handbuch der medizinischen Radiologie*, Band V, *Roentgendiagnostik der Skeletterkrankungen*, Teil 1, ed. L. Diethelm, pp. 3–303, Springer, Berlin, 1976.

From vertebral bodies, bone marrow may leak due to a breach of their rather thin corticalis of compact bone. Especially in large slaughter animals, marrow easily leaks from the vertebral marrow cavities that are opened when a carcass is split at the midline. The characteristics of bone marrow cells offer diagnostic potential that is detailed below (cf. Section 15.4.1.3).

In adolescent animals, all bones have cartilaginous growth zones, most pronouncedly at the epiphyses. In carcasses, *cartilage tissue* is generally connected to bones only (cartilage of joints, scapula, ribs, and sternum, and fibrous cartilage of intervertebral discs and the adjacent surface of joints). Thus, cartilage is as good an indicator for MSM as bone itself [15].

15.4 DIRECT DETECTION OF BONE TISSUE COMPONENTS

Direct methods to detect bone comprise a variety of morphological, histological, chemical, or physical methods, combined with visual methods or automated image analysis, that retrieve cell or tissue components obligatorily connected to bones (Table 15.3). This refers to not only parts of the mineralized bone tissue itself, but also to collateral tissue components like cartilage, bone marrow, firm connective tissue, and nervous tissue of the spinal cord. For bone particles, assessment by a sensory panel also represents an adequate approach. But this potential for direct bone detection appears to be largely unused and is not elaborated any further here.

15.4.1 Morphological Detection of Bone and Collateral Tissue Types

In MSM, cell or tissue structures are often preserved well enough to allow for cytological studies. A number of cytological characteristics can be used to identify MSM: osteocytes, collagen fibers of the tela ossea, components of the bone marrow, cartilage tissue, or firm connective tissue.

15.4.1.1 Morphology of Osteocytes

The lacunae of osteocytes have canaliculi that extend in all directions. Together with mineralized tissue, they form the most assured and distinctive cytological indicators of bone tissue [16]. A presentation of canaliculi succeeds best in the subtle extinction of hematoxylin–eosin staining, or in an unstained preparation, because high-contrast staining masks the faint structures. Especial staining of canaliculi is possible with thionine picric acid according to Schmorl [17,18]. Branscheid [19] used the lacunae and canaliculi to identify minute particles of bone meal deployed as a fertilizer to arable soil, which otherwise would have been difficult to verify (Figure 15.4). For MSM or processed

TABLE 15.3
Direct Detection Methods for Bone Tissue Components in Meat

Detection Method	Evaluation	References
Bone and collateral tissue components		
Morphology of osteocytes and canaliculi	Qualitative	[8,13,20]
Morphological demonstration of bone marrow cells (series of white blood cells, agglomerates of "round" cells).	Qualitative	[15,31,33,34]
Histological demonstration of cartilage tissue by basophile stains (in part metachromatic) and Trichrome staining	Qualitative	[15,32,99]
Morphologic staining of collagen fibers (Calleja, van Gieson, other trichrome stains)	Quantitative, qualitative	[32,41,49,100]
Selective histologic detection of collagen fibers with polarization microscopy, without staining or with Sirius Red staining	Qualitative	[8,21]
Mineralized components		
Various detection methods for mineral components	Quantitative	[6,7] (review), [56,72] (review)
Specific histological demonstration (von Kossa silver impregnation, Alizarin Red)	Qualitative, quantitative	[32,35,36,38–39,41, 44,49,51,65,100–105]
Polarization microscopy of apatite crystals	Qualitative, quantitative	[21]
Histomorphological gravimetric detection (Alizarin Red, Alcian Blue)	Quantitative	[54,55]
Sedimentation after digestion of soft tissue components, flotation technique	Quantitative	[2,38,48,49,57,59–61, 63–65,101,102,105, 108,126]
Radiological detection	Quantitative, qualitative	[38,54,67,106]

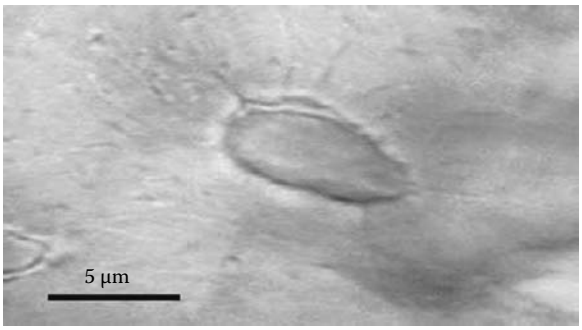
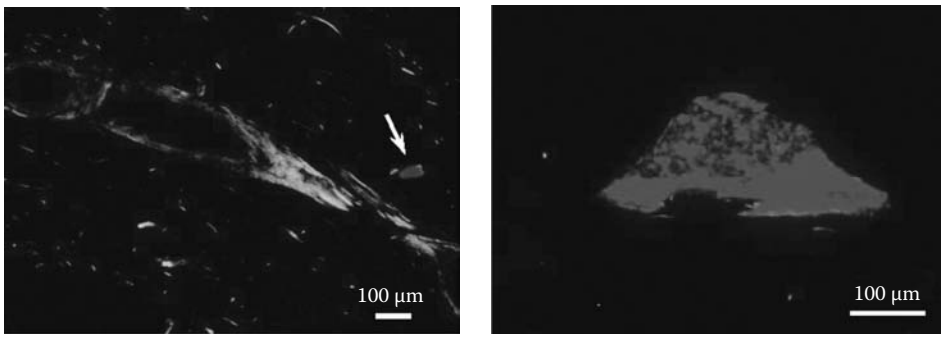


FIGURE 15.4 Bone particle from beef MSM. Osteocyte with clearly visible lacuna and canaliculi. (From Branscheid, W., *Mitteilungsblatt BAFF*, 41, 135, 2002. With permission.)

products, a recourse to osteocyte morphology may be necessary to provide the ultimate identifying feature for bone, especially in cases when selective but not fully specific staining methods are to be made court-proof [20]. But, of course, bone tissue cannot be quantified by this method.

15.4.1.2 Selective Histological Preparation of Collagen Fibers of the Tela Ossea

Collagen fibers form the main organic component of the bone matrix, where they have a pronounced directional course that facilitates their morphological identification. For various meat mixtures, collagen fibers are well presented by specifically adjusted trichrome stainings (Azan, Masson-Goldner, or Van Gieson), or by Calleja staining [17,18]. These methods are especially suited because muscle and connective tissues are sharply differentiated in terms of color. They allow a semiquantitative analysis and under favorable conditions also a histometric–quantitative analysis [10]. But for non-decalcified bones, or for firm connective tissue of tendons and ligaments, these stainings partly fail. Therefore, if bone is to be identified based on collagen fibers, the preparations have to be decalcified previously, e.g., with citrate formic acid [17]. This applies especially to polarization–optical analysis: because the double refractions of hyroxylapatite and collagen fibers interfere, the apatite has to be removed mandatorily. In previous studies, Ebner's phenole reaction was the method of choice for a differentiated analysis of collagen fibers [21]. This reaction even allows quantitative measurements based on optical path differences between fibers after phenole adsorption [22]. However, this is possible at a cytological scale only, not for entire tissue sectioning. Similar quantitative analyses of collagen fibers are possible with the Congo Red topo-optical staining reaction [23]. Nowadays, the best staining for foodstuff histology appears to be Pikrosirius Red, combined with subsequent examination under crossed polars [24,25]. This method provides far more information than the above-mentioned trichrome staining with transmitted-light examination. The Pikrosirius Red method allows to differentiate collagen fiber types I, II, and III, of which only type I occurs in bone. The collagen fiber types differ in polarized colors from green over yellow to bright red, depending on density and packing of the fibers [25]. It has to be considered that the polarized colors also change with the thickness of sections, i.e., they can be interpreted correctly only in case of standardized section thickness [26]. Furthermore, Puchtler et al. [27] point out that bonding to proteins by Picrosirius Red and similar picrostains is not exactly stoichiometric. In meat products, this staining method allows to differentiate native fibers from fibers modified by cooking or thickening, as well as from rind fibers [28,29]. Especially polarization–optical analyses of bone reveal the advantages of the Picrosirius Red staining method. Deepest red polarization colors appear for bone lamellae and allow a selective delineation of minute bone particles even with low microscopic magnification (Figure 15.5). A further advantage is that Picrosirius Red staining can be combined with Alcian Blue staining without any problems [30]. This combination allows to simultaneously mark bone and cartilage tissues in MSM [20]. This allows for cartilage particles to be at least as good an indicator for bone as bone particles themselves, an approach that has often been neglected [15].



(A) (B)

FIGURE 15.5 Microscopy of bone particles and connective tissue with crossed polars after staining with Alcian blue and Sirius red. (A) MSM section including a longitudinal cut of a blood vessel (diagonal structure) and a small bone particle (arrow). Collageneous connective tissue varies in polarization colors from yellow to orange-red, while bone particles have a distinct deep red. (B) Singular bone particle, with partially visible lamellar structure. (From Branscheid, W., *Fleischwirtschaft*, 82, 92, 2002. With permission.)

In transmitted light, the staining sequence produces brilliant contrasts between blue and yellow-red that facilitate a safe differentiation between cartilage and bone tissues. By the way, Alcian Blue also stains plant cell walls and permits their rapid detection (e.g., spicery, [20]).

15.4.1.3 Components of Bone Marrow

The numerically most frequent cells of bone marrow belong to the different series of leukocytes (neutrophilic, eosinophilic, and basophilic series of granulocytes, and lymphocytes) which amount to ca. three quarters of all cells, while the series of erythrocytes amounts to ca. one quarter (Table 15.4). Megakaryocytes would be easiest to identify, but they are hardly ever found in MSM. Mononuclear osteoblasts and polynuclear osteoclasts are other characteristic cells, but

TABLE 15.4
Differential Counts of Bone Marrow Cells from Healthy Male Adults

	Cell Counts (%)	
Cell Type	**Mean**	**(Range)**
Neutrophilic series	54	(49–65)
Eosinophilic series	3	(1–5)
Basophilic/mast cells	<0.1	(0–0.4)
Lymphocytes	16	(11–23)
Plasma cells	1	(0.4–4)
Monocytes	0.3	(0–0.8)
Erythrocytic series	26	(18–34)
Megakaryocytes	<0.1	(0–0.4)
Reticulum cells	0.3	(0–0.9)

Source: Adapted from Perkins, S.L., Examination of the blood and bone marrow. In *Wintrobe's Clinical Hematology*, Vol. 1, 12th edn., J.P. Greer et al. (eds.), Lippincott, Williams & Williams, Philadelphia, PA, pp. 9–20, 2009.

they can only casually be identified at the edge of bone particles and are thus of little diagnostic help. Hence, primarily, the predominant granulocytes qualify for differential diagnosis, especially because their characteristic nuclear shapes support identification. But overall, a detection of bone marrow cells in MSM is difficult, as evidenced by a number of failed attempts [31–33]. Field [34] qualifies microscopic evidence for bone marrow cells as "extremely subjective," although he reports some successful diagnostic attempts from the United States. The difficulties encountered are most probably due to the mechanical disintegration of cells, which affects bone marrow much more than supporting or connective tissues, and which prevails especially in low-quality MSM exposed to high pressure. The ambivalence of cell diagnostics requires unquestionable cytologic traits, otherwise a proof of bone marrow should be discarded. In general, cytological identification of bone marrow is not promising. On the one hand, bone content cannot be quantified by these methods. On the other hand, negative morphological findings of bone marrow cannot prove the absence of bone, but this is prerequisite for a standard test method. In processed meat products, a morphological identification of bone marrow is not possible anyway because cell morphology is destroyed.

15.4.1.4 Cartilage Tissue

Cartilage particles are a regular collateral tissue of MSM, but they are frequently neglected as such. There are many selective staining methods to distinguish them in preparations, allowing to identify them without morphological expertise. Best suited are stainings with basic dyes at low pH (e.g., Alcian Blue at pH 2.5) or with strong metachromasia (e.g., from Toluidine Blue) that stain the matrix material with its sulfated, strongly acidic glycosaminoglycanes (chondroitin sulfate), thus distinguishing cartilage clearly from the background [17,18]. Metachromatic dyes produce particularly fine contrasts. On this basis, at least a manual histometric evaluation is feasible.

15.4.2 Histochemical Detection of Mineralized Components

Mineralization makes bone tissue unique with respect to histotechniques. On the one hand, sectioning of non-decalcified bone is technically demanding. On the other hand, the mineral content enables specific detection methods. Sinell [21] points at the possibility to specifically detect hydroxylapatite by polarization microscopy. To this end, collagen must first be dissolved away from the tissue by potassium hydroxide (KOH). In the dried preparation, bone shows a strongly positive double refraction with air. During the subsequent imbibition sequence, the refraction approaches zero in glycerine, and becomes slightly negative after embedding in Canada balsam. Nowadays, this method will only be useful if applied to few specific samples; for large-sample screening, it is too laborious. For routine applications, histologic staining methods are to be preferred that specifically stain bone because of its mineral Ca-content. This supports an unequivocal identification of bone and allows the application of automated image analysis. Two histochemical staining methods are applicable, namely staining with silver nitrate or with Alizarin Red. For both methods, the chemical background of the staining mechanism is well-known. Both provide a specific identification of calcium within preparations of meat or meat products. The preparations produced by these methods can then be analyzed quantitatively by visual microscopic counting or by automated image analysis.

15.4.2.1 Staining Methods

Silver nitrate (after von Kossa, as modified by [35]) exclusively attaches to mineralized bone particles. These become deep black and differ clearly from other tissues if they are stained with Orange G, a van-Gieson staining, or any other counter staining. The sharp contrast to the black bone particles favors digitized image analysis (cf. Section 15.4.2.2).

Also, the dye *Alizarin Red S* (after [36]) is specific for bone particles and has for long been used for this purpose. The staining mechanism is largely clear and can be regarded as specific for

calcium salts [37]. A brilliantly red color ensues that can well be combined with counter stainings like Calleja, Indigocarmin, Alcian Green, or Alcian Blue. This method is not as laborious, but just as safe as Kossa's silver nitrate staining.

For both methods, bones must not be decalcified. As a consequence, larger particles frequently float from the staining solutions [36,38]. This problem can be solved by coating the slides with celloidin, alias collodion or nitrocellulose ([39], with a detailed description; [35]). But experience shows that this measure prevents subsequent staining with phthalocyanine dyes (e.g., Alcian Blue) because also the celloidin background is stained with these dyes.

15.4.2.2 Quantitative Analysis

During the microscopic examination of preparations, a direct quantification can be made by a *particle counting procedure*. The procedure has to be standardized according to minimum particle size, size of tissue blocks, number of sections from these blocks, and size and number of observation areas from the sections [40]. As a consensus, a minimum of eight sections with 10 observations each is recommended, and all particles >50 µm should be counted [40–42]. Bone particle counting can be combined with cartilage particle counting if the appropriate staining methods have been combined. The results from particle counting are correlated to MSM content (Tables 15.5 and 15.6) and can thus be used to qualify meat products containing different amounts of MSM. While the replicability in one study was high ($r > 0.9$ between operators, Table 15.6), the precision in another study was found to be rather low with variation coefficients of 20%–50% (Table 15.5). The high variation most probably is an effect of inhomogeneous particle distribution within preparations, rather than a technical effect of the counting procedure.

TABLE 15.5
Histometry of Bone Particles in Meat Products Prepared with Known Percentages of MSM

	Number of Particles Counted[b]		
	Mean (Standard Deviation)		
% MSM Added[a]	Pork Shoulder (Pork MSM Added)	Luncheon Meat[c] (Pork MSM Added)	Luncheon Meat[c] (Poultry MSM Added)
0	1 (1)[a]	12 (7)[a]	6 (4)[a]
1	6 (3)[b]	23 (9)[b]	17 (5)[b]
2	—	24 (10)[b]	—
3	—	29 (6)[b]	—
4	—	31 (9)[b]	—
5	81 (37)[c]	32 (6)[b]	—
10	405 (189)[d]	69 (13)[c]	284 (149)[d]
15	—	90 (35)[d]	—
20	—	193 (64)[e]	344 (106)[d]
25	—	234 (41)[f]	—
30	—	245 (56)[f]	—
35	—	340 (73)[g]	—

Source: Adapted from Bijker, P.G.H. et al., *Archiv für Lebensmittelhygiene*, 36, 71, 1985.

Note: (a) through (g) in columns, figures with different superscripts differ significantly with $p < 0.05$.

[a] All MSM levels tested only for luncheon meat with pork MSM.

[b] For each preparation, particles were counted from eight sections per each of 10 samples.

[c] Commercial pork and beef luncheon meat.

TABLE 15.6
Evaluation of Histometrical Particle Counting: Correlation between Number of Bone Particles Pounted and Percentage of MDM Added (Precision) and Correlation between Counts by Two Independent Research Workers (Replicability)

	Precision		Replicability	
Meat Product with Added MSM	***n***	***R***	***n***	***R***
Pork shoulder (pork MSM added)	30	0.82	—	—
Luncheon meat (pork MSM added)	110	0.94	80	0.99
Luncheon meat (poultry MSM added)	30	0.76	40	0.99

Source: Adapted from Bijker, P.G.H. et al., *Arch. Lebensmittelhygiene*, 36, 71, 1985.

Bijker et al. [40] derive a classification of particle counts, based on 8 sections with 10 observations each:

- 0–30 bone particles: no MSM has been used, or at very low percentage only
- 30–60 bone particles: MSM may have been used
- 60 bone particles: MSM has been used

A similar classification by Schulte-Sutrum and Horn [41] includes a legal assessment of the results:

- ≤0.2 bone particles per cm^2: technologically unavoidable, no complaint
- 0.3–1.5 bone particles per cm^2: revision of manufacturing practice
- >1.5 bone particles per cm^2: evidence of MSM, inspection of labeling, and penalty if applicable

Pickering et al. [15] successfully apply particle counting to cartilage tissue, too. In MSM, they find a considerably increased cartilage content compared to hand-deboned meat (HDM). Particle counting has the advantage to be simple and quick and to not require technical effort. However, the counted numbers of bone or cartilage particles do not reveal their actual percentage in terms of weight or volume, nor do they reveal calcium content.

Another way of quantitative evaluation of histologic preparations is the *point counting procedure* [32,43,44]. The principle of this method is to microscopically count point matches between tissue components in question and a point graticule, a point grid, or similar devices with up to a hundred points. This method can be applied with a simple microscope equipped with a point grid, without need for digital support [45,46]. For MSM, Bijker et al. [44] give a detailed protocol to register such tissue hits or point fractions. They demand 3000 counts per section for sufficiently representative results. In addition, they point out that if a given maximum standard error is to be maintained, the number of counts must increase if the proportion of the component of interest declines. Statistical details of point count analysis are given by Eränkö [45], Weiß and Hildebrandt [47], and Elias and Hyde [46]. Motor-driven stages facilitate and standardize the counting procedure, and also improve precision. Besides bone, this procedure is suited to determine any other tissue components [32]. The results refer to percent by volume (% vol.) and offer a better quantification than particle counting. Point counting results for bone or connective tissue are correlated to the corresponding, analytically determined contents of hard bone residue or collagen proteins, respectively, with correlation coefficients around 0.75 [32]. The study of Koolmees et al. [32] also exemplifies the use of point counting to differentiate MSM contents

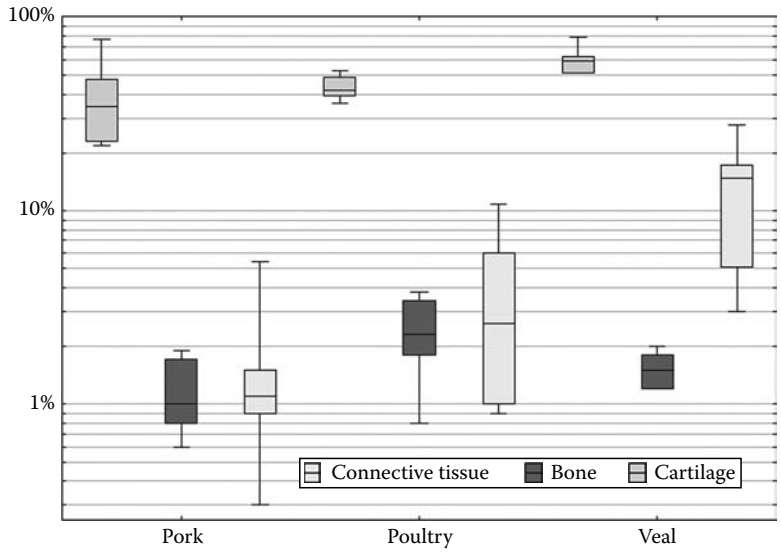


FIGURE 15.6 Particle content (% vol.) determined by histometrical point counting for mechanically deboned pork, poultry, and veal. Boxes are with median and quartiles, whiskers extend from minimum to maximum. Pork and poultry analyses are from seven and six meat plants, respectively, with one sample each; veal analyses are five replicates from one plant. (Original figure from data in Koolmees, P.A. et al., *J. Anim. Sci*. 63, 1830, 1986.)

for various meat types (Figure 15.6). For pork or poultry MSM, bone and cartilage are in the same order of magnitude (mostly <2% or <4%, respectively), while for veal MSM, cartilage contents are generally much higher than bone contents. The highest contents of connective tissue are to be found in veal MSM. For HDM, bone contents lie around 0.5%–0.8% vol. [43].

Video recording of histological sections renders digitized images and offers all possibilities of *computer-aided video image analysis* (VIA). Prerequisite for automated VIA is selective, sharp-contrast staining of tissue components. For bone detection, this can be achieved with Kossa's silver staining or Alizarin Red staining (cf. Section 15.4.2.1). Then, VIA considerably reduces workload due to automation. But its pivotal advantage is the measurement of the complete area of the tissue components in question, compared to the subsampling approach of point counts. Consequently, resultant volume calculations are more reliable. Nowadays, high-quality color video cameras and corresponding commercial analysis programs are available. They offer far more possibilities than the former black-and-white cameras, which are mainly of historical interest. Both approaches are outlined below.

Prerequisite for *black-and-white VIA* are a microscope with movable stage plus a black-and-white camera. The preparation is scanned by moving the stage in x- and y-directions of a given grid (for details, see [10]). Pivotal for black-and-white VIA is an adequate transfer of colors in the histological preparation to well-differentiated gray scale values in the camera image. A continuous monochromatic interference filter is essential to enhance the original color contrasts. Still, the histometric analysis encounters difficulties with typical staining methods for connective tissue. These methods repeatedly produce false-stained spots in the preparation. But Hildebrandt et al. [10] propose viable remedies for this problem that work with various meat products. For bone particles, the situation is far more favorable, especially with silver staining. Deep black bone contrasts superbly with the surrounding tissue that takes light shades of gray, e.g., with Orange G counter staining (cf. [35]). If the histotechnical requirements are observed, black-and-white VIA is an exact method of measurement that provides particle area (in absolute area, e.g., μm^2, or in % of total area), particle number, and the resultant average particle size (e.g., μm^2). Thus, it fulfils the USDA requirements for bone particle characterization (cf. Table 15.1).

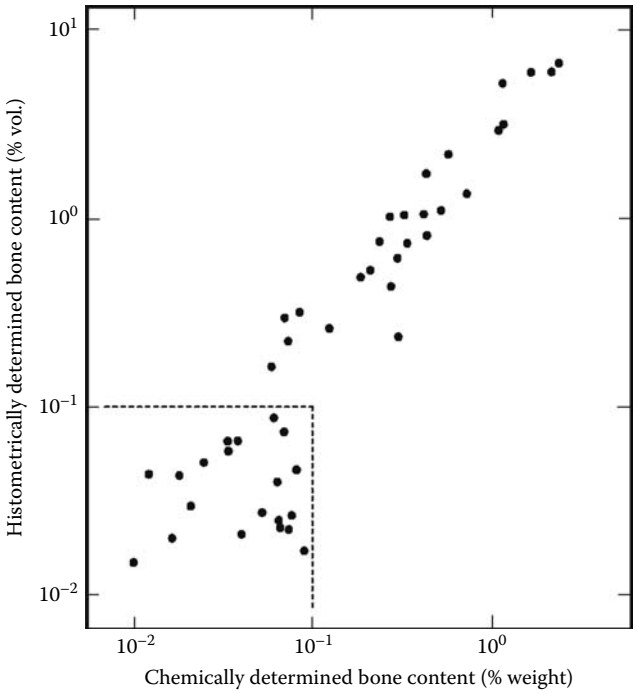


FIGURE 15.7 Relationship between chemical and histometrical determination of bone content in deboned meat or meat products, determined by KOH digestion and by black-and-white video image analysis, respectively. Dashed lines indicate the area of nonlinearity. (Adapted from Hildebrandt, G. and Hirst, L., *J. Food Sci*., 50, 568, 1985. With permission.)

When particle numbers are determined, it has to be decided interactively if adjacent fragments are to be counted as one or as separate particles. Even if not fully reliable staining methods are applied, histometric black-and-white VIA corresponds well with both chemo-physical analysis (atomic absorption spectrometry) and KOH digestion [48,49]. All three methods are highly correlated, with correlation coefficients between 0.97 and 0.98. The histometric method is limited only by low particle contents <0.1%; below this concentration, there is no more linear relation to the chemical analyses (Figure 15.7). This limitation most probably is due to an inhomogeneous distribution of large particles. Inhomogeneity also affects the precision of chemical analyses at low particle concentration, but its effect is attenuated by the larger sample size used with these methods.

The main difference of *color VIA* compared to black-and-white VIA is the camera that transfers the true colors of the preparation into a digital color space. This color space is usually defined in the RGB system, which can be computed to the HSV system (hue, saturation, value) or to the HSI system (hue, saturation, intensity). The HSI system appears to be especially suited for VIA [50]. In the HSI system, hue refers to the color angle between 0° and 360° (where 0° = 360°), while saturation and intensity range from 0% to a maximum of 100%. Color VIA has the advantage to allow automatic clustering of the different color phases that occur in a section. This also allows for a better adaptation to the specific staining conditions of different preparations and enhances the correctness of identifications compared to black-and-white VIA (Figure 15.8). Adjusted phase evaluation can improve the analysis of bone stainings that are pretty distinct. More importantly, it also improves the less distinct trichrome stainings (especially Calleja). Apparently, this can be used for digital analysis of the state of preservation of muscle fibers in MSM [9]. But Tremlova et al. [51] point at difficulties for Calleja staining to determine the fraction of collagen fibers in MSM. In color VIA, Alizarin Red staining is advisable for the detection of bone, in particular, because negative effects of false stainings can better be eliminated for Alizarin Red than for Kossa's silver staining.

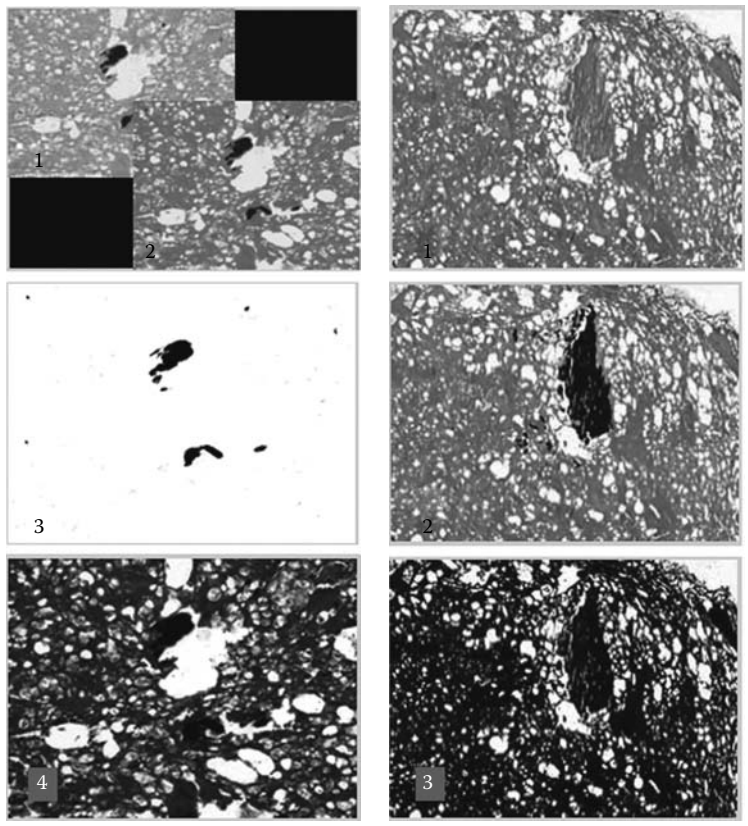


FIGURE 15.8 Outline of video image analysis in black-and-white (A) and in color (B). (A) 1, Kossa stained section; 2, black-and-white image; 3, digital brightening of the non-bone tissue, and determination of the dark bone tissue area; 4, darkening of the non-bone tissue and determination of total dark tissue area. (B) 1, Section stained with Alizarin Red and Indigocarmin; 2, discrimination of the red phase (bone tissue), marking in black, and determination of the marked area; 3, marking of total tissue in black and determination total tissue area. (Adapted from Josefowitz, P. et al., *Fleischwirtschaft*, 87, 122, 2007. With permission.)

As a consequence, Alizarin Red staining yields lower bone particle fractions. Furthermore, silver nitrate impregnation of bone cannot sufficiently take advantage of the potential of the HSI system because black as an achromatic color is difficult to differentiate [7].

Josefowitz et al. [50] extend their digital VIA to measure various criteria for the description of bone particle content:

- Single particle area (μm^2)
- Equivalent circle diameter (μm), i.e., the diameter of a circle with the same area as the particle
- Mean next distance to the proximate particle (μm) as a measure for the tendency to build so-called bone nests
- Particle number (n)

The histometric measurement of bone content based on color VIA corresponds well with chemical calcium determination, even for single values (Figure 15.9). The agreement of methods becomes particularly clear if different MSM origins are compared (Figure 15.10). Bone particle numbers and percent by volume are highly correlated with $r = 0.96$, and they correlate to calcium concentration with $r = 0.92$ and $r = 0.91$, respectively [7].

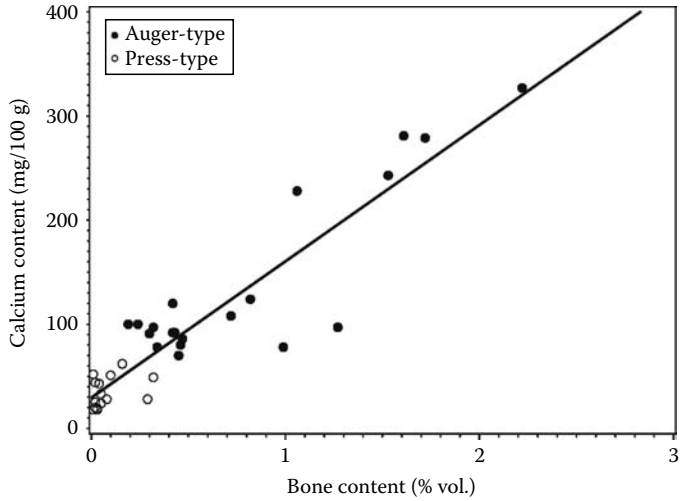


FIGURE 15.9 Relationship between calcium analysis and histometrical determination of bone particle content. Data are for turkey MSM produced with press- or auger-type machines. The straight line is the overall regression Ca (mg/100 g) = 29 + 131 × bone content (% vol.). (Adapted from data in Josefowitz, P., *Histologische, mikrobiologische und chemische Qualitätsmerkmale von Putenseparatorenfleisch*, PhD Dissertation, Freie Universität Berlin, Mensch und Buch Verlag, Berlin, Germany, 2008.)

15.4.3 Morphological and Chemical Isolation of Bone Particles

Bone particles have a distinct tissue structure that allows to separate them well from soft tissues, and to easily isolate them from meat mixtures. There are two approaches to isolate bone particles: by staining or by chemical digestion. Staining has to be followed by manual, macroscopically controlled separation of particles. Chemical digestion of soft tissues allows to segregate bone particles. In either case, recovered particles are simply weighed. Alternatively, the segregated particles can be reduced to ashes, and the remaining ash, or its calcium content, can be quantified. Without any conditioning of samples, bone particles may also be detected by radiologic methods.

15.4.3.1 Histomorphological Gravimetric Detection

Histochemical staining can be applied not only to histological sections, but also to entire organs or small organisms if the staining reaction is sufficiently selective. For many decades, bones have been stained in this manner with Alizarin Red S in order to show fetus deformities in transparent preparations (for a review, see [52]). In these preparations, staining of even minute bone parts is sharp-cut. After staining, soft tissues are cleared by mild KOH saponification and subsequent glycerine impregnation to the extent to be hardly visible any more. Such transparent preparations can still be enhanced by including Alcian Blue staining that selectively shows cartilage tissue [53]. The combined staining with Alizarin Red S and Alcian Blue can be applied to MSM, too. Only, the reactions have to be conducted in test tubes, and liquids have to be exchanged by decantation after short centrifugation (Figure 15.11; [54,55]). Consecutively, separation by forceps and weighing directly quantify bone (originally staining red) and cartilage particles (originally staining blue). If particles are transferred into glycerine, they can be stored permanently, and their numbers or diameters can be determined separately for bone and cartilage. It is advisable to check individual particles microscopically because occasionally mixed particles of bone and cartilage occur, as to be expected anatomically (Figure 15.12). The double staining method has the advantage to require no laborious preparation, and to be realized in just one dye bath fast and without technical difficulties. Only

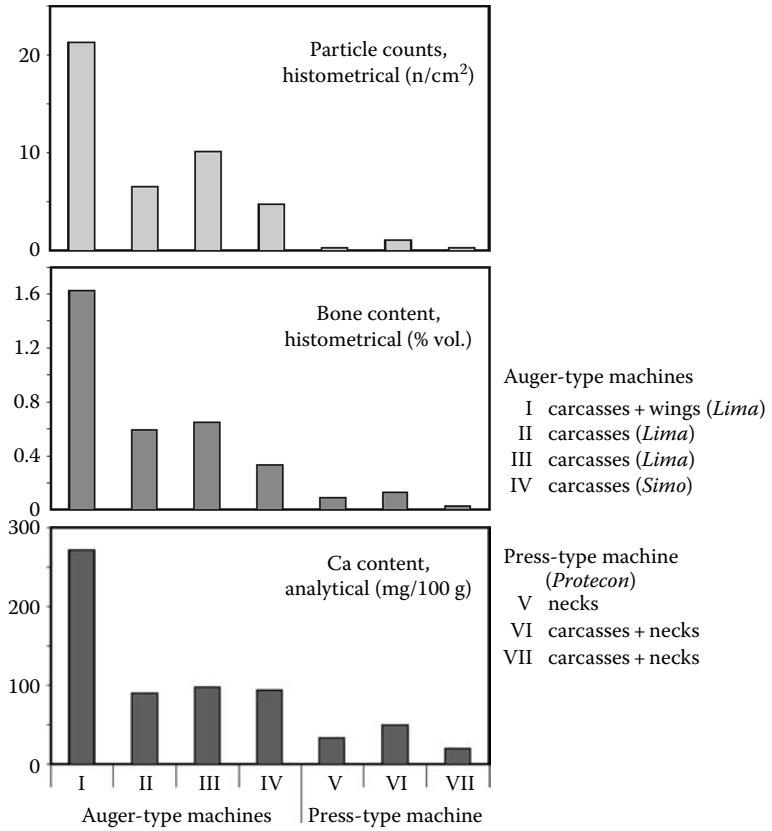


FIGURE 15.10 Analytical methods for bone particle content from turkey MSM produced with different production techniques. Calcium analysis (by inductive coupled plasmaoptical emission spectrometry) is compared to microscopic counting of particle numbers, and to histometrical image analysis. Data are means from five replicate samples of MSM produced by auger-type machines (Lima, Simo) or by a press-type machine (Protecon) from carcasses only (II–IV), from carcasses with wings (I) or with necks (VI–VII), or from necks only (V). (Adapted from data in Josefowitz, P., *Histologische, mikrobiologische und chemische Qualitätsmerkmale von Putenseparatorenfleisch*, PhD Dissertation, Freie Universität Berlin, Mensch und Buch Verlag, Berlin, Germany, 2008.)

particle sorting for weighing takes some time. This histomorphological gravimetry is suited for rather large samples, while the sample quantity that can be analyzed with histological sectioning is inherently very small. Branscheid et al. [55] used weighted samples of 2 g, but this can be increased up to 10 g if particle content is low. Histomorphological gravimetry corresponds well with calcium content if particle content is rather high, as for MSM (Figure 15.13, data point with ca. 300 mg calcium per 100 g, and ca. 800 mg bone per 100 g). For this comparison, calcium content of bone is taken to be 37% (cf. Section 5.1), and calcium determination forms the reference for histomorphological gravimetry. If bone content is below 0.1% wt. (i.e., 37 mg calcium per 100 g), the relation between particle and calcium content is rather erratic (Figure 15.13). This limit for the applicability of histomorphological gravimetry corresponds to the limit for histometrical VIA (cf. Figure 15.7). In either case, the reason is to be found in the combination of small samples and inhomogeneous particle distributions. This inhomogeneity is exemplified by Anhalt et al. [56] who found bone particles in HDM samples at a frequency of only 4%–23% (Table 15.11). Furthermore, particle frequency was not necessarily related to overall (i.e., mean) content (cf. Table 15.11, porcine head and vertebrae). Of course, inhomogeneous distributions pose a problem for any analytic method.

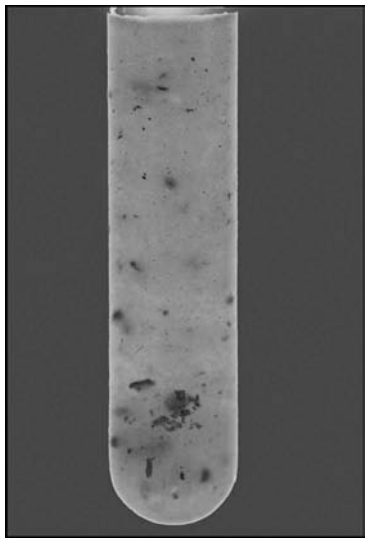
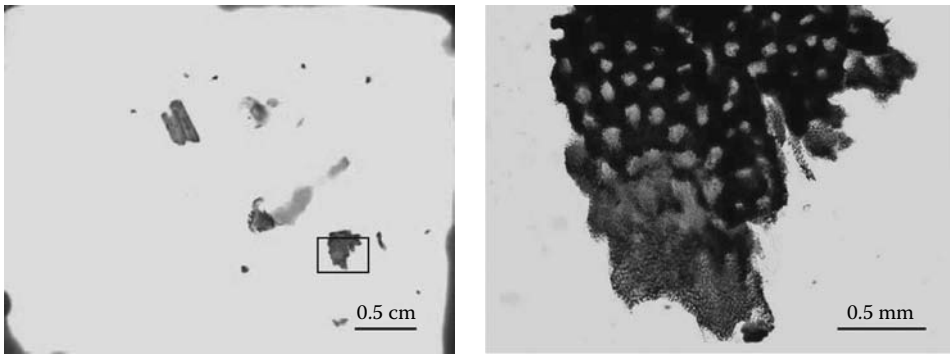


FIGURE 15.11 Histomorphological gravimetric isolation of bone particles (originally staining red) and cartilage particles (originally staining blue) after staining with Alcian Blue and Alizarin Red, presented in 70% ethanol. (From Branscheid, W. et al., *Meat Sci*., 81, 46, 2009. With permission.)



(A) (B)

FIGURE 15.12 Histomorphological gravimetric isolation of bone particles and cartilage particles after staining with Alcian Blue and Alizarin Red, and after clearing with glycerol, embedded on a slide. (A)—overview of the entire sample; (B)—detail of a mixed particle; cartilage–black, bone–light gray (cf. rectangle in (A)). Particle content of the original sample: 0.2% bone and 0.7% cartilage). (Unpublished figures from material described in Branscheid, W. et al., *Fleischwirtschaft*, 11, 106, 2008.)

15.4.3.2 Chemical and Physical Isolation Methods

The mineral and organic fractions can be separated with appropriate reagents. In principle, all methods comprise the isolation and subsequent rinsing as well as drying of residual particles (Table 15.7). The basic difference relates to the medium used for isolation, which can be achieved through digestion with proteases (particularly papain), digestion with a hot alcoholic solution of caustic potash, or sedimentation in saturated salt solutions (Table 15.8). Papain digestion [57] is the most laborious method, but it is frequently recognized as standard. It implies lavation with carbon tetrachloride, a substance that should be avoided with regard to the problem of ozone depletion. The method detects only 80% of bone particles present, obviously because smaller particles get lost during the procedure [2]. Furthermore, papain digestion has the disadvantage that particles agglomerate and counting is impaired. This can be avoided by using mixtures of enzymes (papain

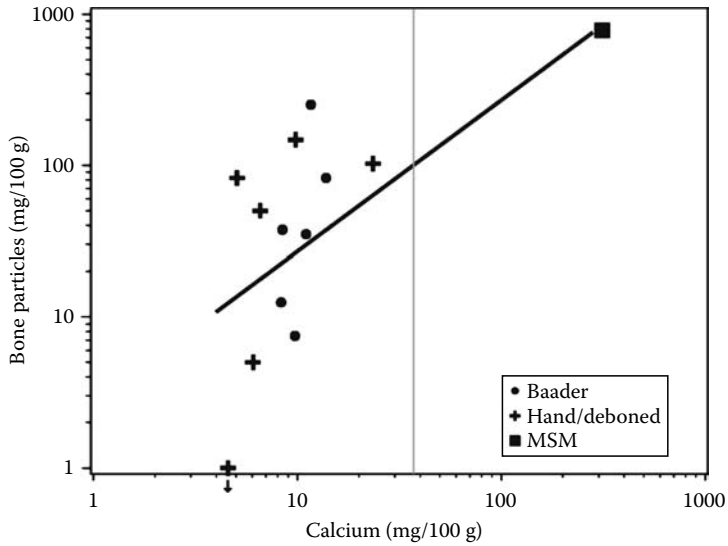


FIGURE 15.13 Relationship between calcium content and bone particle content from broiler meat, produced as hand-deboned minced meat from thigh, as meat separated from wishbone (furcula) with a Baader machine, or as conventional MSM. Values are averaged for two replicate samples each; for hand-deboned and for Baader-deboned meat, data are from two different plants and from three different times during the production cycle. The straight line is the identity for bone with 37% calcium content. The reference line at 37 mg calcium per 100 g corresponds to 0.1% wt. overall bone content. The data point at 1 mg bone per 100 g actually has zero bone content. (Modified from Branscheid, W., *Fleischwirtschaft*, 11, 106, 2008.)

and ficin [58]; papain, ficin and bromelein [59]). Negatu et al. [59] optimized the enzymatic digestion method and improved matches between expected and analyzed bone content data without the use of carbon tetrachloride. Subject to direct comparison, the modified enzymatic method appears to have lower losses than the KOH method or sedimentation methods (Table 15.8). However, an advantage of the KOH method is its most simple performance. Because KOH simultaneously removes protein and fat, no subsequent defattening of particles is required. Froning et al. [58] propose to reduce digestion time significantly to no more than 10 min, which secures the KOH method against material loss and helps to yield satisfactory results under practical conditions. Sedimentation with hot guanidine chloride [60] or in a saturated solution of magnesium sulfate [61] has not been reported in recent literature. But particularly the latter method could be a valuable approach also nowadays because the sediment is available for the analysis of ash and calcium contents (cf. [62]). Overall, results of isolation methods can differ markedly and are difficult to compare [62]. Sinell [63] suggests to combine methods as a mutual check of their validity, exemplified for CCl_4 and KOH separation.

A comparison of isolation methods is often impaired because results are presented in different ways. Some studies report the raw weight of dry isolated particles (e.g., [57–59]). Other studies use bone-type specific conversion factors that have to be determined empirically (e.g., [64,65]). Such factors are used to recalculate fresh bone weight, which allows to present "clean bone" content (Figure 15.14). Bijker et al. [65] make the reservation that a determination of conversion factors for all bone types would require considerable additional research effort. Therefore, results given as hard bone residues should be sufficient. For MSM, they propose that hard bone residue should not exceed 0.4%, with 90% of the particles being less than 1 mm and none greater than 3 mm.

The sediment from physical isolation can by analyzed for calcium (e.g., by atomic absorption spectrometry), if applicable after morphological examination for alien particles. From the calcium content of the sediment, fresh bone content can be calculated [2,60]. Incineration of the sediment is analytically simpler, but it produces good results on the assumption that the entire ash is from bone [63].

TABLE 15.7
Essential Steps of Different Digestion Methods for the Determination of Bone Particles in Meat and Meat Products

Reference	Sample Pre-Treatment	Digestion	Washing and Defattening	Analytics	Measurement
[57]	Autoclave	0.1%–0.25% papain; 37°C	Acetone; carbon tetrachloride and acetone	—	Crude bone (% wet weight)
[101]	—	0.1%–0.25% papain; 37°C	Acetone; carbon tetrachloride + acetone	—	Crude bone (% wet weight)
[59]	—	0.5% papain + 0.5% bromelain + 0.002% ficin; 37°C	Water; autoclaving; water	—	Crude bone (% wet weight)
[63]	Defattening (ether); drying (120°C)	10% KOH in water; boiling for 2 h	Water	Bone ash content (combustion for 20 min)	Bone content (% fat-free dry weight) with empirical correction for non-bone ash
[64]	—	8% KOH in ethanol (96%); Heating at 95°C –100°C for 60–90 min	Acetone	—	Crude bone (% wet weight) calculated by empirical factors (cf. Fig. 15.14)
[58]	—	ca. 7% KOH in ethanol; Boiling for 15 min	Ethanol (50%); acetone; petrol ether (hexane)	—	Crude bone (% wet weight)
[61]	Mincing twice	Saturated $MgSO_4$; Room temperature for 2 h	Water; drying at 105°C for 24 h	—	Crude bone (% wet weight)
[60]	—	Guanidine hydrochloride, concentrated urea + H_2O_2; 100°C–103°C, overnight	Acetone	Ca-content of particles (AAS)	Bone content (% fresh weight) calculated from Ca-content of sediment

From calcium and ash contents, bone content can be recalculated, again with empirical factors (cf. Figure 15.19). But such factors highly depend on the characteristics of the material analyzed. Conversion factors, e.g., to fresh bone weight, may therefore yield results that differ at an order of magnitude. For this reason, Arneth [62] proposes to avoid any conversion to fresh bone, but to refer to the detected bone fraction as dry fat-free bone.

Overall, the methods used for particle isolation are the best if particle dimensions and particle numbers are to be measured. In particular, particle diameter depends on separator type and species provenance of meat (Table 15.9). For the same separator type, MSM from turkey has particles that are a multiple larger than particles from beef MSM. For turkey, particles from HDM are twice as large as from MSM. But the average values for turkey from Table 15.9 may be extreme because, in other studies, the proportions of bone particle size classes vary more between bones within species than between species (namely, poultry, pork, and veal; Table 15.10). Thus, the anatomical origin of bones has a major effect on the composition of the sediment from physical isolation. For a specific type of deboning machine, the fraction of large particles produced is critical. Consequently, in pork, the vertebral, costal, and head bones are more critical than others if a press-type machine is used for

TABLE 15.8
Evaluation of Isolation Methods for Bone Particle Determination: Comparison of Expected and Determined Bone Content

	Bone Content (%)	
	Expected	Determined (Mean [Standard Deviation])
Standard enzymatic method [57]		
Data adapted from Field et al. [2]	3.8	2.9
Material: beef, single samples	4.2	3.3
	4.6	3.5
	4.6	3.9
	5.2	4.0
Modified enzymatic method [59]		
Data from Negatu et al. [59]	1.5	1.1 (0.1)
Material: rabbit meat, single samples	3.0	2.8 (0.2)
	4.5	4.4 (0.3)
	6.0	5.2 (0.4)
Sedimentation method [61]		
Data from Schulze [108]	0.0	0.2 (0.1)
Material: pork, means of 15 samples respectively	1.0	0.8 (0.3)
	2.0	1.3 (0.2)
	4.0	2.7 (0.5)
	7.0	4.8 (0.5)
KOH method [64]		
Data from Schulze [108]	0.0	0.02 (0.02)
Material: pork, means of 15 samples respectively	1.0	0.6 (0.1)
	2.0	1.3 (0.2)
	4.0	2.4 (0.2)
	7.0	4.4 (0.2)

deboning (Table 15.10). Data for poultry and veal point at press-type machines to be generally more critical than auger-type machines (Table 15.10).

Bijker et al. [66] compare particle isolation with the histometric method. Particle size classes >1000 µm are more reliably determined by physical isolation, and particles >2000 µm are virtually entirely missed by the histometric method. One reason may be that, in histological sections, large particles are seldom encountered in a way to identify their full dimension. But, in the end, large particles are crucial for consumer perception. This situation is a severe argument in favor of particle isolation methods.

The inhomogeneous distribution of bone particles within samples poses another fundamental problem [56]. For HDM, which is characterized by low bone particle content, numerous repeated measurements from identical samples yielded bone sediment in only 4%–23% of the measurements (Table 15.11). Consequently, a safe evaluation of bone particle content requires a sufficiently high number of replications. Of course, this holds for other analytic methods, too.

15.4.3.3 Radiologic Detection of Bone Particles

Bone is the classic object of radiologic studies. But even purpose-made devices can detect only rather large particles due to limited resolution. Therefore, radiologic analysis primarily is a screening method that allows to detect those particles that affect sensory quality. For such

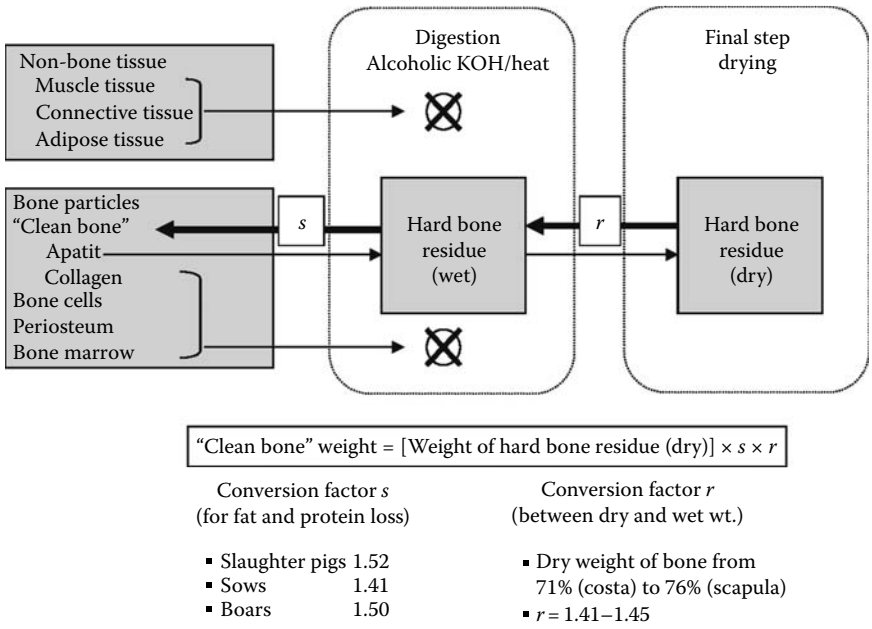


FIGURE 15.14 Calculation of "clean bone" weight from hard bone residue by empirically derived conversion factors, as applied with KOH digestion. (Adapted from Bijker, P.G.H., Methods to determine the bone content and the size of bone particles in mechanically deboned pork M.D.P., *Proceedings of the 25th European Meeting of Meat Research Workers*, Budapest, Hungary, August 27–31, 1979, pp. 843–849, 1979.)

TABLE 15.9
Bone Particle Size from Beef and Turkey Meat Sources

	Diameter (μm)	
	Mean	**Range**
Beef[a] neck MSM[c]	90	10–450
Turkey[b] MSM[c]	410	140–1570
Turkey[b] hand-deboned meat	850	290–2860

[a] Adapted from Field, R.A. et al., *J. Food Sci*., 42, 1406, 1977.
[b] Froning G.W., *Poult Sci*., 58, 1001, 1979.
[c] Beehive mechanical deboner.

screening, qualitative testing is sufficient [38,67]. However, radiologically detected particles can be counted without any problems, and results are comparable if particle numbers refer to sample weight, e.g., particles per 100 g [68,69]. To this end, samples have to be spread in standardized thickness, e.g., 3 mm. Good contrast even of small particles can be achieved with devices adjusted for soft tissue analysis, e.g., in mammography (Figure 15.15; [69]). As an advantage, such devices are preset for digital analysis of samples. They can detect particles >0.1 mm^2, but larger particles are detected more reliably.

Also x-ray computed tomography (CT) is capable to detect bone—and also cartilage—particles (Figure 15.16). Prepared samples with given bone particle contents produce different density spectra

TABLE 15.10
Frequency of Bone Particle Size Classes in MSM from Pork, Poultry, and Veal Bones (% Total Number)

		Bone Particle Size Class			
	Deboner[a]	**100–1000 µm**	**1000–2000 µm**	**2000–3000 µm**	**>3000 µm**
Pork					
Costae, vertebrae	Protecon	91.6	7.0	0.5	0.9
Pelvis, scapula, long bones hind/forelimb	Protecon	95.2	4.3	0.5	—
Heads	Protecon	81.8	16.7	1.3	0.2
Poultry					
Carcasses, backs	Protecon	84.8	11.2	4.0	—
Carcasses, necks	Paoli	96.0	2.0	1.5	0.5
Carcasses, backs, necks, wings	Beehive	97.5	2.5	—	—
Veal					
Femur, tibia, fibula	Protecon	89.0	11.0	—	—
Pelvis, vertebrae	Protecon	92.5	7.5	—	—
Heads	Beehive	100.0	—	—	—
Pork					
KOH-method	N/A	90.6	7.6	1.1	0.6
Histometry	N/A	97.5	2.4	0.1	—

Source: Koolmees, P.A. et al., *J. Anim. Sci*., 63, 1830, 1986; Bijker, P.G.H. et al., *Tijdschrift voor Diergeneeskunde* 105, 440, 1980.

[a] Deboner machines are press-type (Protecon) or auger-type (Paoli, Beehive).

TABLE 15.11
Average Bone Content and Frequency of Bone in Hand Deboned Pork and Beef after Isolation with Saturated $MgSO_4$ Solution

	Average Bone Content (% Fresh wt.)	**Replicate Samples (*n*)**	**Frequency of Samples with Bone (%)**
Porcine			
Head	0.31	290	17
Vertebrae (neck)	0.05	299	18
Forelimb	0.01	298	7
Hindlimb	0.01	299	10
Bovine			
Head	0.07	300	18
Vertebrae (neck)	0.17	297	23
Forelimb	0.00	297	4
Hindlimb	0.01	299	7

Source: Anhalt, G. et al., *Arch. Lebensmittelhygiene*, 28, 202, 1977.

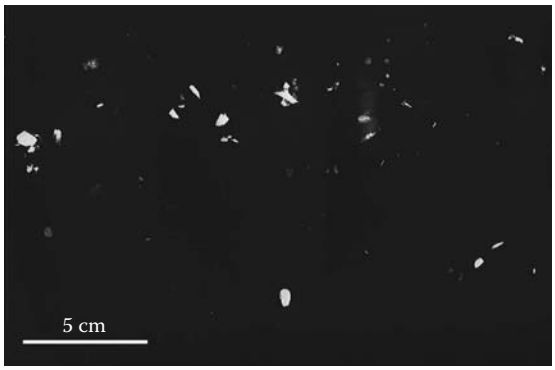


FIGURE 15.15 Radiography of a MSM preparation with numerous large bone particles, taken with a device adjusted for mammography. Bone particles are projected sharply onto photographic film, and particles down to ca. 40 µm diameter can be identified. They can be counted and measured manually or with appropriate software. (From Nitsch, P., *Fleischwirtschaft*, 85, 90, 2005. With permission.)

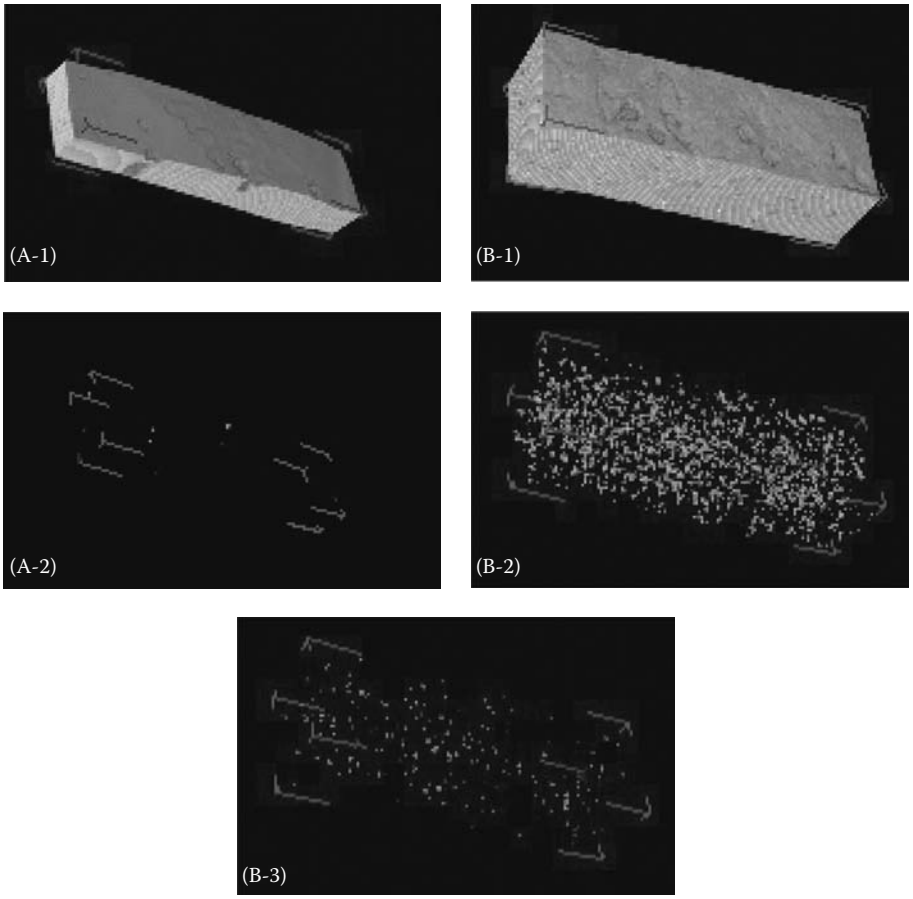


FIGURE 15.16 Detection of bone and cartilage particles by x-ray computed tomography. CT scans were taken of hand-deboned minced meat (A) and of MSM (B), with reconstructions of entire meat samples (1), of voxels filtered at >120 Hounsfield units, which reveals cartilage and bone particles (2), and of voxels filtered at >200 Hounsfield units, which reveals bone particles only (3). Particles can be counted and measured at the voxel resolution of $0.25 \times 0.25 \times 1\,mm^3$. (Unpublished figures from material described in Branscheid, W. et al., *Mitteilungsblatt Fleischforschung Kulmbach*, 46, 175, 2007.)

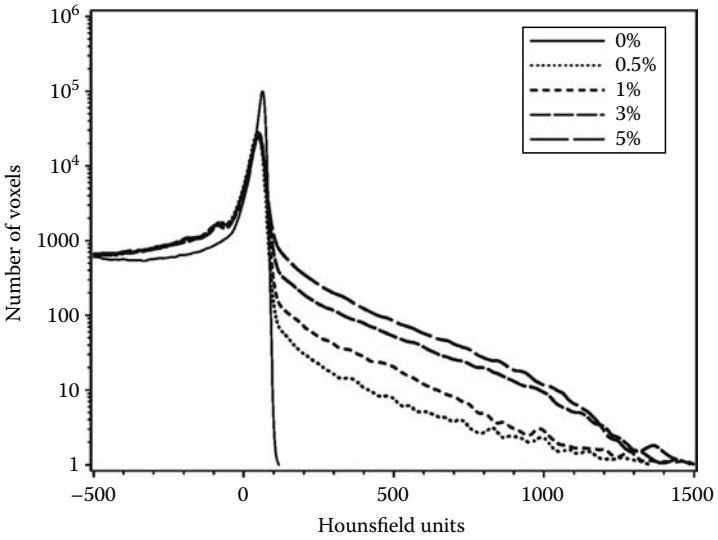


FIGURE 15.17 Quantitative analysis of bone particle content from prepared minced meat samples by x-ray computed tomography. Lines show the frequency distribution of voxels with specific Hounsfield units (HU) for pure minced meat, and for minced meat mixed with 0.5%–5.0% wt additions of finely ground bone. HU values increase with tissue density and correspond to grayscale values of CT images. Pure meat volume corresponds to the peaks around ca. 70 HU. Bone particle volume can be quantified by the total volume >120 HU, the maximum for pure minced meat. Samples were 100 g for pure minced meat, and 50 g for samples mixed with bone particles. (Modified from Branscheid, W. et al., *Mitteilungsblatt Fleischforschung Kulmbach*, 46, 175, 2007.)

of CT scanned samples (Figure 15.17). The larger the particle content, the larger the proportion of voxels with high Hounsfield units, i.e., high density, in the sample. For known meat–bone mixtures, CT analysis can be calibrated and yields precise estimates of bone content in % weight [54]. But it is not generally possible to apply this method for unknown meat–bone mixtures because CT analysis suffers from the problem of mixed voxels, which may have different effects on CT spectra in different mixtures. In this context, Branscheid et al. [54] propose to improve the spatial resolution of CT scans to well below 0.25 mm in order to come along with quantitative bone particle detection.

15.5 INDIRECT DETECTION OF BONE COMPONENTS IN MEAT

Indirect methods to detect bone do not identify bone as a physical substrate. Instead, they analyze for chemical components of bone (ash, calcium) or of collateral tissue (bone marrow, spinal cord). These components also occur in other tissue types, for which reason, it may be difficult to determine, in particular cases, which fraction actually comes from bone. On the other hand, the advantage of indirect analytic methods is to provide immediate quantitative results that have a low methodological error. As for direct detection methods, indirect methods differ in their applicability to the various tissue components (Table 15.12). Analytically, most important are not only mineralized components of bone but also bone marrow and its characteristic components (minerals, proteins, nucleotides). Tissue of the central nervous system (CNS), in particular from the spinal cord, can be used as an indicator of MSM from the spine. Following the bovine spongiform encephalopathy (BSE) epidemic, CNS tissue, particularly from beef cattle, has become a major concern for product safety and consumer protection. Consequently, both in the United States and in the EU, MSM from beef spines is not allowed for human consumption.

In the following, indirect analytic techniques are not detailed because—contrary to direct methods—they are not specific for meat analysis. Furthermore, these methods are hardly ever under technical consideration. What concerns here are problems caused by physiological variability of

TABLE 15.12
Indirect Detection of Bone Particles in Meat

	Detection Method[a]	References[b]
Mineralized components		[76,79]
Calcium (also as ash)	AAS; ICP-OES; ICP-MS	[8,48,103,110,114]
Bone marrow		[34]
Iron	AAS	[82,86]
Hemoglobin	CGE	[87]
Purin bases including pyrimidines	Column chromatography; HPLC	[82,85,90]
pH	pH-meter	[77,115,116]
Protein profile	Gel electrophoresis; SDS-PAGE; IEF	[85,125]
Antibodies	ELISA; Polyclonal antiserum against a specific marrow cell protein	[88]
Tissue of central nervous system		
Antibodies–polyclonal antiserum against neuron-specific enolase or glial fibrillary acidic protein	ELISA; Monoclonal antibodies	[91–94]

[a] Abbreviations: AAS, atomic absorption spectrometry; CGE, capillary gel electrophoresis; ELISA, enzyme-linked immunoabsorbent assay; HPLC, high-performance liquid chromatography; ICP-OES, inductive coupled plasma optical emission spectrometry; ICP-MS, inductive coupled plasma mass spectrometry; IEF, isoelectric focusing; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis.

[b] Reviews are in bold.

the components under study (e.g., calcium), or by external intrusion (cf. [42]). For the analysis of meat and meat products, approved routine methods are available for laboratory practice (for general compilations, see [70,71]). Calcium analysis is of special concern and has repeatedly been described in detail [72–75].

15.5.1 Analysis for Calcium and Ash Content of MSM

Bone has a high content of hydroxylapatite, and hence of calcium and ash, too (Table 15.13). For mammal species (cattle, pig, sheep), ash content of dry fat-free bone ranges between 50% and 60%, the corresponding calcium content between 19% and 24%. For poultry, the respective values are always some percentage points lower. For all animal species, ash and calcium contents increase considerably with age. Also, bone type has a major effect: femurs and other long bones have particularly high contents, while small bones have rather low ones. If calcium is related to bone ash, this calcium content of ash is remarkably constant at 36.5%–38.5%, which gives an overall average of 37%. The standard error within animal species is largely no more than 1%. This constancy led Arneth [62] to define calcium content of bone ash as most adequate for the presentation of calcium analysis results. In North American studies, calcium content is mostly related to dry fat-free bone, which is likewise adequate. It is less useful to relate calcium content to fresh bone weight, which varies more than dry bone weight and may be biased by preparation. Surprisingly, in many studies, it is not evident from the description of methods what calcium content is related to.

In MSM, bone with high mineral content is mixed into raw meat that is poor in calcium but not calcium free. The range of calcium content in pure muscle tissue is rather wide, both within and between species (Table 15.14). On average, it ranges from 4 mg/100 g to 15 mg/100 g, with extreme values up to 37 mg/100 g (ground beef samples) and down to non-detectable amounts (specific pork

TABLE 15.13
Composition of Bovine, Porcine, Ovine, Chicken, and Turkey Bones from Animals of Different Age Classes and for Different Bone Types

	Dry Matter (% of Fresh Bone)[a]	Composition of Dry Fat-Free Bone (%)[a]			Calcium in Bone Ash (%)[a]
		Ash	Calcium	Hydroxyproline	
Bovine					
Age in months					
2–3	44.9 (1.4)	49.8 (0.9)	18.4 (0.4)	3.7 (0.1)	36.9 (0.0)
12–24	68.0 (1.2)	56.3 (0.7)	20.8 (0.3)	3.9 (0.1)	36.9 (0.3)
48–96	73.0 (1.4)	63.9 (0.9)	23.9 (0.4)	4.0 (0.1)	37.3 (0.4)
Bone					
Lumbar	58.4	55.2	20.4	4.0	37.0
Cervical	60.4	53.4	19.5	3.8	36.5
Ribs	62.6	58.9	22.1	3.9	37.5
Femur	66.2	59.2	22.1	3.7	37.3
	(1.5)	(1.0)	(0.4)	(0.1)	(0.4)
Porcine					
Age in months					
6–8	63.4	52.5	19.3	3.9	36.7
24–48	74.7	57.3	22.2	3.9	38.6
	(0.7)	(0.6)	(0.3)	(0.1)	(0.3)
Bone					
Lumbar	68.7	53.9	20.2	3.8	37.4
Cervical	66.1	52.9	20.0	4.1	37.6
Ribs	67.5	54.9	20.4	3.9	37.3
Femur	74.2	57.8	22.3	3.7	38.4
	(1.0)	(0.8)	(0.4)	(0.1)	(0.5)
Ovine					
Age in months					
5–6	60.7	54.0	20.3	3.7	37.6
10–12	67.3	57.2	21.5	3.8	37.6
48–96	77.5	62.9	24.1	3.6	38.4
	(0.8)	(0.7)	(0.3)	(0.1)	(0.3)
Bone					
Lumbar	66.8	56.0	21.1	3.8	37.8
Cervical	62.8	55.6	21.3	3.8	38.2
Ribs	68.5	58.0	21.8	3.6	37.6
Femur	75.8	62.4	23.7	3.7	38.0
	(1.0)	(0.8)	(0.4)	(0.1)	(0.4)
Chicken					
Age in months					
2–3	49.9	47.0	17.5	3.8	37.3
12–13	61.0	54.7	21.0	3.5	38.4
	(0.9)	(0.6)	(0.3)	(0.1)	(0.7)
Bone					
Synsacrum	52.2	47.6	17.8	3.7	37.2
Cervical	54.8	52.9	20.1	4.0	37.9
Ribs	58.3	50.3	18.7	3.7	37.2
Femur	56.4	52.6	20.6	3.3	39.1
	(1.2)	(0.9)	(0.5)	(0.1)	(0.9)

TABLE 15.13 (continued)
Composition of Bovine, Porcine, Ovine, Chicken, and Turkey Bones from Animals of Different Age Classes and for Different Bone Types

	Dry Matter (% of Fresh Bone)[a]	Composition of Dry Fat-Free Bone (%)[a]			Calcium in Bone Ash (%)[a]
		Ash	Calcium	Hydroxyproline	
Turkey					
Age in months					
5–6	54.1	54.5	20.4	4.2	37.5
12–13	59.6	55.4	21.3	3.6	38.6
	(0.8)	(0.6)	(0.3)	(0.1)	(0.3)
Bone					
Synsacrum	53.2	54.3	20.6	4.0	38.0
Cervical	58.8	56,0	21.4	4.1	38.2
Ribs	55.6	50.2	19.1	4.0	37,9
Femur	59.8	59.1	22.5	3.3	38.1
	(1.1)	(0.9)	(0.4)	(0.2)	(0.4)

Source: Adapted from Field, R.A. et al., *J. Anim. Sci*., 39, 493, 1974.

[a] Least squares means and (*standard errors*).

muscles). Consequently, it is problematic to define limiting values of physiological calcium content that would allow to differentiate technically unavoidable muscle calcium from elevated calcium content due to bone particles. Stenzel and Hildebrandt [76] evaluated deboned meat cuts and minced meat to derive an empirical limiting value. They propose 20 mg/100 g (i.e., 200 ppm) as a threshold to indicate MSM because 92%–98% of beef and pork samples had calcium contents <20 mg/100 g (Table 15.15). For poultry, all of a small number of samples had <15 mg/100 g. But at least for beef and pork, these results point at a problem in defining a limiting value: a certain rate of false-positive MSM indication has to be taken into account. In the case of beef cuts, this amounts to 8% non-MSM samples with >20 mg/100 g (cf. Table 15.15). On the other hand, there is the problem of false-negative results. In an interlaboratory test, not all meat preparations exceeded the limiting value of 20 mg/100 g, even when high amounts of MSM had been added (Figure 15.18). In spite of these caveats, and in view of the available data (Table 15.14), a limiting value of 20 mg/100 g for technically unavoidable calcium content of raw meat appears to be a practicable approach [76]. For processed meat products, a considerably higher limiting value (e.g., 35 mg/100 g [76]) would be applicable because a number of materials added to meat products have substantial calcium contents [60,62]. Regulation (EC) 853/2004 (annex II, sect. V, chapt. III, No. 3) prescribes that MSM I calcium content must not noticeably exceed that of minced meat (cf. Figure 15.1). Consequently, a limiting value in the order of magnitude of 20 mg/100 g would be applicable for MSM I. But in fact, regulation (EC) 2074/2005 (annex IV, No. 1) prescribes a limiting value of 100 mg/100 g for MSM I. This results in a large tolerance for technically unavoidable compared to physiological calcium content. Even large MSM additions cannot be detected with this limit (cf. Figure 15.18). U.S. regulations set still higher limits (cf. Table 15.1). For AMR, the product of which is similar to minced meat and needs not to be declared, the limit for calcium is 150 mg/100 g. Novel separation methods easily comply with this value [77]. For MSM as defined by U.S. regulations, the limits for calcium content are much higher, yet, depending on the species of origin. This MSM has only limited usability and has to be declared correspondingly (cf. Table 15.1).

However, benchmark for MSM is not fresh meat taken from primal cuts but material produced by hand deboning. This has somewhat elevated calcium contents compared to fresh meat. But HDM is mostly far exceeded by MSM (Table 15.16). Depending on separation methods, calcium content of

TABLE 15.14
Calcium Content in Fresh Meat from Various Species

	Ca Content (mg/100 g)[a]	Reference
Beef		
Muscles only	4.7 (3.9–5.6)	[117]
9 muscles	4 (1–13)	[118]
Separable lean with fat	8	[119][c]
Regular ground beef	8–37	[34]
Muscles only	5.7 (4.0–5.9)	[120]
Muscle	5.7	[121]
Veal		
7 muscles	6 (2–17)	[118]
Muscle only	13 (11–14)	[120]
Pork		
10 muscles	5 (0[b]–34)	[118]
Separable lean with fat	5	[121][c]
Muscle only	5.1 (4.1–6.2)	[120]
Muscle	5	[124]
Chicken		
Flesh and skin	11	[122][c]
Chicken for roasting	14	[120]
Breast and thigh muscle	5.6–6.7	[114]
Turkey		
Flesh and skin	15	[122][c]

[a] Means and ranges.
[b] Below detection limit.
[c] Means and ranges.

TABLE 15.15
Frequency of Low, Medium, and High Calcium Content in Meat Cuts and Minced Meat from Various Species

		Frequency (%) of Calcium Content		
	N	<15 mg/100 g	15–20 mg/100 g	>20 mg/100 g
Meat cuts				
Beef	83	70	22	8
Pork	230	96	3	1
Minced meat				
Pork/beef	101	88	8	4
Chicken	16	100	—	—
Turkey	3	100	—	—

Source: Adapted from Stenzel, W.-R. and Hildebrandt, G., *Fleischwirtschaft*, 86, 96, 2006.

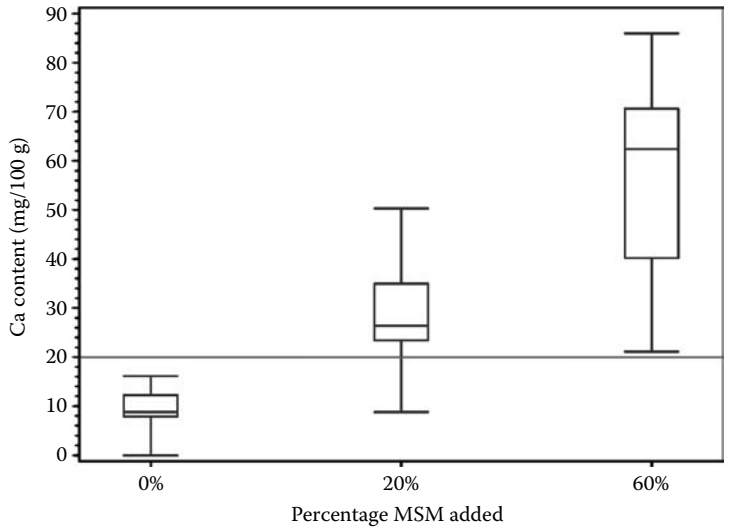


FIGURE 15.18 Analytical determination of calcium content from samples with known additions of MSM. Results are from an interlaboratory test with 13 participants. (Adapted from Schulte-Sutrum, M. and Horn, D., Eignungsprüfung Lebensmittelhistologie/Nachweis von Separatorenfleisch, *Protokoll der 55. Arbeitstagung des Arbeitskreises Lebensmittelhygienischer Tierärztlicher Sachverständigen ALTS*, 113–125, 2002.)

MSM can be 50- or 100-fold compared to HDM. Contrariwise, for low-pressure methods, the difference can be insignificant. MSM calcium content depends on the pressure used for separation, and thus on the concomitant meat yield [78]. Not surprisingly, increased calcium content is associated with an increased content of palpable bone particles. After all, this is the background for the good agreement of calcium analysis and histometric methods (cf. Figure 15.10). In summary, calcium content is a useful criterion to differentiate between MSM and fresh meat. Only low-pressure separation methods (like Baader machines) and adjusted mixtures of MSM and fresh meat cannot be detected for sure. Therefore, U.S. regulations follow a pragmatic approach and, from the first, do not regulate products with calcium contents less than 150 mg/100 g any further, while they extensively account for morphological characteristics of bone particles (cf. Table 15.1).

Also, ash content can be used for MSM detection because calcium is a rather constant fraction of bone ash (37%, cf. Table 15.13). Ash analysis is far less laborious and, thus, is suited for less-equipped laboratories as an alternative to calcium analysis. The intercorrelations of bone, ash, and calcium contents have been analyzed by Field [79], who proposes respective conversion factors (Figure 15.19). These factors are also suited to approximately convert bone contents that were determined directly (cf. Section 15.4) to ash or calcium contents. But for exact results, it is necessary to determine specific conversion factors adapted to the respective material. Generally, only calcium content is used as a parameter for the indirect determination of bone content, although ample information is available for ash content of fresh meat, MSM, and HDM ([56,62,80–82]; reviewed by [79]). A key factor for this preference is legislation that exclusively refers to calcium. But also methodological aspects argue for a preference of calcium rather than ash analysis for the determination of MSM bone content. In pure muscle tissue, calcium content generally does not exceed 0.008% (Table 15.14), while ash content is in the order of magnitude of 1% [79]. The relation of calcium content in lean meat compared to fresh bone is 0.008:22 (i.e., 1:2800), the respective relation of ash content is 1:60 [79]. Consequently, variation in meat calcium does not affect overall calcium content, while variation in meat ash may have an effect on overall ash content. Hence, calcium analysis is better suited than ash analysis for the detection of MSM bone particles in meat mixtures [79]. Therefore, preference should be given to calcium analysis despite the simplicity of ash analysis.

TABLE 15.16
Calcium Content in MSM of Various Species and Parts, Compared to the Respective Hand Deboned Meat

	Calcium Content (mg/100 g Fresh Weight)[a]					
	Hand Deboned		MSM		Separator[b]	Reference
Beef						
Neck	56		1060		Bee	[81]
Neck bone	24	(2)	72	(2)	BFD	[77]
Neck bone	24	(2)	73	(1)	DMM	[77]
Neck bone	21	(2)	80	(1)	DMM	[77]
Plate	51		1490		Bee	[81]
Leg	28		269	(204–306)	MRS	[124]
Shoulder	30		369	(289–419)	MRS	[123]
Head	109		508	(398–621)	MRS	[123]
Aitch bone	38		88	(20)	Pro	[82]
Beef (*cow*)						
Rib, plate	13		1550		Bee	[81]
Rump	83		1550		Bee	[81]
Short loin	14		1500		Bee	[81]
Veal						
Shoulder	35		1760		Bee	[81]
Frames	45		710		Bee	[81]
Backs	42		540		Bee	[81]
Pork						
Ham	29		1390		Bee	[81]
Picnic	43		1220		Bee	[81]
Boston butt	79		730		Bee	[81]
Leg	26		121	(85–158)	MRS	[123]
Head	78		183	(87–291)	MRS	[123]
Shoulder	27		152	(139–191)	MRS	[123]
Shoulder	55		70	(6)	Pro	[82]
Chines	242		206	(31)	Pro	[82]
Pork (*sow*)						
Loin	37		410		Bee	[81]
Lamb						
Neck and ribs	30		33	(6)	Pro	[82]
Chicken						
Breast	18		113	(106–124)	MRS	[123]
Neck	28		139	(125–158)	MRS	[123]
Backs	54		84	(16)	Pro	[82]
Back parts	16		36		Aug	[114]
Back parts	16		25		Baa	[114]
Breast parts	31		122		Aug	[114]
Breast parts	31		34		Baa	[114]

TABLE 15.16 (continued)
Calcium Content in MSM of Various Species and Parts, Compared to the Respective Hand Deboned Meat

	Calcium Content (mg/100 g Fresh Weight)[a]				
	Hand Deboned	MSM		Separator[b]	Reference
Turkey					
Large frames	20	44	(8)	Pro	[82]

Note: Baa, Baader machine; Bee, Beehive Mechanical Deboner—0.46 mm holes (auger-type); BFD, Protecon, Model BFD (Townsend)—166 bar of pressure; DMM, Desinewated Minced Meat DMM 10 machine (Townsend)—95 bar of pressure; MRS, Seffelaar and Looyen BV unit, type MRS 40; Pro, Protecon (press-type).

[a] Mean and (standard deviation) or (minimum–maximum).

[b] Aug: Auger-type deboner (unspecified).

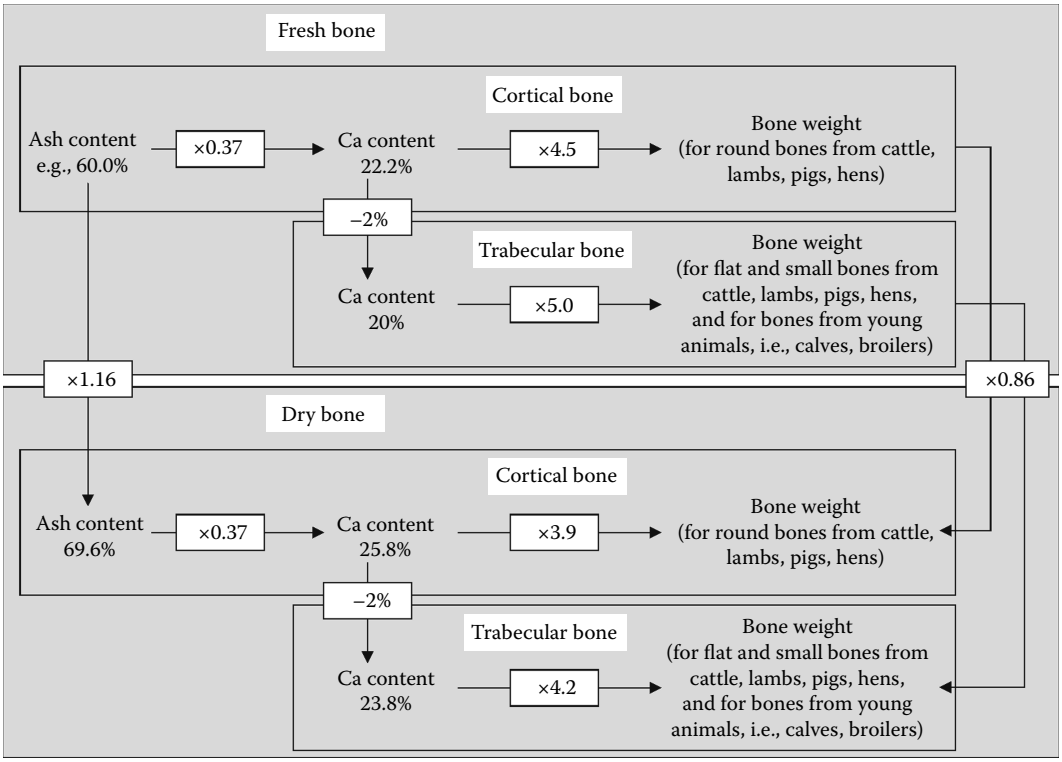


FIGURE 15.19 Conversions between bone weight and ash or calcium content in fresh or dry bone, differentiated for cortical and trabecular bone. Contents are given for a realistic example of 60.0% ash content measured from fresh bone. Example application of conversion factors: the official USDA limit for Ca content from advanced meat recovery is 0.15% Ca content; this corresponds to 0.15% × 5.0 = 0.75% wt as a limit for fresh trabecular bone content. Fresh-to-dry conversion factors for ash content and for bone weights follow from average 13.8% moisture of fresh bones. (Adapted from Field, R.A., *Meat Sci*., 55, 255, 2000.)

15.5.2 Analysis for Collateral Tissue Types

Only bone marrow and parts of the central nervous system that come from the spinal cord are relevant for indirect detection of bone (Table 15.12). In practice, major interest is in bone marrow whose skeletal occurrence has been described above (cf. Section 15.3).

15.5.2.1 Bone Marrow

Some characteristics of bone marrow provide an opportunity for identification even if it is diluted in meat mixtures. Basically, suitable characteristics are the elevated ferreous content, immunospecific proteins, and nucleic acids and their associated purines.

Preeminent is the ferreous content, because iron is accumulated in hematopoetic red marrow for the formation of erythrocytes, but ferreous content is not elevated in yellow marrow containing mainly fat cells. Red bone marrow proper has 13–23 mg iron per 100 g fresh weight, typical commercial beef MSM has 4.3–6.6 mg/100 g, and hand-deboned beef meat or minced meat have only 1.2–3.1 mg/100 g [80,82–85]. Values for pork are comparable [86]. Ferreous content of poultry is lower, with values down to 1.5 mg/100 g, which hardly differ from HDM [82,85]. As to be expected, most of the ferreous content of MSM is heme iron [86]. But to sum up the available data, ferreous content hardly qualifies for a reliable detection of bone tissue or MSM because differences between the means for MSM and HDM are too variable and too small. Ferreous content is rather a quality criterion that indicates dietary value as a source of iron. It may also indicate a reduced shelf life of the raw product because iron catalyzes fat oxidation and deterioration [86]. The rationale for iron analysis also applies to hemoglobin, which can be analyzed, e.g., with gel electrophoresis. Hemoglobin relations to muscle proteins (i.e., myoglobin, actin, myosin) appear to be most informative [87]. But no convincing results have been obtained with methods based on this relation [85]. Field et al. [84] point at an age effect on iron and hemoglobin contents, which are increased in elderly animals and interfere with the respective analyses.

Bone marrow from MSM is also associated with other characteristic components. Specific proteins can be identified by immunological methods [88] or by electrophoresis [127]. Furthermore, nucleic acids can be analyzed (reviewed by [34]), or purines that have high contents in proliferating bone marrow [85,89]. But all these methods are affected by disturbing factors that interfere with unequivocal detection of bone. This is exemplified by purines that are not only released under pressure from bone marrow but come from muscle tissue, too [90]. Thus, bone detection through purine analysis alone is hampered and requires additional information or analyses.

15.5.2.2 Central Nervous System

Fractions of the CNS can be found in MSM if vertebrae have been deboned. These come from the spinal cord and adjoining spinal ganglia. Because of this contamination, vertebral columns from beef are legally not allowed for processing in the United States or in the EU (cf. [91]). Therefore, CNS analysis in beef is relevant exclusively for hygienic reasons. For pork, CNS analysis can be used to indirectly identify spinal bone particles [92,93]. There are two methodological approaches to CNS identification: cholesterol analysis and immunological analysis, both reviewed by Lücker et al. [91].

CNS has a high content of *cholesterol* that can still be detected if diluted in MSM. Cutoff points for CNS proof in meat products are 115 mg/100 g for emulsion-type sausages, and 181 mg/100 g for liver sausages [91]. These cutoff points provide a high reliability, but sensitivity is rather low. The analytical detection limit for additions of nervous tissue to meat products is 0.5%. This makes the cholesterol method primarily suited for screening [91]. Results are biased if high-cholesterol additives, e.g., yolk, are used for the meat products. Incidentally, also bone marrow has relatively high cholesterol content, which may elevate MSM cholesterol content considerably depending on the type of separated bones (cf. [34] for a summary).

Immunological analysis for CNS is more sensitive and more specific than cholesterol analysis. Both neuron-specific enolase and the glial fibrillary acidic protein are suitable substrates, but the latter has a somewhat higher sensitivity [91]. Nowadays, effective commercial analysis kits are available for glial fibrillary acidic protein detection [92–94]. But CNS detection is not specific for vertebral bone in the case of sausages for which the use of brain is permitted under certain conditions. Furthermore, immunoreactivity declines rapidly with the duration of heating [91]. In cooked sausages, glial fibrillary protein detection is possible only if, during thermal treatment, the F-value did not substantially exceed 0.4 [92]. While in cooked sausages ("Kochwurst"), the limit of detection of nervous tissue, based on glial fibrillary protein detection, is around 0.1%; it is 0.01% in boiled sausages ("Brühwurst"). Test kits are more responsive to spinal cord tissue than to brain, hence detection limits may be considerably lower than 0.1% as reported by the manufacturer [93,94]. The content of glial fibrillary protein is directly proportional to the content of MSM added [93]. Therefore, MSM detection via glial fibrillary protein analysis may be safer than direct determination of bone particles.

15.6 BONE IN MEAT—LITTLE MORE THAN THE NEEDLE IN THE HAYSTACK

Bone detection in meat mixtures has two objectives that are fundamentally different. On the one hand, it serves as a control tool against consumer deception if food contains undeclared MSM. To this end, detection methods must allow exact quantification because inspection authorities take action if limiting values for bone content have been violated. Approved methods are calcium analysis, histologic bone detection, and bone particle isolation through digestion or staining. If bone content is rather high, all these methods give comparable results, as evidenced by comparative trials. If bone content is low, an appropriate analysis has to be chosen that allows the analysis of sufficiently large samples to overcome the problem of inhomogeneous particle distributions in meat mixtures.

On the other hand, bone detection serves as a tool for sanitary consumer protection to safeguard against food production with MSM from risky bone material. In this context, particularly beef vertebral columns are of major concern, as evidenced by the BSE crises. Ultimately, bone detection is an instrument to prevent food contamination with presumed infectious agents, namely prions. Thus, bone detection is not an end in itself but a means to identify critical collateral tissues. This does not require a comparison with limiting values but a fundamental yes-or-no decision if the critical tissue is present or not. This procedure is exemplified by the most important identification of nervous tissue from the spinal cord, which can be realized by immunological methods with high sensitivity. Simultaneously, this method can also be regarded as a highly sensitive, qualitative proof of vertebral bone that is little affected by inhomogeneous particle distributions.

To sum up, bone detection in meat samples resembles a search for the needle in the haystack. As for the needle, there is no full reliability to find bone particles that are present. But if there is an encounter, the effect is likewise resounding: one large bone particle, whether from hand-deboned meat or from MSM, drastically alters the calcium content of a meat sample. Contrariwise, modern deboning techniques, like the Baader machine, are so far improved that the separated meat cannot be distinguished from fresh meat, whether by bone content or by other criteria, e.g., morphology of muscle fibers or microbiological hygiene (cf. [8]). U.S. legislation accounts for such technological improvement by allowing the use of MSM for meat products, without limitation and without an obligation of declaration, as long as it complies with the respective limiting values (cf. Table 15.1). This is the reasonable, formal consequence of the fact that limitations and controls only work if coherent evidence is feasible. In the case of bone detection, only high contents provide court-proof conclusiveness. This puts clear limits to all methods described here. MSM addition may be suspected only if natural contents of analyzed substrates are exceeded. In the case of calcium content, natural limits can be specified at 20 mg/100 g wet weight for meat samples, and at 35 mg/100 g wet weight for meat products (cf. [76]). EU and U.S. regulations considerably exceed these natural limits. The official

calcium limits of 100 and 150 mg/100 g, respectively, work reliably also with methods other than calcium analysis. At this order of magnitude, error introduced through the conversion from direct bone detection to calcium analysis has hardly any consequences, even if no effort is made to determine specific conversion factors. Consequently, the original objective of bone detection, namely identification of MSM, can be achieved by various methods of equal value. Direct detection methods simply have the advantage to be more concrete. But none of the methods described stand out as an unequivocal "gold standard."

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16 Detection of Adulterations: Identification of Animal Species

Johannes A. Lenstra and Stephan A. van der Heijden

CONTENTS

16.1 INTRODUCTION

Identification of the species origin of animal products is important for several reasons. First, consumers are to be protected against economic fraud by partial or complete substitution of the declared species with material of cheaper origin. Second, religious rules forbid the use of material from certain species, such as pork for Jews or Muslims and beef for Hindus. Third, unintended consumption of material from certain species may cause allergic or other idiosyncratic reactions that affect the health of susceptible individuals.

The development of methods for species identification has become a most active field of research, with now a heavy emphasis on DNA analysis. Table 16.1 gives a short description of the current DNA methodology. Previous reviews of species identification are summarized in Table 16.2. Here, we review and evaluate the most recent advances. We describe new methodologies, provide a list of the several recently developed dedicated methods, and mention current trends. In addition, molecular-genetic approaches to traceability at the level of domestic breeds are discussed.

The DNA sequence contained in an animal or plant genome does not depend on the tissue and survives most treatments inherent to food manufacturing and preparation. Therefore, the same analysis methods may be applicable to fresh tissue, blood, dairy products, other edible by-products, and even to highly processed feedstuff.

16.2 ADVANCES IN METHODOLOGY

Although the vast majority of methods are now DNA based, ELISA or other immunochemical methods may still have the advantage of convenience.[1–4] However, antibodies with the required species specificity are not always available. A bioinformatical approach has been used for the selection of tissue-specific peptide sequences in order to generate monoclonal antibodies.[5]

TABLE 16.1
DNA Methods Currently Used for Species Identification

Method	Description
Microarray	Miniaturized array of DNA spots, usually on glass slides. If used for species detection, each spot contains DNA specific for a given species. Binding of homologous DNA from the sample to one of more DNA spots generates a fluorescent signal that identifies the species origin of the sample.
Microsatellite	(Also SSP for simple-sequence polymorphism). A tandem repeat of 2–5 nucleotides as the sequence CACACA…CA. Such sequences occur at several positions in the DNA of most eukaryotes. Since the number of repeat unit for is many microsatellite loci variable within a species, the length of a PCR fragment containing a microsatellite is also polymorphic. Amplification of about 12 different microsatellite creates for each individual a unique DNA profile, which is now commonly used for forensic purposes. Microsatellite profiles also allow the assignment of an individual profile to a population or animal breed.
Multiplex PCR	PCR reaction with more than one pair of primers, each specific for a different sequence. For species identification, each primer pair is specific for one of the species to be detected. Often, the primer pairs share one of the primers that is generic and binds to the DNA of all species to be detected.
PCR	Polymerase chain reaction, a universally used method that, starting with a complex DNA sample (the template), amplifies a specific DNA fragment of 100–2000 bp by (typically) 30 cycles of (1) DNA denaturation (separating the complementary strand), (2) annealing (binding of two oligonucleotide primers that flank the DNA fragment of interest and bind on the two different DNA strands), and (3) extension of the primers by a heat-stable DNA polymerase. Extension of one primer creates a binding site for the other primer, and so the extension product serves as template for the next cycle. This results in an exponential chain reaction. For species identification, primers are either species-specific (bind only to DNA sequence of the species to be detected, so the PCR reaction only occurs if DNA from this species is present) or generic (amplifies DNA of more species), in which case the species identification depends on the analysis of the PCR product, for instance, by PCR-RFLP, primer extension, SSCP, or sequencing.
PCR-RFLP	PCR-restriction enzyme length polymorphism, detection of a DNA substitution that either creates or removes a recognition sequence of a restriction enzyme and then changes the cleavage pattern.
Primer extension	Detection of a DNA substitution by binding of an oligonucleotide primer adjacent to the position of the mutation and, depending of this mutation, extension of the primer with a labeled nucleotide by the action of DNA polymerase.
Real-time PCR	PCR reaction during which the accumulation of the PCR product can be followed by generation of a fluorescent signal, for instance, via the specific binding of the compound SYBR Green to the double-stranded PCR product. The PCR cycle at which the signal is a quantitative detectable is a measure for the initial amount of template DNA.
SSCP	Single-strand conformation polymorphism, detection of a mutation by denaturation of the PCR product, and electrophoresis under conditions at which the mobility of the single strands depend on the mutation.

Alternatively, species-specific proteins may be detected by mass spectrometry, as shown by the identification of specific peptide biomarkers originating from different fish parvalbumins[6] or from the arginine kinases of shrimp species.[7]

Although the most essential ingredients of routine DNA technology, DNA extraction, hybridization, PCR, and gel electrophoresis, are established all around the world, reports describing new methodological variants continue to appear. Farouk et al.[8] describe a fast DNA extraction using microwave radiation for the detection of meat species. Chapela et al.[9] compared different methods for extraction of DNA from canned tuna.

The classical "universal" mitochondrial DNA (mtDNA) PCR primers[10] were shown to amplify different bovine cytochrome *b* fragments,[11] which is most likely explained by coamplification of nuclear mtDNA copies. Selection of PCR primers specific for species or groups of species is

TABLE 16.2
Reviews on the Methodology of Species Identification

Taxonomic Range	Scope	Reference
Animals, plants	DNA methods	49
Animals, plants	DNA methods, subspecies level	38
Animals, plants	PCR methods	50
Animals	General	25
Animals	Quantification	51
Fish	PCR methods	52
Fish	ELISA	1
Fish	DNA methods	53
Fish	Application of DNA methods	54
Fish, seafood	DNA methods	55
Fish, seafood	Application of DNA methods	56

facilitated by a new computer program.[12] Pun et al.[13] developed universal primers for the mtDNA D-loop that generated amplicons with a species-specific length.

PCR-RFLP is still the most widely used, convenient, if low-throughput method for the detection of species-specific mutations. For the separation of PCR-RFLP fragments, lab-on-a-chip capillary electrophoresis has been demonstrated[14] as an alternative to the universally used agarose gel electrophoresis. Other protocols for mutations detection are primer extension[15–17] or single-strand conformation polymorphism analysis via an optimized, but nonstandard electrophoresis protocol.[18,19] Kitaoka et al.[20] demonstrated a quantification of the ratio of sequence variants by an elaborate ribonuclease protection assay.

Three reports have described the use of microarrays for species identification. Simultaneous differentiation of the mtDNA cytochrome *b* fragment of 30 fish, avian, and mammalian species can be achieved by the commercially available bioMerieux FoodExpert® system.[21] Teletchea et al.[22] supplied proof of concept for the identification of 71 out of 77 species, identification for 6 species being hampered by errors in Genbank entries. Development of a microarray for fish species identification is described by Kochzius et al.[23]

Another fundamental innovation is isothermal amplification with species-specific primers combined with electrochemical DNA detection.[24]

16.3 DEDICATED SPECIES IDENTIFICATION ASSAYS

Table 16.3 surveys the most recently developed methods for species identification. Most reports target a restricted number of species, but specific detection methods are now available for the majority of the animal edible species. For the most common species, several tests are available. In the case of criminal prosecution, confirmation of fraudulent species admixture or substitution by a different method would strengthen the evidence. On the other hand, the current redundancy of independently developed protocols not only impedes an across-laboratory standardization and validation, but also generates papers that are mostly repetitive.

Although the reported methods may be expected to meet their specifications, two caveats should be mentioned. First, PCR amplification with species-specific primers (Table 16.3, second column) and qualitative endpoint detection (most often by agarose gel electrophoresis) is especially vulnerable to contamination of samples and reagents. Second, simultaneous analysis of several species by multiplex PCR with either species-specific or "universal" primers may very well be biased toward the sequences with the most efficient amplification, for instance, by an exact match of primers. As a consequence, correct analysis of mixed samples should be validated.

TABLE 16.3
Dedicated Methods for Species Identification

Taxonomic Range[a]	Method[b]	References
Vertebrates		
30 species	MA	21
71 species	MA	22
Generic	S	57
Generic	S	29
Common Meat and Dairy Species		
5, *Ec*	M, Q	27
5, *Ec* in fish meal	R	58
5, *Ec*, *Mg*, and *Ap*	Q	28
3	O[c]	24
3	P	59
3, *Ec*	M	60
3, *Mg*	M, Q	26,34
3, *Oa* in feed	Q	61
3, *Oa* and *Ec* in minced meat	M	62
3, *Oa*, *Ec*, and *Mg*	Q	63
3, *Bb*, *Ch*, quail, and rabbit	R	64
Bt	Q	65
Bt	Q	66
Bt in feed	Q	36
Bt, *Oa*, *Ss*, mammals, and generic avian, in feed	Q	67
Bt, *Ss*	Q	33
Bt, *Ss*, *Oa*, and *Ch*	R, CE	14
Bt, *Ss*, *Oa*, *Ch*, and *Bb*	PE	16
Bt, *Ss*, *Oa*, *Ch*, *Ec*, elk, and deer in feed	O[d]	68
Bt, *Ss*, *Oa*, *Ch*, *Ec*, elk, and deer in feed	SSCP	19
Bt, *Ss*, *Oa*, *Ch*, *Ec*, dog, and cat	P	69
Bt, *Ss*, human, dog, and cat	P	70
Bt, *Oa*, *Ch*, *Gg*, *Mg*, and *Aa*	Q	37
Bt and yak	M	71
Bt in *Ch* milk	M	72
Bt in *Ch* milk	M	73
Bt in yak milk	M	74
Bt in *Bb* cheese	Q	75
Bt in *Bb* cheese	R	76
Bt in *Bb* cheese	O[e]	77
Bt in *Ch* and *Oa* milk	Q	78
Ch in *Oa* milk	*Q*	79
Ec	P	80
Gg	P	81
Gg	P	82
Gg, *Ss*, and ruminants	P, M	83
Gg, *Ss*, *Ap*, and pigeon	P	84
Gg, *Mg*, *Ap*, and *Aa* in feed	P	85
Gg, *Mg*, *Ap*, guinea fowl, and quail	R	86
Mammalian, generic	O[f]	13
Ruminant, generic in feed	Q	35

TABLE 16.3 (continued)
Dedicated Methods for Species Identification

Taxonomic Range[a]	Method[b]	References
Ruminants, *Ch*, *Oa*, and *Ss* in feed	P	12
Ss	R	87
Ss	P	88
Ss	P	89
Ss in feed	Q	90
Ss, wild versus domestic	R	91
Ss, *Gg*, and *Oa*	P	8
Ss, *Ec*, and *Ea*	P	92
Ss, *Ec*, and *Ea*	Q	93
Other Meat Species		
Cat, dog, rat, and mouse in feed	P	94
Cetacean and generic in feed	P	95
Chamois, ibex, red deer fallow, fallow deer, and roe deer	R, CE	14
Chamois, ibex, and mouflon	R	96
Chamois, ibex, and mouflon in feed	P	97
Chamois, ibex, and mouflon	Q	98
Crocodile	R	99
Deer (red, fallow, and roe deer)	Q	100
Deer (red, fallow, and roe deer)	P	101
Deer	Q	102
Duck, 4 species	P	103
Game bird, 8 species	R	104,105
Game bird, 4 species	P	106
Peacock	R	107
Rabbit in feed	P	108
Aquatic Species		
11 fish species	MA	23
25 fish species	MS	6
10 fish families	S	31
Fish species	S	32
Fish species in surimi	S	30
Albacore, bluefish, eel, and tuna	FRIP	20
Anglerfish	R, S	109
Anchovy	S	110
Anchovy	R	111
Billfish	R	112
Bivalve	S	113
Bonito	M	114
Catfish	I	115,116
Cod, fish (generic)	Q	117
Dolphin	M	118
Flatfish	S	119
Gadoids	P	120
Grouper, wreck fish, and perch	P, I	2,3,121,122
Grouper and wreck fish	I[g]	4
Hake	SSCP	18

(*continued*)

TABLE 16.3 (continued)
Dedicated Methods for Species Identification

Taxonomic Range[a]	Method[b]	References
Hake	PE	17
Hake	Q	123
Hake and cod	R, S	124
Mackerel and Mullet	M	125
Mitten crab	S	126
Razor clam	R	127
Razor clam	M	128
Salmon and trout	S	129
Salmon, trout, and bream	R, S	130
Scallop	M	131
Scallop	O[h]	132
Skipjack, tuna	M	133
Scombroid	S	134
Shark	S	135
Shrimp, prawn	R	136–138
Shrimp, prawn	S	139
Shrimp, prawn	MS	7
Sturgeon, paddlefish	S, R, RAPD	140
Spiny lobster	R	141
Squids	R, S	142
Tuna	PE	15
Tuna	Q	143
Tuna	R	144
Tuna	FRIP	145

[a] **3**, the three major meat species (cattle, pig, and chicken); **5**, the 'big five' livestock species (cattle, sheep, goat, pig, and chicken); *Aa*, *Anser anser* (goose); *Ap*, *Anas platyrhynchos* (duck); *Bb*, *Bubalus bubalis* (water buffalo); *Bt*, *Bos taurus* (cattle); *Ch*, *Capra hircus* (goat); *Ea*, *Equus asinus* (donkey); *Ec*, *Equus caballus* (horse); *Gg*, *Gallus gallus* (chicken); *Mg, Meleagros gallopavo* (turkey); *Oa*, *Ovis aries* (sheep); *Ss*, *Sus scrofa* (pig).

[b] CE, capillary electrophoresis; FRIP, fluorogenic ribonuclease protection assay; I, Immunochemical assay; M, multiplex PCR; MA, microarray; MS, mass spectrometry; O, other method; P, PCR with species-specific primer; PE, primer extension; Q, quantitative real-time PCR; R, PCR-RFLP; S, PCR-sequencing; RAPD, random amplified polymorphic DNA; SSCP, single-strand conformation analysis.

[c] Isothermal amplification and electrochemical DNA detection.

[d] Cloth hybridization.

[e] Casein analysis.

[f] mtDNA D-loop variation.

[g] Immunostick.

[h] Microsatellite genotyping.

See tables in previous reviews (Table 16.1) for surveys of earlier work.

Several Spanish laboratories have contributed to the methods mentioned in Table 16.3. As noted previously,[25] separate publications by the same group may describe different methods for detection of the same set of species, sometimes without adequate cross-referencing or comparison of the methods. Clearly, much is to be gained by combining results in one paper with a wider methodological and taxonomic scope.

However, several efforts are undertaken to develop methods with comprehensive species coverage. Multiplex real-time PCRs have been developed for the detection of four[26] or six[27] species. Laube et al.[28] developed a ready-to-use reaction plates for the detection of seven meat species. We already mentioned the use of microarrays,[21–23] which allows even more species to be tested in one experiment.

Especially for the detection of aquatic species, a straightforward differentiation of species is accomplished by sequencing of mtDNA PCR products.[29–32] Formerly denoted by "FINS" (forensically informative nucleotide sequencing), it is now referred to by "DNA barcoding." It can only be applied to samples of single origin[32] but may by phylogenetic analysis also pinpoint a species for which no sequence data are available.

Another drawback of several studies is that most methods are used mainly, if not exclusively, in the laboratory where they have been developed. However, ring trials have been described in which aliquots of the same samples are distributed over different laboratories for across-laboratory validation of quantitative real-time assays.[33–37]

16.4 BREED IDENTIFICATION

For all domestic species, different breeds have been developed, which by human selection have acquired different production traits. Like species identification, traceability of the breed of origin[38] serves the protection of consumers against economic fraud. Nearly always, genetic diversity across breeds is only slightly higher than within breeds and breed-specific DNA traits are rare. However, assignment of individuals to a breed can be accomplished by genotyping a panel of genetic markers and Bayesian estimation[39] of the probability that the individual belongs to a breed for which allele frequencies are available. This was accomplished with AFLP,[40] SNP,[41,42] and microsatellite[43–45] markers. Sasazaki et al.[46] used a combination of SNPs in mtDNA in a Y-chromosomal gene and in the MC1R coat color gene as well as three AFLP markers converted to SNPs to discriminate between Japanese domestic and imported beef. As an alternative, Watanabe et al.[47] identified combinations of markers that are in linkage disequilibrium and succeeded in distinguishing Japanese Black cattle from other breeds. Alves et al.[48] tested the use of maternal mtDNA markers for genetic traceability of European wild boars and Iberian and Duroc pigs.

16.5 CONCLUSION

The last decade, diverse, and versatile methodologies for species identification have been established. Considering the current redundancy of the many in-house protocols and the repetitive character of several publications, we recommend that new manuscripts are critically evaluated with regard to originality and progress relative to earlier work. At this point, priority should be given to standardization and across-laboratory validation of the most suitable assays. Ideally, these would quantitatively detect all relevant species in samples of mixed or single origin. Identification of the breed origin via a genetic rather than phylogenetic approach is technically feasible but requires comprehensive datasets of allele frequencies per breed.

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17 Detection of Neuronal Tissues and Other Non-Muscle Tissues with Respect to TSE

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CONTENTS

17.1 INTRODUCTION

Nearly two and a half decades after the emergence of a new transmissible spongiform encephalopathy (TSE) in bovines of the United Kingdom, the ban on so-called specified risk material (SRM) remains the most important measure to effectively reduce any potential oral human TSE exposition risk from the food chain. Already, in 1988, milk from clinically TSE-affected dairy cows was prohibited in the United Kingdom as a measure of preventive consumer protection in absence of any scientific prove [67]. In the end of 1989, the so-called specified bovine offal was legally defined and banned [68]. Due to sparse knowledge on this new disease, data from scrapie of sheep were used for estimating the potential risk and defining respective risk tissues. Consequently, this definition was imperfect and subject to change on the basis of new scientific data on TSE. Tissue diversity of TSE infectivity in bovines proved to be more restricted than in scrapie, especially excluding lymphoreticular tissues such as spleen or lymph nodes. Unfortunately, this early legal definition proved to be rather ineffective in practical circumstances, in particular the removal of the brain from the skull, which was still introduced into the food chain via production and use of mechanically recovered meat. As a consequence, the definition was broadened to such material that could probably be contaminated by risk tissues. Today, the SRM (18, last amended on 20/04/2009) is legally defined in bovine animals as (1) the skull excluding the mandible and including the brain and eyes and the spinal cord of animals aged over 12 months; (2) the vertebral column excluding the vertebrae of the tail, the spinous, and transverse processes of the cervical, thoracic, and lumbar vertebrae and the median sacral crest and wings of the sacrum, but including the dorsal root ganglia, of animals aged over 30 months; and (3) the tonsils, the intestines from the duodenum to the rectum, and the mesentery of animals of all ages. In addition, when harvesting the tongue of bovine animals (of all ages) for human consumption, only its part rostral to the lingual process of the basihyoid bone is to be used.

TABLE 17.1
Total BSE Infectivity in Tissues of a Clinically BSE-Affected Bovine according to Comer and Huntly [15], Modified as Pertaining to Ileum Weight [46]

Tissue	Infectivity Density ($CoID_{50}/g^a$)	Weight (kg) per 537 kg Animal	$CoID_{50}$ per BSE Case	% of Total Infective Load per Animal	Cumulative Load (%)
Brain	50	0.5	25,000	66.0	66.0
Spinal cord	50	0.2	10,000	26.4	92.5
Trigeminal ganglia	50	0.02	1,000	2.6	95.1
Dorsal root ganglia	50	0.03	1,500	4.0	99.1
Tonsils	0.005	0.05	0.25	0.0	99.1
Ileum	5	0.07[b]	350	0.9	100.0

Sources: Comer, P.J. and Huntly, P.J., *J. Risk Res*., 7, 523, 2004; Lücker, E. and Zetzsche, K., *Fleischwirtschaft* 5, 44, 2007.

[a] $CoID_{50}$: cattle orale infectious dose (50%).

[b] Original value of ileum weight: 0.8 (see text).

SRM in ovines and caprines is legally defined as (1) the skull including the brain and eyes, the tonsils, and the spinal cord of animals aged over 12 months or which have a permanent incisor erupted through the gum and (2) the spleen and ileum of animals of all ages. This extremely heterogeneous legal definition includes a vast variety of tissues, only certain species, and, in some cases (e.g., brain and spinal cord), age limits. Further, the origin of animals has to be taken into account as the SRM specification does not apply to animals with origin from states with an officially recognized status of negligible TSE risk in bovines, presently with the exception of member states of the European Union [18]. Thus, the analytical identification of SRM is rendered impossible. Table 17.1 demonstrates that TSE infectivity in bovines concentrates on the central neuronal tissues (CNT) brain and spinal cord including some neuronal tissues closely situated in the vicinity of the CNT, such as trigeminal ganglia. Non-neuronal SRM such as tonsils and ileum can be analytically neglected due to low or extremely low infectivity densities, low mass, and insignificant technological use [20,53]. Up to date, the ileum weight was approximated by 800 g; however, the real weight was shown to be one order of magnitude lower [46]. Histopathological lesions are restricted to the CNT where massive PrP^{Sc} accumulation is correlated to increasing infectivity. The Scientific Steering Committee of the European Commission estimated about 95% of total TSE infectivity in bovines to be concentrated in the CNT [65]. More recently, the reduction of TSE infectivity by removal of CNT-based SRM was estimated to be in the range of up to three orders of magnitude [38]. Thus, the analytical detection of SRM could be in part mastered by concentrating on CNT-based analytes. In addition to the food safety aspect, methods for the detection of CNT contribute to the authorities' ability to detect and deter deviations from food labeling regulations [3,17].

In a "TSE-road map," the European Commission expressed the need for a future lifting of the SRM ban in view of the overall and constant reduction of the frequency of bovine TSE cases as well as the very high costs of preventive measures [19]. However, some major scientific uncertainties on TSE cannot be clarified on the short run, such as infectious dose for humans and incubation period as a function of repeated small doses. Moreover, new insights into atypical cases of bovine TSE [33,47] indicate a constant probability for introducing sporadic TSE into the food chain.

Altogether, this does not argue in favor for a total lift of the ban, in particular without establishing alternative measures in order to guarantee the same level of protection as currently instigated. As such, suitable analytical methods for the detection of CNT-based SRM would be very welcome. As shown in Table 17.2, a variety of analytical methods for the detection of CNT as risk material on meat and in processed meat were developed since the emergence of the new variant

TABLE 17.2
Overview and Tentative Rating of Methods for the Detection of CNT (SRM) on Meat and in Processed Meat

Method	Marker	PY	Stab.	Spec.	Sens.	DL	Species	Age	Avail.	Pract.	Valid.
Photomomtric enzymatic	Cholesterol	1997	+++	+	+	—	—	—	+++	++	+/–
Gaschromatography (GC)	Cholesterol	(2000)	+++	+	++	—	—	—	++	++	+/–
GC mass spetroscopy	Fatty acids (patterns)	(2001) 2004	+++	+++	++	0.01	+	+	++	++	+/+
Histology, CNT	Morphological	—	+	—	—	—	—	—	+++	++	—
Histol., non-CNT	Morphological	—	+++	+++	+	—	(+)	—	+++	++	—
Immuno histology	NSE, NF, …	1999	+	++	++	1	—	—	+	+	+/–
IC, Westernblot	NSE	1998	+	++	++	0.01	—	—	+	++	+/(+)
IC, Westernblot	GFAP	1999	+	++	+	—	—	—	+	++	–/–
IC, Westernblot	PLP	2007	+	++	++	0.01	—	—	+	++	+/–
IC, ELISA	GFAP	1999	+	+++	++	0.05	—	—	+++	+++	+/+
IC, ELISA	PrP^{Sc}	2002	+++	+++	+	0.25	(+)	(+)	++	++	+/–
IC, ELISA	MBP, Syntaxin, …	2002	+	+++	++	—	—	—	++	++	(+)/–
RT-PCR	mRNA (GFAP, …)	2003	+?	+++	+++ (?)	0.1	+	—	++	++	+/–
RT-PCR quant.	mRNA (GFAP, …)	2005	+?	+++	+++ (?)	0.01	+	—	++	++	+/?

CNT, central nervous tissue; SRM, specified risk material; IC, immuno chemistry; ELISA, enzyme-linked immunosorbent assay; RT-PCR, reverse transcriptase polymerase chain reaction; quant., quantitative; NSE, neuron-specific enolase; NF, neuro filamanet; GFAP, glial fibrillary acidic protein; PLP, protein lipoprotein; PRP^{Sc}, pathoisoform of the prion protein; MBP, myelin basic protein; mRNA, messenger RNA; PY, year of first publication; Stab., stability of marker; Spec., specificity; Sens., sensitivity; DL, limit of detection (CNT %) as reported by the authors, species/age, species/age of the animal the CNT originated from; Avail., availability; Pract., practicability; Valid., validation, within laboratory/ringtest between laboratories; +, low; ++, medium; +++, high; —, not applicable or not performed, not known; and ?, subject to further studies.

of Creutzfeldt–Jacob disease. All of the many scientists involved in this method development and validation state that the analytical detectability of the banned CNT-based SRM would significantly contribute to preventive consumer health protection against oral exposure to TSE risks.

In the following, the current state of the analytical detection of neuronal tissues (Table 17.2), other tissues, and infectivity as related to TSE risk is presented in chronological order of publication.

17.2 ENZYMATIC–PHOTOMETRIC CNT DETECTION

Cholesterol was the fist marker to facilitate the detection of CNT in heat-treated meat products [39]. The cholesterol content of CNT substantially exceeds that of the major meat technological relevant tissues, such as muscle or adipose tissues. The cholesterol content in brain of about 2000 mg/100 g (wet weight) is about 40 times higher than that of muscle tissue (aprox. 50 mg/100 g wet weight). To facilitate the evaluation of current potential introduction of CNT in the food chain at the time of the emergence of a new variant of CJD, which confirmed the concerns about transmissibility of bovine TSE to humans, a readily available method was urgently needed. Thus, it was beneficial that quantification of cholesterol, first, can be easily achieved by the use of an enzymatic photometric method and, second, that the principal method had already been validated for cholesterol quantification in eggs and egg derived products by the Federal German Health Authority in a ring trial at that time [12]. The test principle is based on the quantitative oxidation of the 3-β hydroxy group of cholesterol to delta4-cholesten-3-on using cholesterol oxidase, which is followed by photometric detection and quantification of a lutidine color reaction at 405 nm. Only some minor modifications of the method were needed, in particular as pertaining to sample preparation [42]. However, as cholesterol is also present in non-CNT, cutoff values for normal (CNT-free) cholesterol contents in meat products have to be established. In emulsion-type sausages, it was possible to detect CNT (brain) at levels of about 1% with a 99.9% statistical security [42], whereas, in certain cooked sausages, due to the addition of high amounts of cholesterol containing liver, cutoff levels were increased [45]. When analyzing sausages with an addition of tissues with relevant cholesterol contents, such as liver, kidney, or egg products, either an increased number of false-positive samples have to be tolerated or higher cutoff values have to be applied, which consequently leads to an increased number of false-negative results. Overall, the cholesterol quantification as a method for the detection of CNT in meat products is still of some interest, not only for laboratories with basic instrumentation and low budgets but also as a secondary method supplementing fast immunological screening methods (see below) as pertaining to heat-treated samples with low but conspicuous results [45]. Cholesterol as marker for CNT also offers potentials for the construction of a low-cost miniaturized rapid test kit for the detection of surface contamination by CNT in the slaughtering process where tissues with high cholesterol content will not interfere.

17.3 IMMUNOCHEMISTRY: WESTERN BLOT

The first immunological procedure for the specific detection of traces of CNT in meat products was reported in 1998, using the western blot technique and neuron specific enolase (NSE) as a marker [41]. The method applied poly-, later on monoclonal anti-NSE antibodies in a western blot after protein extraction and sodium dodecyl sulfate–polyacrylamide gel electrophoresis separation [43,45]. Detection limits—as demonstrated by blind testing using standard emulsion-type sausages with varying brain contents—were estimated to be as low as 0.01% CNT [43]. In combination with cholesterol quantification, the NSE-western blot (integrated CNT-detection procedure) was applied in a screening of retail meat products from German food outlets. In 1998, the first CNT positive sample was detected: A high quality liver sausage product showed strong immunoreactions in the NSE western blot along with a significantly increased cholesterol content. The use of brain was not labeled. Analytical finding was shown to be correct by analyses of samples from different production batches, which were also CNT-positive, and official inquiries that revealed that the respective

TABLE 17.3
Own Results of CNT-Detection by NSE-Western Blot and Cholesterol Quantification in Different Groups of Meat Products from Retail Outlets (Mainly) in Germany 1998 to April 2001

Meat Products	Samples	CNT-Positive (NSE, Cholesterol)	
Group (main component)	(n)	(n)	(%)
Raw sausages	28	3	10.7
Emulsion-type sausages	215	7	3.3
Cooked sausages (blood)	46	0	0
Cooked sausages (fat)	77	16	20.8
Cooked sausages (gel)	44	0	0
Cooked sausages (liver)	316	40	12.7
Others	48	0	0
Total	774	66	8.5

manufacturing process entailed the use of 4% porcine brain. Overall, this study with NSE-western blot and cholesterol quantification demonstrated a far higher frequency and a more widespread use of CNT in meat products than was suspected at this time (Table 17.3). NSE western blotting was officially introduced in Swiss import control [11], and later it was established in the United States [57]. In Germany, a commercial NSE-western blot became available in 2001 (ScheBo-BioTec, Gießen, Germany). Several lab-internal (e.g., 2001) and inter-laboratory validation studies were conducted using the NSE-western blot, some of them in comparison with other CNT-detection methods, which became available in the meantime [3,27,31]. While these yielded positive validation results, NSE-western blotting was only applied temporarily in routine control in Germany, due to the low degree of both availability in official laboratories of food control and practicability when compared to other IC techniques. In particular, the standardization of the analytical procedure and the interpretation of the immunoblots appeared not to be error proof. In addition, presumably false-negative results were reported for products containing avian matrices [11,57]. Indeed, avian tissues can cause nonspecific immunoreactions in close vicinity of the NSE band [58]. In view of the superior practicability of the enzyme-linked immunosorbent assay (ELISA) technique, a survey of available und newly produced monoclonal anti-NSE antibodies was conducted. This study, however, failed to identify suitable antibodies for the ELISA technique with respect to the detection of CNT from heat-treated matrices.

In addition to NSE, a large panel of antigens were tested for their suitability to detect CNT in fresh and heat-treated meat products by western blotting, starting with glial fibrillary acidic protein (GFAP) [43] and including myelin basic protein (MBP), neurofilament, syntaxin and others [52]. However results, albeit partially promising, did not induce further development.

Myelin proteolipid protein (PLP) was identified as a further suitable CNT marker in meat products or on meat (PLP). Western blot analysis of PLP detected CNT contamination selectively and sensitively [70]. Bäuerlein et al. [8] developed a rapid dot blot assay using an anti-PLP antibody. The detection limit was reported to be 0.01% fresh bovine brain in minced bovine muscle. The assay can be applied in a swab test allowing a detection of down to 0.5 mg CNT on meat or other surfaces.

An altogether different analytical approach was taken by trying to directly detect PrP^{Sc} as a marker for TSE infectivity [16] in meat products [26,44]. Several methods for the detection of PrP^{Sc} in CNT matrix are commercially available, validated, and officially approved for purpose of TSE surveillance [18]. In view of bio-security restrictions, however, it appeared to be impossible to test their applicability for the matrices of meat products. Such studies would have to be based

on standards of meat products with bovine TSE-positive brain. This problem was solved by the development of a microtechnology for simulating meat production on a low scale (gram range), thus enabling the transfer of standard production (kilogram range) into high-security laboratory conditions [44]. First results applying the Bio-Rad Platelia BSE purification and detection kits gave close linear relations between TSE-positive bovine brain content in standards of emulsion-type microsausages, which were linear in a range of up to 10% brain content and indicated detection limits as low as 0.25% brain. In follow-up studies, the applicability of two novel immunoassays for the direct detection of PrP^{Sc} in meat products was tested.

17.4 IMMUNOCHEMISTRY: ELISA

Detection of CNT in meat products by enzyme-linked immunosorbent assay (ELISA) technique was first performed using astrocytic GFAP, a major protein constituent of glial filaments in differentiated astrocytes, as a marker for CNT [59]. In this study, a modification of a colorimetric enzyme immunoassay originally designed for clinical purposes (GFAP-ELISA) was applied. It included a sandwich of polyclonal and monoclonal anti-GFAP antibodies for the detection of CNT in mixtures with fresh muscle tissue and blood. The limit for detection of GFAP was reported to be as low as approx. 1.0 ng. This was still improved to 0.2 ng GFAP by introducing a fluorescent ELISA (F-ELISA) detection system, the GFAP F-ELISA [61]. GFAP was not detected in skeletal muscle and blood clots and only in traces in the sciatic nerve (nanogram per milligram range) while brain and spinal cord each showed content in the microgram per gram range [59]. The GFAP ELISA is commercially available in two versions in Germany since 2001 (R-Biopharm, Darmstadt, Germany; ScheBoBioTech, Giessen, Germany). Applicability of the GFAP-ELISA was demonstrated also for heat-treated meat products and validation studies including comparisons with other CNT markers and detection systems showed altogether good to excellent results [3,4,31,32,56,60]. The principle of CNT detection by means of GFAP-ELISA was validated and introduced as an official method for food control in Germany in 2004 [13]. Recent studies focused on the detection of non-heat-treated CNT such as contamination of fresh meat or of carcass-splitting band saw blade surfaces and ground meat or advanced meat recovery samples [55]. In comparison, the GFAP F-ELISA proved to be superior to the commercial GFAP-ELISA version and the immunohistochemical GFAP detection in terms of sensitivity and repeatability [30]. According to the authors, all three methods are widely used in the United States. In contrast to these studies that demonstrated the CNT-specificity of GFAP, false-positive results were obtained in two cases [63]. Blood samples taken *intra vitam* from clinically healthy cattle frequently showed positive immuno-reactions in the range of up to 0.2% CNT using the commercial GFAP-ELISA. In contrast, these reactions were not detected when using other CNT detection methods, in particular the laboratories own newly developed GFAP-ELISA [36], which became commercially available in 2004 (ScheBo-BioTec, Gießen, Germany). Currently, the GFAP-ELISA can be seen as the standard procedure of CNT detection for screening of fresh and heated meat products and for the control of CNT contamination on meat surfaces or equipment.

MBP was shown to be a suitable CNT marker involving immunochemical detection by an indirect ELISA technique [29] and by western blot [52]. Moreover, these studies indicated MBP to be potentially species specific and thus enabling us to differentiate between bovine and porcine or avian CNT. Protein gene product 9.5 (PGP 9.5) might become another suitable antigen for immunological CNT-detection in heat-treated meat products as recent results on the development of a sandwich ELISA indicate [21].

The direct PrP^{Sc} detection from the matrix of meat products was also possible by using a sandwich immunoassay (Bio-Rad TeSeE test), and a two-sided immunoassay using two different monoclonal antibodies directed against two epitopes presented in a highly unfolded state of bovine PrP^{Sc} (Roboscreen Beta Prion BSE EIA Test Kit). In comparison to the originally applied western blotting, these tests showed increased sensitivity, robustness, and applicability [25]. Further studies for optimizing and validating the analytical procedure prior to its application in official food control are needed.

17.5 MICROSCOPY AND IMMUNOHISTOLOGY

According to Tersteeg et al. [66], early studies on microscopic identification of CNT (brain) in complex matrices such as meat products gave contradictory results, which might be due to varying technological treatment. In studies of the authors, brain was not detectable in emulsion-type sausages, not even in substantial amounts exceeding 10% and more brain content. Specific staining of the CNT (e.g., silver impregnation) did not help to improve results [71]. Consequently, the authors tested several antibodies specific to CNT proteins in the first immunohistological approach for the detection of CNT in meat products. These studies showed the suitability of NSE (gamma-enolase) for CNT-detection in heat-treated meat products, whereas other markers failed to work [71]. Another study, which tested GFAP, neuron filament (NF), NSE, and MBP demonstrated the interdependence of heat treatment and type of antibody/epitope used for the detection [66]. Here, the selected anti-MBP antibody turned out to be of superior applicability, whereas a further study showed an anti-NF antibody to be more suitable than antibodies against NSE, GFAP, MBP, or peripherin [7]. NF facilitated the detection of 1% CNT addition in heat-treated meat products as well as the differentiation between nerve fibers and CNT by using morphological criteria. Currently, the U.S. Department of Agriculture, Food Safety Inspection Service recommends an immunohistological procedure for GFAP [69], which, however, was shown to produce inconsistent results [30]. Herde et al. [28] report the suitability of certain anti-GFAP and anti-MBP antibodies for the detection of bovine brain (8%) in heat-treated meat products. Anti-synaptophysin antibodies were used for CNT detection in immunohistological studies with non-heated matrices (e.g., [14]), and anti-S100ß-protein immunostaining gave first proof for micro-embolization of brain tissues due to captive bolt stunning [6].

Immunohistological methods offer the combination of micromorphological criteria with specific antigen detection. For instance, morphological criteria might facilitate the differentiation between central and peripheral nervous system tissues when using antibodies of respective low specificity. Moreover, non-CNT based SRM, such as lymphatic tissues or intestines, can be detected by micromorphological analysis as suggested by Koolmees and coworkers [34]. In this context, the micromorphological approach offers the potential for species differentiation. The animal species of a certain risk tissue, which is morphologically identified, e.g., spleen, could be characterized in a second step by in situ species specific antigen staining or hybridization.

17.6 MOLECULAR BIOLOGY: PCR

Detection of CNT-based SRM by means of molecular biological analytical tools is highly attractive in view of their extreme specificity and sensitivity. In principal, they also offer a species identification of the detected CNT. This analytical approach is highly sophisticated as mRNA suitable for species specific CNT detection has to be identified and concerns about mRNA stability in meat products and quantitative extractability as well as contamination aspects have to be met. First, reports on the principal suitability of mRNA-based CNT detection were published by two working groups already in 2003 [37,64].

Seyboldt and coworkers [64] used a 168 bp CNT specific GFAP mRNA target and reverse transcription polymerized chain reaction (RT-PCR) for tissue specific CNT detection followed by RFLP in order to discern between bovine and non-bovine CNT species. The method was successfully applied along with a commercial GFAP-ELISA in one further study on CNT detection in retail liver sausages. The authors concluded that their PCR assay would be useful to characterize the CNT species in CNT-positive meat products as detected by immunological screening [51].

Lange and coworkers [37] identified suitable target mRNAs for CNT. The selected primers for $GFAP_{87}$ and MBP_{51} facilitated the detection of CNT in raw sausages and non-heated and heated standards of emulsion-type sausages with defined addition of brain by RT-PCR. Results indicated ample stability against meat technological influences such as storage, temperature, and ripening. RT-PCR with $GFAP_{87}$ facilitated the detection of CNT without species specificity, whereas MBP_{51}

enabled the selective detection of CNT of bovines, ovines, and caprines but not of CNT of porcines and poultry.

Gout and coworkers [22] designed an assay using methylation specific PCR (MSP). The authors isolated GFAP promoter fragments and identified key differences in the methylation patterns of certain CpG dinucleotides in the sequences from bovine and sheep brain and spinal cord and the corresponding skeletal muscle in order to specifically amplify the neuronal tissue derived sequence and therefore identify the presence of CNT tissue.

Abdulmawjood and coworkers [1] report the development of a quantitative real-time RT-PCR for the detection of CNT in meat and meat products. This real-time RT-PCR assay used encoding gene sequences of bovine, ovine, and caprine GFAP as markers. The mRNA assay facilitated the highly species specific detection of bovine, ovine, and caprine CNT tissues in meat and meat products. Bovine brain dilutions as low as 0.01% could be detected. The real-time RT-PCR assay was not affected by meat technological procedures, in particular heat treatment. The first part of a national collaborative trial involving liquid extracts of meat products with varying brain content showed good reproducibility and detection limits as low as 0.1% [2]. Further in-house studies on the validation and standardization of this real-time RT-PCR assay revealed high bovine tissue specificity and sufficient mRNA marker stability [62]. The authors concluded that their real time RT-PCR assay appears to be a suitable tool in routine diagnostic testing for the illegal use of tissue of the central nervous system.

In a further approach Nagarajan and coworkers [48] presented a quantitative real-time RT-PCR detection of CNT using GFAP-mRNA as marker and in vitro GFAP-RNA as internal calibration standard. They report stability of this marker at 4°C for up to 28 days, a potential detection limit of 0.01% CNT, and a cutoff value of 0.025% CNT.

The methods offers one-step species detection. However, this approach was tested only with raw matrix and is thus not applicable for heat processed products.

While interlaboratory validation of extraction procedures and stability of mRNA markers will have to be further addressed in detail, the RT-PCR assays offer a promising analytical approach for the detection of tissues of the central nervous system with potential for species specificity and high sensitivity.

A combination of IC and PCR was reported by Kuczius and coworkers [35]. Their immuno-PCR is based on a normal sandwich-ELISA using an anti-GFAP antibody in combination with a secondary antibody bound DNA-fragment and PCR amplification. This promising approach might facilitate an increase of ELISA sensitivity by one to two orders of magnitude. However, no heat-treated standard material/samples have been tested so far.

17.7 GAS CHROMATOGRAPHY/MASS SPECTROMETRY

Cholesterol as a marker for CNT can easily be detected by gas chromatography (GC) [38]. Analytical results were by far more precise and reliable than those obtained with the screening method (photometric–enzymatic). However, this analytical approach was not further developed, in particular as costs were disproportionate in view of the limited specificity of this marker. Nevertheless, markers from the lipid fraction of CNT remained extremely promising, and today, certain fatty acids (FAs) of the central nervous system are important markers for CNT, showing a high potential for further analytical discrimination according to the complex legal definition of SRM. Sphingolipids, such as galactocerbrosides, gangliosides, and sphingomyelins constitute highly interesting markers for CNT in meat products, particularly with regard to their specificity for and high content in myelinized nervous tissue. In modern food control, laboratory methods for the detection of such compounds—such as gas or liquid chromatography and mass spectrometry (MS)—are readily available. A further promising characteristic is the potential stability of most of the compounds from the lipid fraction as they proved to be much more stable than those of the protein or nucleic acid fraction of the CNT used as markers in other phenotypic or in genotypic methods. Niederer and Bollhalder [49] were the first

to make use of this novel analytical approach in CNT detection. They identified certain FAs within the complex lipid fraction, in particular nervonic acid, to be absolutely specific for CNT. Following solid phase extraction and acid derivatization to the respective methyl esters, the FA methyl esters were separated and quantified by means of GC/MS. Detection limits were reported to be as low as 0.01% CNT in standards of meat products. They reported that the relation between the *cis*- and *trans*-isomeres of the "nervonic acid" offered the possibility to categorize the detected CNT as pertaining to the species and age of the animal from which the detected CNT originated. Although first reproduction of the proposed method indicated that the general potential of this analytical approach was valid, certain drawbacks became apparent [5,9,40,50]. Mainly, most non-neuronal tissues used in meat technology showed baseline contents of the presumed CNT-specific FAs, including nervonic and cerebronic acid. Thus, it was necessary to establish cutoff values as was the case with cholesterol. Fortunately, the specificity of CNT-FAs is still distinctly higher than that of cholesterol to the effect that detection limits can be achieved, which are one to two orders of magnitude lower. Moreover, structural characterization studies by retention time and dimethyl disulfide adduct profiling proved the previously designated "trans-nervonic acid" (*trans*-15-tetracosenic acid) to be *cis*-17-tetracosenic acid [10]. Nevertheless, small amounts of trans-isomers of some long chain FAs were detected, which might improve efficiency of the differentiation between non-SRMs and SRM according to the legal definition. In a further study, the ratio of ω7-/ω9-isomeres of tetracosenic acid ([15c-C24:1]/[17c-C24:1]) was shown to be well suited to differentiate between the non-SRM and SRM status of CNT traces in meat products [40]. Besides some methodological optimizations, which resulted in the presentation of a standard operating procedure, the authors proposed an analytical strategy essential to the quantification of the CNT. It became apparent that the content of all relevant FAs (C22:6, C24:1 ω 9, C24:1 ω 7, C24:0, C24-OH) in the respective CNT varies as a function of species and age. This necessitates an analytical step-by-step strategy, which starts with the nonspecific identification of the presence of CNT in a sample (step 1). In case of a CNT-positive sample, the second step involves the analytical identification of species and age of the CNT (step 2); only then it becomes possible to quantify the CNT as a species- and age-specific calibration has to be applied (step 3). Cerebronic acid turned out to be the most suitable FA for both CNT identification (step 1) and CNT quantification (step 3). Following sylilation, cerebronic acid showed maximum absolute differences as well as the best ratio between contents of CNT-free samples and CNT.

In spite of its analytical complexity, it was demonstrated—within an externally controlled blind test—that the present approach using FAs as markers and GC/MS is a highly sensitive and robust method, which facilitates the identification of the SRM status of CNT traces in meat products [23]. Within the observed range of 0.5%–3% CNT addition, neither the animal species of the main component (muscle tissue) nor severe heat treatment (up to 133°C, 3 bar, 40 min) had any influence on the capability to identify the SRM status of the detected CNT traces. Further research activities deal with optimizing the analytical procedure, in particular the sample extraction [54], as well as the statistical data evaluation as pertaining to CNT-species and CNT-age differentiation, which depends on a comprehensive data base of CNT-FAs and multifactorial data analyses of FA-patterns [24]. Poerschmann and coworkers [54] demonstrated that sequential pressurized liquid extraction can prove to be superior to the originally used exhaustive lipid extraction followed by solid-phase extraction regarding lipid recoveries and clear-cut boundaries between lipid classes. Alcoholysis using trimethylchlorosilane/methanol facilitated complete transesterification of lipids and quantitative formation of methyl esters. Grießbach and coworkers [24] showed that differentiation of CNT species and CNT age must be founded on a comprehensive data base of CNT-FAs and can be significantly enhanced by multifactorial data analyses of FA patterns. Finally, the present GC/MS approach was validated with full success during an official ring test using emulsion-type sausage standards with varying addition of brain from different animal species and ages (bovine, ovine, porcine, and avian). In the first part of the ring trial (0% and 3.0% CNT), 96.4% of SRM-positive and 98.2% of the SRM-negative samples (additional samples: 90% and 100%, respectively) were correctly identified by 14 participating laboratories. In the second

part of the ring trial (second part of ring trial: 0%, 0.5%, and 1.0% CNT), 86.5% and 98.9% of the respective SRM-positive and SRM-negative samples were correctly identified by 13 participating laboratories. In this part of the ring trial, the characteristic standard deviation of repeatability and comparability was 0.118% and 0.162% CNT (m/m).

For the time being, the analytical GC/MS approach using FAs as markers for the detection of CNT in meat products is the only method that facilitates the differentiation between non-SRM and SRM status of the detected CNT. Only this analytical approach allows species and age categorization of the animal from which the detected CNT was derived from. Furthermore, the extraordinary heat resistance of these FA-CNT markers could be applied as a basis for further research in characterizing CNT contamination not only in meat products but also in meat and bone meal.

While the analytical GC/MS strategy and procedure is very complex, time, and cost-intensive, it could be best put to use as future reference method, in particular to validate and more closely characterize positive results of immunochemical screening methods or within the scope of method development.

17.8 CONCLUSION

A still decreasing rarity of bovine TSE and extraordinary costs on the one hand and atypical/sporadic TSE on the other hand may soon necessitate the substitution of present preventive TSE measures, in particular the ban on the so-called SRMs, by an analytical approach at the level of the final product. Furthermore, methods for the detection of CNT are needed by official food control with respect to food labeling regulations. Of all methods presented here, the GFAP-ELISA, a fast, inexpensive, and easily practicable method, and the analytical GC/MS approach using heat-resistant FAs and their patterns for the detection of CNT and categorization of species and age are presently the most advanced and validated analytical approaches.

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18 Residues of Food Contact Materials

Emma L. Bradley and Laurence Castle

CONTENTS

18.1 INTRODUCTION

The main function of food packaging is to protect and preserve the food, to maintain its quality and safety, and to reduce food waste. There can be a downside, however, and this is contamination of the food by chemical migration from the packaging.

Nearly all the food and drink that we buy and then consume is packaged in some way. It is very likely that it has also made direct contact ("touched") with other materials during harvesting, production, transport, storage, preparation, and cooking. A food contact material (FCM) is any material or article intended to be placed in contact with foodstuffs. Food-packaging materials are the most obvious example of this, but the term also includes parts of food processing equipment, storage containers, kitchen utensils, etc. These FCMs can be made from a range of different materials such as plastics, paper or cartonboard, metals, glass, ceramics, cork, and rubber. To these primary packaging materials also have to be added the adhesives, inks, varnishes, and coatings needed to make final materials and articles in their usable form. There are literally thousands of different chemicals needed to make this variety of materials and give them the characteristics and properties that are needed to perform their function.

This chapter deals with the transfer of these chemicals to edible animal by-products (EABPs) when they touch the FCMs. Depending on the nature of the EABP, the transfer of some chemicals may cause taint or odor problems. If the chemical is hazardous to health (meaning toxic), and if the concentrations are high enough, then this may even make the product unsafe to eat. So, in order to maintain both the quality and the safety of the food, it is important to understand how this contamination can be tested for and kept under control by the proper selection and use of packaging materials. The chapter aims to give the reader this understanding and includes some examples that are illustrative of the main scientific and technical issues. It will start first with the chemical and physical processes that underlie this contamination process, which is called chemical migration. This is because it is the migration phenomenon that makes testing of products for chemical residues from FCMs, and testing the FCMs themselves, a rather specialized topic.

In considering this, it is sensible to first consider the range of EABPs involved. The characteristics of these food products strongly influence the types of packaging materials and other FCMs that are suitable for them. There are many possible lists of EABPs, some coming from legislative definitions, but this simple list serves our purpose here. As we shall see later, the physical and chemical characteristics of each EABP along with the typical storage conditions used, are major determinants of the migration process.

- Liver
- Heart
- Kidney
- Tongue
- Stomach (tripe, haggis)
- Blood (sausage, pudding)
- Brains
- Sweetbreads (thymus, pancreas)
- Stock (e.g., from bones)
- Meat extracts
- Trimmings (jowl, tail, feet, skin)
- Spleen
- Gelatine (skin, hair, bones)
- Rendered fat (lard)
- Intestines (casings)
- Testicles
- Poultry giblets

18.2 FOOD CONTACT MATERIALS AND CHEMICAL MIGRATION

Chemical migration can be defined as "the mass transfer from an external source into food by submicroscopic processes." The extent to which any chemical substance migrates into a foodstuff is controlled by processes that are subject to both kinetic and thermodynamic control. For processes under diffusion control, they can be described by Fick's second law. Some migration processes are not under simple diffusion control; for example, if there is chemical attack at a surface, it possibly involves not only physical processes but also chemical decomposition processes. For processes under thermodynamic control, solubility is the key determinant. Putting the detailed mathematical

description of the migration process to one side, the extent of any chemical migration is dependent on the following factors:

- The nature of the food contact material
- The nature of the foodstuff
- The nature of the migrating substance
- The extent of direct or indirect contact between the food contact material and the foodstuff
- The temperature of the contact
- The duration of the contact

18.2.1 The Nature of the Food Contact Material

Migration from a material occurs at its interface with the food. Chemical migration depends on the concentration of the substance in the food contact material and on the diffusion characteristics of the substance within the material and away from the surface of the food into which it migrates.

Impermeable materials: For a material with a low diffusivity, the rate at which the surface is replenished with the migrant will be slow or even effectively zero. Migration from materials such as glass, ceramics, or metal occurs only from the surface of the material (Figure 18.1); no diffusion of substances will occur from within these materials to the surface, within a normal timeframe.

Permeable materials: Plastic and rubber materials exhibit diffusivity to different extents depending on their structure, crystallinity, etc. Some are hard and glassy (polycarbonates, thermosets) and others are soft and rubbery (plasticized PVC). However, in all cases, diffusion of migratable substances from within the material to the food contact surface can occur (Figure 18.2).

Porous materials: Fibrous materials such as paper, board, and woven plastic fibers provide practically no resistance to the movement of some substances within the matrix. The migration of substances occurs from the food contact surface, from within the material as well as any substances contained in inks and coating applied to the nonfood contact surface (Figure 18.3). This is also true for some plastics in the category above. For example, a thin film of a low barrier plastic can allow adhesive or ink components from an outside self-adhesive label, to pass through the film and into the packed food. Porous substrates offer practically no resistance to the migration of small organic molecules. Larger molecules and inorganic salts may be impeded or stopped from migration.

Multilayer packaging materials are also commonplace where a barrier layer such as aluminum foil is included in the packaging structure. In these cases, any migratable substances on the nonfood side of the aluminum foil layer will not be able to pass through this barrier layer and therefore

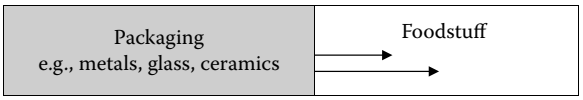


FIGURE 18.1 Depiction of chemical migration from an impermeable material.

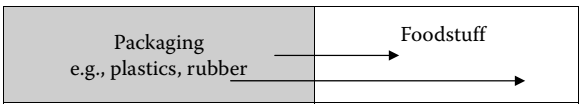


FIGURE 18.2 Depiction of chemical migration from a permeable material.

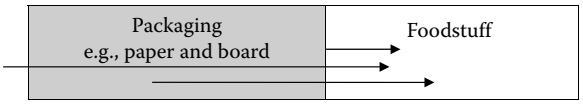


FIGURE 18.3 Depiction of chemical migration from and through a porous material.

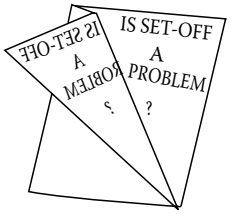


FIGURE 18.4 Depiction of setoff.

migration of such substances into the foodstuff will not occur by this mechanism. However, if a material has been rolled (reeled) or stacked such that the food contact surface is stored in contact with the nonfood contact surface, then transfer of chemicals between the two can occur (Figure 18.4). In such cases, even the presence of a functional barrier such as a layer of aluminum foil is not sufficient to ensure that no migration will occur. This transfer process in known as setoff and it is especially important when evaluating inks and varnishes.

18.2.2 The Nature of the Foodstuff

When considering migration, foodstuffs are conventionally split into five categories that describe their physical and chemical properties: aqueous, acidic, alcoholic, fatty, and dry. The solubility of the migrating substance in the foodstuff will influence the extent of the migration. Lipophilic ("fat-loving") substances have a greater solubility in fatty foods or foods with free fat on the surface and so the migration of such substances into these food types will be greater than that into an aqueous foodstuff. Conversely, polar or ionizable molecules and salts are more soluble in aqueous media and less soluble in fatty foods.

18.2.3 The Nature of the Migrating Substance

As well as considering if the characteristics of the chemical are a good match with the food or not, its compatibility with the FCM should also be considered. A substance that is incompatible with the FCM could "bloom" to the surface resulting in it being readily available to transfer to the foodstuff. Conversely, any strong interaction that occurs between a substance and the material containing it will reduce migration.

18.2.4 The Extent of Contact with the Foodstuff

Furthermore, if the foodstuff interacts strongly with the FCM, it can cause swelling at the surface that increases the rate at which chemicals are released. The greater the surface area of the material in direct contact with the foodstuff, the greater the potential for migration. Similarly, where intimate contact is made as opposed to point contact, for example, liquid or semisolid foods including sauces and pastes, compared to solid foods, the potential for migration also increases.

18.2.5 The Temperature of the Contact

As migration is a diffusion process that occurs more rapidly at elevated temperature, the extent of the migration increases with increasing contact temperature.

18.2.6 The Duration of the Contact

The longer the material is in contact with the foodstuff, the greater the extent of the migration that will occur. Migration kinetics are normally first order, which means that the extent of any migration increases relative to the square root of the contact time.

As a simple example of the interplay between these parameters, consider the case of ox tongue or a similar EABP. At the abattoir or processing plant, it may be in contact with a collection bin or a conveyer belt for just a few second or minutes at ambient or chilled temperature. The same product may then be canned, heated in the can up to 130°C for cooking and sterilization and then stored for several months or even years before it is eaten.

18.3 WHY TEST AND WHAT TO TEST FOR?

18.3.1 The Purpose of Testing

By the processes described above, any substance present in a material placed in contact with a foodstuff has the potential to migrate. This migration can impact the safety of the food because some substances used to make food contact materials may be harmful if consumed in sufficient amounts. Migration can also have an adverse effect on the quality of the food because the transfer of sensorially active substances may impart a taint or odor that is offensive to the consumer. The need to control the effects of food contact materials on both of these aspects is considered in legislation. For example, in the European Union, the Framework Regulation (EC) No. 1935/2004 covers all food contact materials. It states in the general requirements of Article 3 that

> Materials and articles, including active and intelligent materials and articles, shall be manufactured in compliance with good manufacturing practice so that, under normal or foreseeable conditions of use, they do not transfer their constituents to food in quantities which could: (a) endanger human health; (b) bring about an unacceptable change in the composition of the food; (c) bring about a deterioration in the organoleptic characteristics thereof.

A similar philosophy on the need for controls operates in the United States, Japan, and in other countries although the detailed legislative and technical instruments used differ. Extensive references to the legislation in Europe, the USA, and other countries can be found in Ref. [1].

18.3.2 What Residues Need Testing For?

As mentioned previously, a range of different chemicals are needed to make materials intended for food contact. There are several thousands of chemicals in inventory lists used by producers and of these probably several hundred chemicals find regular use. They include monomers and other starting substances needed to make plastics, catalysts, and production aids to make plastics and paper, additives to modify the properties of the finished products, ingredients of inks and adhesives, etc. Since most chemical migration is a diffusion phenomenon, it is the small, low-molecular-weight substances that tend to migrate fastest. This is certainly true for the monomers used to make high-volume plastics and coatings such as vinyl chloride, 1,3-butadiene, acrylonitrile, and styrene. Additives on the other hand must remain in the finished material in order to have a technical effect and so they tend to be higher-molecular-weight substances to reduce their loss. Finally, as producers strive to make materials with lower migration properties, they are turning to so-called polymeric additives of molecular weight of 1000 Da or more. This range of substances means in turn that the full range of analytical methods are deployed in testing for these residues; with headspace gas chromatography–mass spectrometry (GC-MS) for the volatiles, GC-MS for the semivolatiles, and increasingly liquid chromatography (LC)-MS for the nonvolatiles and the polar residues. The detection level needed depends on the toxicological or organoleptic properties of the substances, but typically it is in the range of a few parts per million (ppm, mg/kg) down to ca. 10 parts per billion (ppb, μg/kg) in the food.

18.4 TESTING STRATEGIES

The food itself can be tested for undesirable chemical residues. Alternatively, the packaging material can be tested before it is used to ensure that it does not contain residues that could migrate at levels that could cause problems. Finally, uniquely for food contact materials, the packaging can be tested for its suitability before use by employing food simulants that are intended to mimic the migration properties of different categories of foods.

18.4.1 Overall Migration and Total Extractables

By way of an example, the EU plastics directive imposes an overall migration limit to ensure that materials are acceptably inert and do not transfer large quantities of substances which, even if they are not unsafe, could bring about an unacceptable change in the food composition. The total amount of all migrating substances is limited to 60 mg/kg of food. This is tested for using food simulants and a set of test methods is available as European standards. Because a test for overall migration using food simulants is entirely conventional, i.e., the test result depends on the method used, the standard test procedures have to be used and followed exactly. In countries such as the United States and Japan suitability end tests of materials may use extraction solvents rather than food simulants.

18.4.2 Specific Migration Limits

Again, by way of an example, the EU plastics directive contains a positive list of monomers and additives permitted for use in the manufacture of plastic for food contact. This list contains limits on the migration of individual or groups of substances—limits that have been assigned following the toxicological assessment of these substances. Similar lists exist such as the National Legislation in European and other countries for the chemical ingredients used to make paper, silicones, inks, adhesives, coatings on metal, etc. The form of any restrictions—as specific migration limits or limits on the solvent-extractable substance or on the total content in the material—differs from country to country and for different material types.

18.4.3 Extraction Tests Followed by Estimation of Migration Levels

Compliance of a material with a specific migration limit or some other migration restriction can be tested by extracting the material to determine the concentration of the substance(s) of interest. Alternatively, the concentration in the packaging ($c_{p,0}$) may be available from formulation details provided by the producer. Then the migration expected into food can be estimated either by assuming total mass transfer (a worst case 100% migration scenario) or by using mathematical models. A number of commercial and freeware software packages are available to predict the extent of migration from the $c_{p,0}$ value. They have been validated for plastics only. All are based on the diffusion theory and a consideration of partitioning effects. The underlying key parameters are the diffusion coefficient of the migrant in the plastic (D_P) and the partition coefficient of the migrant between the plastic and the food or food simulant ($K_{P,F}$). These models have been tuned to provide an overestimation of migration in the majority of cases so that they can be used with confidence in compliance testing.

18.4.4 Using Food Simulants

Food simulants are an important tool for testing the suitability of materials for the food that are intended to be placed in contact with. Again, the EU system for plastics is taken as an illustrative example. Simulants intended to mimic the migration from plastics into foods were introduced in the early 1980s along with the rules for using simulants. Simulants are specified for the five food categories described earlier.

Food Type	Food Simulant
Aqueous foods of pH ≥ 4.5	A—distilled water
Acidic foods of pH < 4.5	B—3% acetic acid solution
Alcoholic foods	C—10% ethanol solution (or higher)
Fatty foods	D—rectified olive oil or similar oil
Dry foods and frozen foods	No migration testing is specified

Simulants were introduced at a time when instrumentation and analytical methods were not available to test foods for all the substances of interest at detection levels of mg/kg to μg/kg. Simulants also provide a means to test for broad food categories rather than having to test individual food items. However, as methodology and instrumentation have advanced, our ability to measure migration into foods has evolved rapidly. It has become clear that in some circumstances the simulants may not overestimate migration (as designed) but may underestimate migration into foods, and so some revision is necessary. For example, under some circumstances, there can be measurable migration into dry and frozen foods and a simulant may need to be specified. Similarly, some low-fat foods such as milk can elicit higher migration than conventional simulants and so this area may need revision too.

18.5 EXAMPLES OF PACKAGING TYPES AND ANALYTICAL METHODS

We shall demonstrate some packaging types and analytical methods by way of a commentary on some types of everyday packaging used for EABPs. For this, we have chosen the product category of stock, gravy powders, and similar preparations made using (among other ingredients) animal fat, bones, giblets, and the like.

18.5.1 Packaging Example 1—Figure 18.5

Layer 1a. Small plastic pot. These pots are normally made of thermoformed polystyrene or occasionally polypropylene. The relatively large ratio of contact area to food mass means that migration concentrations can potentially be high, although once the concentrated stock is diluted with water for use the concentration in the stock as consumed will be diluted accordingly. For polystyrene, the migrants of interest will be additives such as mineral oils used to help the plastic flow during thermoforming or injection molding as well as styrene monomer itself.

FIGURE 18.5 Stock concentrate in plastic pots.

Plastics are the most commonly used material type for packaging foodstuffs and EABPs are no exception. Examples of plastics used to package chilled foods such as offal include containers made of high-density polyethylene, polypropylene, or polystyrene. The film lidding materials used with these containers are usually polyester or ethylene vinyl acetate copolymer, often laminated to aluminum foil. If packed in store, offal would often be in an expanded polystyrene tray with a paper-based soak pad to absorb excess liquid and overwrapped with a plasticized PVC clingfilm. The different types of plastics and the typical monomers and additives used in their production have been reviewed elsewhere (see Ref. [2]). Analytical method example 1 describes a typical procedure for testing for a volatile migrant, in this case styrene monomer from individual portion packs of stock concentrate.

Layer 1b. Foil lidding. The metal foil lid on the pot has a thermoplastic heat-sealable coating. This could be coated onto the meal foil as a waterborne dispersion or a solvent-based coating or it could be extrusion-coated from the melt. Being naturally flexible and not exposed to light or to view, this coating does not need high levels of additives (such as plasticizers or light stabilizers) nor decorative inks and so the migration potential is low. Metals are generally inert but they can suffer from corrosion by moist or liquid foods and especially those that are acidic or high in salt. The heat-sealable plastic layer will provide a degree of protection of the aluminum foil in this case.

Layer 2. Cartonboard box. Unbleached cartonboard box plus adhesives and inks. In this example, the main food packaging is likely to provide an effective barrier preventing migration from the outer box. As a general rule, however, the nature and chemical composition of indirect packaging and secondary packaging does need to be considered to ensure that they do not contaminate the food, especially by low-molecular-weight volatile substances that can migrate via the gas phase.

18.5.1.1 Analytical Method Example 1—Testing for a Volatile Migrant Using Headspace-GC-MS: Styrene—Figure 18.6

Purpose: Individual packs of food packed in polystyrene are tested for migration of styrene.

Procedure: The following is an outline of a procedure used in our laboratories. The product is mixed and poured directly (5 g) into a headspace vial (10 mL capacity). The samples are analyzed in three ways: (a) as received (no additions), (b) with a d_8-styrene internal standard added at 200 µg/kg (duplicates), and (c) with d_8-styrene internal standard added at 200 µg/kg and with a 100–600 µg/kg spike of styrene added (all in duplicate). The specimens are incubated at 90°C for 30 min and a portion of headspace gas (1 mL) is analyzed by GC-MS. Mass spectrometric detection is conducted in the selected ion mode (*m/z* 78 and 104 monitored for d_0-styrene and *m/z* 84, and 112 monitored for d_8-styrene).

Results: The concentration of styrene in the foodstuff is then quantified using the standard addition calibration curve generated by plotting the peak area ratio of the d_0-styrene to the d_8-styrene against the concentration of styrene added to the sample and extrapolating the curve obtained. In

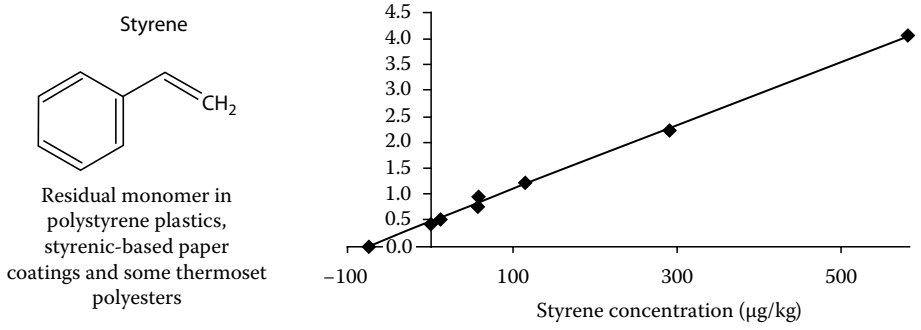


FIGURE 18.6 Styrene—HS-GC-MS standard addition line (ion 104 for styrene versus 112 for d_8-deuterated styrene, internal standard) in a fatty food matrix—the extrapolated intercept shows the sample-contained styrene at ca. 75 µg/kg.

this example (Figure 18.6), the interception of the standard addition line indicates that the sample contains styrene at 75 µg/kg. The presence of styrene should be confirmed on the basis of the GC retention time and by the ratio of the *m/z* 78 and *m/z* 104 ions.

Interpretation: If high migration levels are determined for such products, it is probably due to the high ratio of surface area to volume for these small pack sizes and the long storage time. High migration of styrene can also be attributed to the high fat content of the stock and the nonpolar (lipophilic) nature of styrene. Polystyrene plastics contain a rather high residual monomer content (ca. 500 mg/kg in the plastic) that cannot be removed completely by vacuum stripping, because styrene can be reformed on thermal processing into materials and articles by the unzipping of the polystyrene chain.

18.5.2 Packaging Example 2—Figure 18.7

Layer 1a. Spiral-wound body. In this example, the body of the container is made of a strip of brown (unbleached) paperboard that is spiral wound to make the cylinder. The paperboard will probably have either a very thin plastic layer or some chemical treatment to make it grease resistant. Neither a thin plastic layer nor a chemical treatment will completely prevent migration from the paperboard. Commonly, the paperboard is made using recovered (recycled) paper and this can contain chemical residues from inks and adhesives that the recycling process does not remove completely. Because of their high surface area and fat content, gravy powders and granules can cause high migration. Problems found in the past have been the migration of diisopropylnaphthalenes (DIPNs), mineral oils, and phthalates, especially dibutyl phthalate (DBP) and di-2-ethylhexyl phthalate (DEHP).

In these pack formats, the strip of spiral-wound paperboard may have an aluminum foil as the inside layer. This does help to reduce the potential for migration although the process of winding the cylinder does not provide a complete barrier of foil.

Layer 1b. Peelable film lid. The film lid is a thin sheet of bleached paper with an even thinner film of thermoplastic so that the lid can be heat sealed onto the container body.

Layer 1c. Container bottom. In this example, the circular bottom of the container is also paperboard (although this is not shown in the photograph). It is white and clearly a different grade compared to

FIGURE 18.7 Gravy powder in cartonboard.

the paperboard used to make the body. Spiral-wound paperboard containers can also be fitted with metal bottoms.

Layer 1d. Resealable plastic lid. As the consumer starts to use the product, the disposable lidding is removed and the resealable plastic lid is used in its place. The lid is low density polyethylene. It does not make direct contact with the gravy product unless the container is inverted or placed on its side. Gas-phase migration is possible but the migration potential is generally low.

Layers 2 etc. Inks etc. As with most packaging, the outside of this container is heavily decorated with inks and an over-varnish has been applied. The ink and the varnish may be cured conventionally or may be cured using UV light. Residues of UV photoinitiators may persist and permeate through some paper and board packaging materials. Analytical method Example 2 describes a typical procedure for testing foods for benzophenone.

18.5.2.1 Analytical Method Example 2—Testing for a Semivolatile Migrant Using GC-MS: Benzophenone—Figure 18.8

Purpose: Foods packaged in printed cartonboard or printed plastics or plastic films with a printed label attached are tested for any migration of inks components, in this case, the uv-photoinitiator chemical benzophenone.

Procedure: The following is an outline of a procedure used in our laboratories. A specimen of the food (5.0 g) along with internal standards (1.5 µg d_{10}-benzophenone) is extracted twice by shaking with acetonitrile:dichloromethane (1:1, 10 mL). The extract is evaporated to dryness and then partitioned between hexane (5 mL) and acetonitrile (5 mL) to remove the fat. The acetonitrile extract is analyzed using GC-MS, monitoring ions *m/z* 77, 105, and 182 for benzophenone and *m/z* 110 and 192 for the internal standard (Figure 18.8). Benzophenone is confirmed as present if it meets three criteria: (a) ion ratios for *m/z* 182/77 and 182/105 to be within ± 20% of standards, (b) retention time relative to internal standard within ± 2% of standards, and (c) the full scan mass spectrum should contain no additional ions (<20%) not seen in standards.

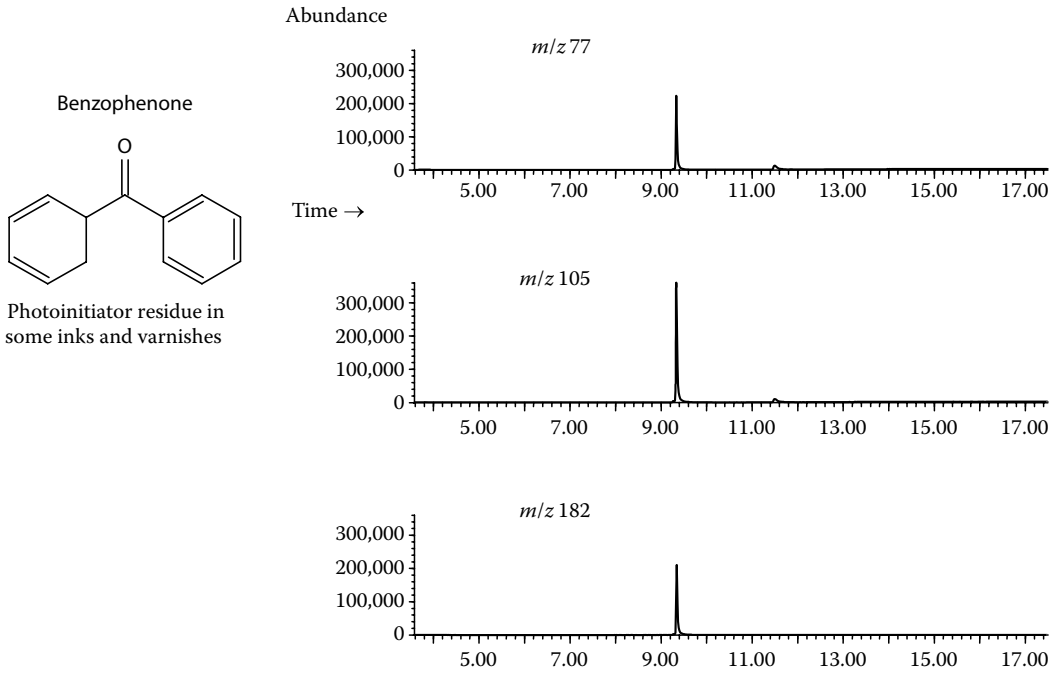


FIGURE 18.8 GC-MS traces for benzophenone in a spiked high-fat sample.

Results: The limit of detection is 0.04 mg/kg or better, depending on food type. In a survey, benzophenone was present in 61 of the 350 samples (17%) packaged in cartonboard. The mean concentrations ranged from 0.029 to 4.5 mg/kg.

Interpretation: In the analysis of foods of animal origin, the ability to isolate and preconcentrate the analyte(s) of interest is often limited by the fat content of the sample. In this example, advantage is taken of the differential solubility of the analyte (benzophenone) and fat in the two immiscible liquids acetonitrile and hexane. Most of the positive samples identified in the aforementioned survey were in printed cartonboard. Interestingly, one of the samples tested was gravy granules packed in a spiral-wound cylindrical tub. The migration was very low and the concentration of benzophenone in the gravy granules was just 0.01 mg/kg. This was so despite the fact that the area of board was 475 cm^2, the board contained benzophenone at 9.3 $\mu g/cm^2$, and the food weight was 500 g. So there was the potential for migration (total mass transfer) of up to 8.8 mg/kg. The fact that only less than 0.1% did migrate was interpreted as being because the board was especially dense and thick at 740 gsm (grams per square meter), which was by far the highest grammage of the samples tested. Therefore, it seems likely that the benzophenone had not permeated through the thick board from the printed outside to the inside to be available for migration.

18.5.3 Packaging Example 3—Figure 18.9

Layer 1a. Glass bottle. Glass is considered to be an inert material, and this is generally true although it does depend on the grade and chemical composition of the glass. Glass itself is a completely inorganic material and so if any leeching of constituents could occur then the main food types are acidic and high salt foods. Some glass containers may be surface treated with organic reagents. The most common is treating the outside of bottles and jars to provide scuff and scratch resistance for refillable bottles or to reduce friction and scratching as bottles jostle together on fast-filling lines.

Layer 1b. Metal lid with an internal coating and plastic sealing gasket. Although the lid is at the top of the container, it does make direct contact with the food contents during transportation and handling, as the picture illustrates. For a sticky product, as in this case, once the lid is fouled with food, it does not drain away quickly and so migration is a possibility. Gaskets have been problematic in the past with high migration of plasticizers, epoxidized soybean oil (ESBO) and

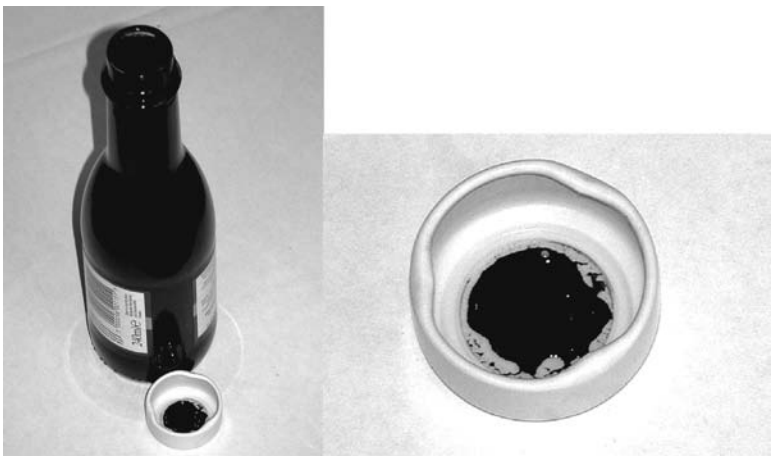


FIGURE 18.9 Gravy mix in glass bottle.

semicarbazide (SEM). The gasket must be deformable and elastic to provide a microbiologically safe seal. In some aspects, therefore, the gasket behaves like a viscous liquid and high migration can occur, especially in the case where there is high temperature processing of food in glass jars with metal lids.

The use of metals to package EABPs is rather limited. Examples include aluminum foil—either plain or varnished or laminated to plastic and paper films, metal closures for bottles and jars, and some food cans. In most cases, the metal food can or lid is coated. This coating is intended to form a barrier between the food and the metal surface. The coating protects the food from the metal substrate as well as the metal substrate from the potentially corrosive foodstuff contained within.

The major types of can coatings are made from epoxy resins. These coatings exhibit a combination of toughness, adhesion, formability, and chemical resistance under the conditions that the coated metal is subjected to. As well as the epoxy resins, hardeners, such as acid anhydrides, aminoplasts, or phenolplasts may also be included in the formulation as well as additives, such as pigments, fillers, wetting and flow aids, defoamers and lubricants, and any reaction/breakdown products formed from these starting materials. As mentioned previously, migration is influenced by both contact temperature and time. Most canned foods are sterilized (e.g., at 121°C for 1 h) and they also have long shelf lives (up to several years) so the migration conditions in canning are severe. Consequently, coatings manufacturers are constantly striving to produce "cleaner coatings" with fewer low–molecular-weight migratable substances. Analytical method Example 3 describes a typical procedure for testing for a specific substance of interest—bisphenol A.

Layer 2–4. Adhesive label (*adhesive, paper, inks*). Since glass is an effective functional barrier to migration, the label and the adhesive and ink used on the label do not need to be considered for their migration potential.

18.5.3.1 Analytical Method Example 3—Testing for a Fluorescent Migrant Using HPLC-FLD: Bisphenol A—Figure 18.10

Purpose: The internal surface of metal food cans and closures (lids, caps) used on bottles and jars are commonly protected using an epoxy-phenolic coating ("lacquer"). Any residues of incomplete polymerization of this coating may migrate into the food product. In this example, the migration of bisphenol A (BPA) is tested for. BPA is used in the manufacture of the bisphenol A diglycidyl ether starting substance used in the preparation of the epoxy can coating.

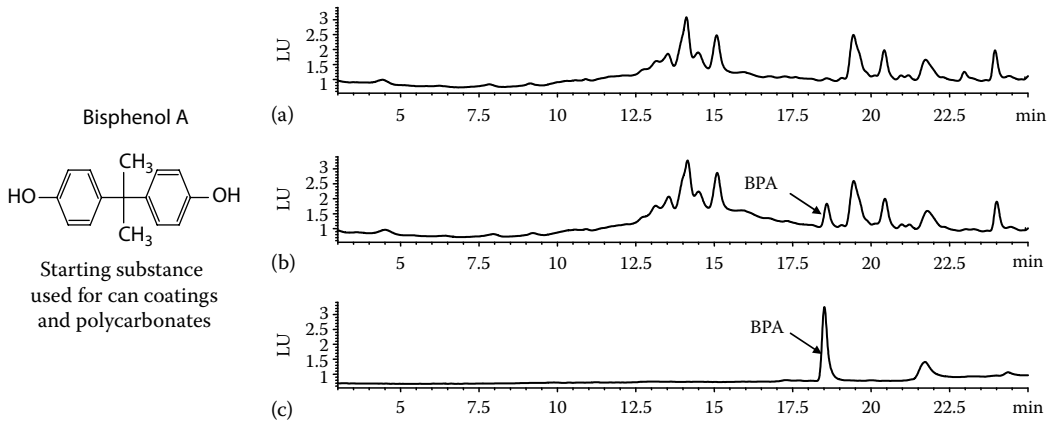


FIGURE 18.10 HPLC-FLD chromatograms of (a) sample extract, (b) sample spiked with BPA at 2 μg/kg, and (c) solvent standard of BPA.

Procedure: The following is an outline of a procedure used in our laboratories. A portion of the food is extracted with acetonitrile. The extract is evaporated to dryness under a gentle stream of nitrogen and reconstituted in a 1:1 mix of water:acetonitrile. The solvent is separated from the foodstuff and is frozen to precipitate the fat from the solution. The supernatant is analyzed by high performance liquid chromatography with fluorescence detection (excitation 235 nm, emission 317 nm). Duplicate samples are extracted and analyzed. Overspiked samples, spiked with BPA at 0.5, 1, 2, and 20 µg/kg (each in duplicate) are extracted and analyzed in the same way.

Results: In this example (Figure 18.10), the solvent standard establishes the retention time for BPA at 18.5 min (lower panel). The extract of the food sample shows no evidence of BPA at this retention time (upper panel). Spiking the food sample with BPA at a level of 2 µg per kg demonstrates that the test method would detect BPA if it were present (middle panel).

Interpretation: The detection limit is estimated to be 1 µg/kg or better. It is concluded that the sample contains no detectable BPA, no more than 1 µg/kg. If the sample was tested positive and if the quantitative result needed further investigation, then LC-MS would be used and an isotope-labeled internal standard would be added before the food sample was extracted with solvent. The use of MS as the detector gives extra confidence in the correct identification of BPA in the sample.

18.5.4 Packaging Example 4—Figure 18.11

Layers 1-x. Multilayer packaging material. Many types of packaging materials consist of more than one layer. This is especially true for flexible packaging films where the combinations of toughness for protection, barrier properties against gases (e.g., modified atmosphere packaging) or odor, printability, heat sealability, economy, etc., can be provided by combining several layers in a multilayer structure. The layers may be joined by coextrusion processes (from the melt) by coating processes or by lamination of films using adhesives. As well as the potential migrants derived from the individual materials that make up the different layers, the potential also exists for migration of components

FIGURE 18.11 Liquid stock in a sachet with a bottom gusset (also known as a stand-up pouch).

present in any adhesives used. Typically, reactive adhesive systems are used that are polymerized *in situ*. They include polyurethanes and to a lesser extent epoxy adhesives. A very common multilayer film would be nylon or polyester (for toughness and barrier properties) laminated using reactive polyurethanes to a polyethylene film (for heat sealability) and printed on the outside or reverse printed with inks inside the laminate sandwich (for decoration and consumer information). Any residues of incomplete polymerization of the adhesive or any reaction by-products may remain in the food contact material and may then migrate into a foodstuff on contact. Analytical method Example 4 describes procedures for testing foods and food simulants for primary aromatic amines (PAAs) that are hydrolysis products of some aromatic isocyanates used to make polyurethane adhesives.

Some food products are retorted at high temperature in the pouch. These retortable pouches clearly need to withstand these high temperatures, and also, given the strong influence of temperature on migration, must be formulated in a way that does not give migration under the demanding conditions of use.

18.5.4.1 Analytical Method Example 4—Testing for a Decomposition Product Using a Colorimetric Test Backed Up by LC-UV or LC-MS: Primary Aromatic Amines—Figure 18.12

Purpose: PAAs may be migrate from multilayer packaging films and from colored food contact articles as impurities of azo dyes. Many PAAs are toxic, some have carcinogenic potential to harm humans, and so the migration of PAAs into food is subject to very strict legal limits.

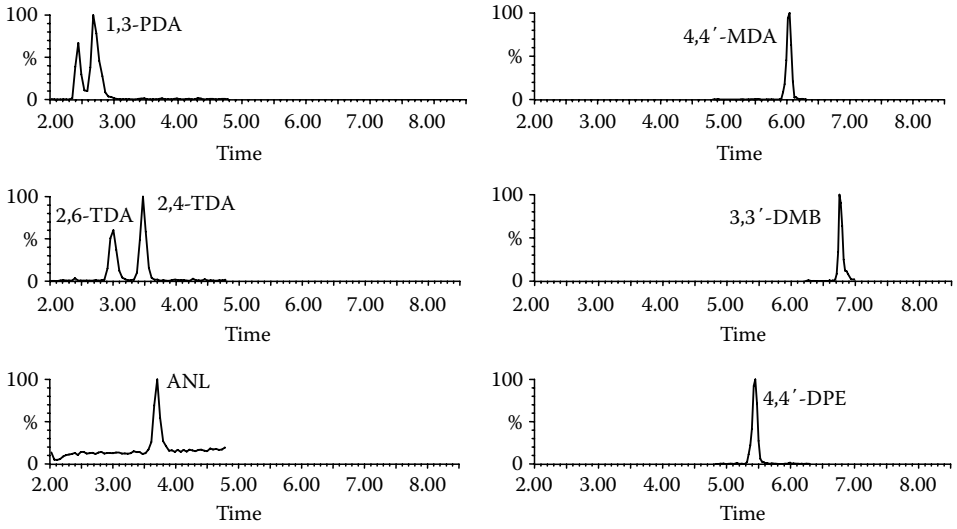
Procedure: The analysis consists of two parts. Part A is applicable only to simple food simulants. It is a colorimetric screening method to estimate the total concentration of all PAAs. PAAs are subjected to diazotation using hydrochloric acid and sodium nitrite. Ammonium sulfamate is added to protect the nitrosated PAAs that are then coupled with N-(1-naphthyl)-ethylenediamine dihydrochloride to give a purple-colored derivative. This is concentrated on a solid-phase extraction (SPE) column and the color estimated by UV spectrometry at 550 nm. Calibration is realized using aniline. The method may be subject to interferences, and so it can pass a sample (a negative result), but any positive result must be confirmed using a more specific method of test.

Part B is an HPLC-based method for confirmation purposes using either UV (for simulant analysis only because of interferences) or using LC-MS (for simulants and foods). Aqueous simulants from the migration test are concentrated using a cation-exchange column and the concentrated solutions analyzed by HPLC with UV detection. Low detection limits are achieved by using a large injection volume and concentration of this volume by a precolumn back-flush procedure. Confirmations of identity of the PAAs are carried out by diode array detection.

As an alternative to UV, LC-MS can be used. The LC-MS method can determine up to 30 PAAs of interest with acquisition in the multiple reaction monitoring (MRM) mode using the primary $M^{+\cdot}$ (positively charged molecular ion) or the MH^+ (protonated molecular ion) as the precursor ions. Figure 18.12 shows the structures and the LC-MS traces for some of the PAAs most commonly found in our laboratories.

Results: In a small survey of commercial films, all of the samples were within legal limits. An undercured film was obtained from a manufacturer and the migration of certain PAAs (derived from aromatic isocyanates) was high. As expected, considering the mode of formation of PAA from isocyanate precursors and considering also the basic nature of PAAs, the simulant 3% acetic acid gave the highest test results.

Interpretation: The results indicated the importance of allowing full cure to take place before the laminate is used to pack food. During the cure process, the isocyanates react with polyols to make the polyurethane adhesive stick. If there is significant unreacted isocyanate, it can hydrolyze to PAA and contaminate the packed food. The recovery of certain PAAs spiked into fatty foods was low, and this indicates that reaction with food components such as free fatty acids may occur.



Name	Abbrev.	Structure	MW
1,3-Phenylenediamine	1,3-PDA	a	108.1
2,6-Toluenediamine*	2,6-TDA	b	122.1
2,4-Toluenediamine*	2,4-TDA	c	122.1
Aniline	ANL	d	93.1
4,4′-Methylenedianiline*	4,4′-MDA	e	198.3
3,3′-Dimethylbenzidine	3,3′-DMB	f	285.2
4,4-Diaminodiphenylether	4,4′-DPE	g	200.2

* The methylene diphenylisocyanate (MDA) and toluene diisocyanate (TDA) isomers are the residues most commonly found. 2,2′-methylenedianiline and 2,4′-methylenedianiline are not readily available as analytical standards, and so they are normally made as standards by the hydrolysis of the corresponding diisocyanates.

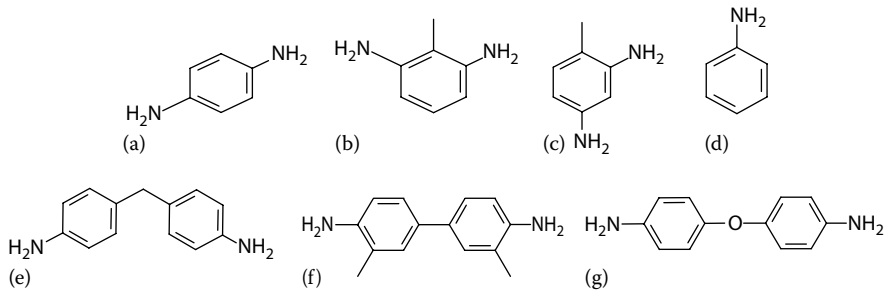


FIGURE 18.12 LC-MS traces (selected ion vs. retention time) for the PAAs derived from hydrolysis of the aromatic isocyanates used in some packaging adhesives.

18.5.5 Packaging Example 5—Figure 18.13

18.5.5.1 Testing for the Unexpected

As well as testing for known ingredients used to make food contact materials, a proper safety assessment must go further. For example, the fourth amendment to Directive 2002/72/EC includes the explicit provision that there is a general requirement to assess the safety of all potential migrants. This includes what have become known as the non-intentionally added substances (NIASs) such as impurities, reaction, and breakdown products. The example of PAAs used above is one such case of a type of NIAS. The onus is placed on the business operator to make this assessment. Again, although this directive is applicable only to plastics, it can also be used as a guide for other food contact materials. To demonstrate their safety, these NIASs should be assessed in accordance with international risk assessment procedures. Figure 18.13 illustrates the complexity of chemicals that can be extracted from FCMs. In this case, a paperboard sample was extracted but similarly complex mixtures can be obtained from plastics, coatings, adhesives, and inks. Depending on the food contact application, some of these chemicals may migrate.

A risk assessment of the NIAS should have three components: (a) identification of the substances present in the material, (b) estimation of their migration level leading to an estimate of possible consumer exposure, and (c) risk assessment that considers the potential exposure in context with any hazard (nature and potency) posed by the chemical. This requirement to identify substances places emphasis on the information-rich separation techniques using mass spectrometry as the detection system; i.e., GC-MS and LC-MS (/MS). Increasingly, testing laboratories will turn to LC-time-of-flight (TOF)-MS to get accurate mass information on molecular ions and fragment ions to gain further confidence in substance identification. Analytical method Example 5 describes procedures for testing for NIASs.

18.5.5.1.1 Analytical Method Example 5: Identifying Potential Migrants Including Unknowns in an FCM—Figure 18.14

Purpose: When assessing the safety of food contact materials, the concentrations of both known ingredients and any impurities, reaction products, or breakdown products (non-intentionally added substances or NIASs) migrating into the food or food simulant should be considered.

Procedure: The following is an outline of a procedure used in our laboratories. Identification of the potential migrants can be achieved through the application of a suite of analytical methods focusing on the analysis of substances with molecular weights below 1000 Da. This molecular weight

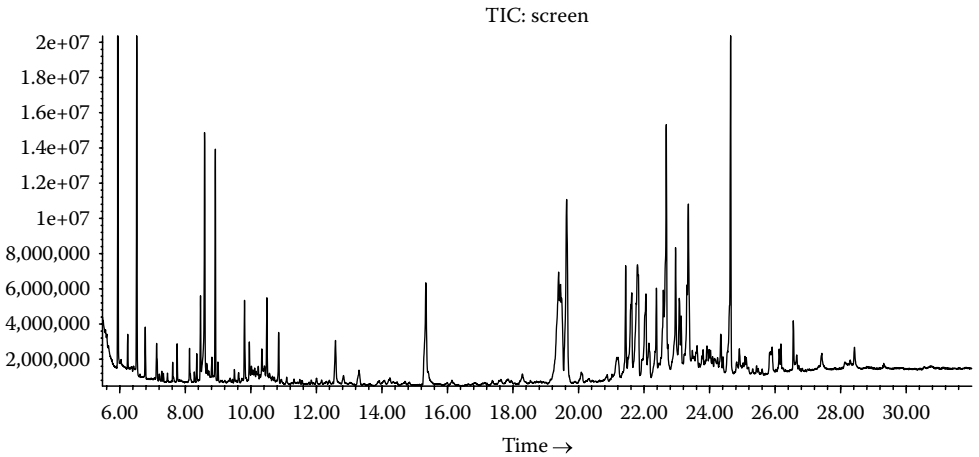


FIGURE 18.13 GC-MS total ion chromatogram obtained from the analysis of an ethanol extract of a food contact paper/cartonboard.

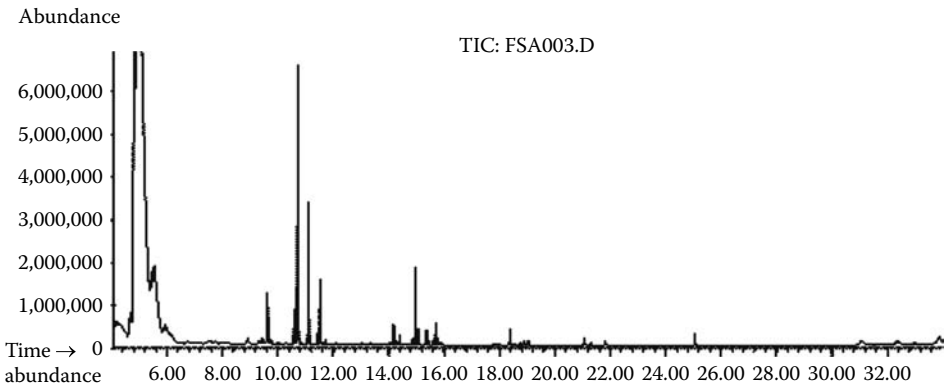


FIGURE 18.14 Thermodesorption GC-MS chromatogram obtained from the analysis of the polymer.

cutoff is chosen in view of toxicological significance—larger molecules tend not to be absorbed in the stomach or the GI tract. Analysis of solvent extracts of an FCM is made using thermodesorption GC-MS to detect very volatile substances, GC-MS and GCxGC-time-of-flight (TOF)-MS to detect semivolatile substances and liquid chromatography (LC)-TOF-MS, or LC-Fourier transform (FT)-MS to detect more polar and nonvolatile substances.

Results: To assess the safety of an FCM, the analytical suite of methods described above was applied. A thermodesorption GC-MS chromatogram obtained from the analysis of the FCM is shown (Figure 18.14). GC-MS and LC-TOF-MS chromatograms obtained from the analysis of solvent extracts of the same material were also obtained (not shown). Each of the peaks in the chromatograms corresponds to substances in the FCM. These were identified using library spectra for GC-MS and by the accurate mass determination for LC-TOF-MS or LC-FT-MS.

Interpretation: In this example, many substances were detected and identified. For illustration, consider the presence of two. Butanol was detected at low levels. It was not a known ingredient of the plastic. It was attributed to either an impurity or a breakdown product derived from the additive acetyltributylcitrate used as a plasticizer in the plastic tested. 2-Ethylhexanol was also detected at low levels. It was attributed to either an impurity or a breakdown product derived from the additive di-*n*-octyl-bis(ethylhexylthioglycolate) used in the plastic formulation as a stabilizer.

18.6 OTHER PACKAGING CONCEPTS

18.6.1 Active and Intelligent Packaging

One of the most innovative developments in food packaging in recent years is the use of active and intelligent packaging. Active packaging materials can be defined as "*food packaging which has an extra function, in addition to that of providing a protective barrier against external influence*." It is intended to change the condition of the packed food, to extend shelf life or improve sensory properties while maintaining the freshness and the quality of the food. This can be achieved through the removal (scavenging) of substances that have a detrimental effect on food quality. Examples of active absorbers and scavengers include

- Oxygen scavengers
- Moisture absorbers
- Ethylene and off-flavor scavengers
- Acetaldehyde scavengers
- Amine scavengers
- Sulfide scavengers
- Bitter taste removers

Active packaging systems can also aim to emit substances that improve the foodstuff. Examples of active releasing substances include

• Carbon dioxide regulating systems	• Antioxidant releasers
• Antimicrobial releasing systems	• Sulfur dioxide releasers
• Nitrogen releasers	• Flavor releasers

Intelligent packaging materials can be defined as, "*concepts that monitor to give information about the quality of the packed food*." Examples of monitoring systems used in food contact applications include

Time and/or temperature indicators	Oxygen indicators
Freshness and ripening indicators	Carbon dioxide indicators

Consequently, for active packaging, the packaging is intended to influence the food and for intelligent packaging, the food is intended to influence the packaging. Intelligent packaging systems to more closely monitor and indicate the freshness and shelf life of packs may have a role to play in ensuring freshness while reducing food waste. These packaging concepts do cost money of course, and they are currently limited mainly to food products that can command a price premium and those that are perishable and have a short shelf life. They have limited application at present for EABPs. See Ref. [2].

18.6.2 Surface-Active Biocides

A number of products have come into the market in recent years with surface biocidal properties. These include conveyor belts, cutting boards, and the inside linings of commercial and domestic refrigerators. These surface-active biocidal materials should not be confused with active packaging because there is no intention that the biocidal agent has any preservative effect on the food. Rather, the intention is that the biocide remains in the food contact material, perhaps concentrated at the surface, and improves the surface hygiene and cleanability. Surface-active biocidal materials may have benefits especially for parts of complex food processing machinery that are difficult to clean effectively in place (in situ).

A common biocide used for this is silver in a number of chemical forms. Silver ions are antimicrobial to all microbial species that are likely to be found in a food environment, including against Gram-negative bacteria, Gram-positive bacteria, moulds, and yeast. Another biocide used is 2,4,4′-trichloro-2′-hydroxydiphenyl ether that seems to have a less uniform activity against bacteria, moulds, and yeast.

Although these surface-active biocides are not intended to migrate into the food and exert any preservative effect, some level of migration is inevitable and should be tested for as for any other substance used in food contact materials. In the two examples given, the inorganic silver compounds may be expected to migrate mostly into aqueous and acidic foods, whereas the organic substance 2,4,4′-trichloro-2′-hydroxydiphenyl ether is expected to migrate more into fatty foods.

Since these materials are intended to complement and not replace normal cleaning and hygiene procedures, hard facts and data on efficacy are difficult to find, especially in the open literature.

18.6.3 Nanomaterials

Developments in nanotechnology promise a technological revolution in a number of industrial sectors. Nanotechnology represents a broad assemblage of processes, materials, and applications that span physical, chemical, biological, and electronic science and engineering fields. The common theme is that they involve manipulation of materials at a size range in the nanometer scale. Materials smaller than about 100 nm in one or more dimension are collectively termed as nanomaterials.

Nanomaterials could help meet some of the performance requirements of food packaging materials and other materials used in food contact such as machine parts, valves, etc. Some nanotechnology-derived products are near to a commercial reality. Examples include increased mechanical properties to allow further light weighting and using nanocomposites and nanocoatings to improve gas barrier properties. There are also a number of nanoproducts under development for active packaging as described above, as releasers or absorbers of chemicals or as intelligent (smart) packaging materials using nano-based sensors. The same comment made for active packaging above can also be made here for nanomaterials; they will cost money of course, and it seems unlikely that packaging for EABPs will be in the vanguard of such applications. See further reading [3].

18.7 CONCLUSION

Chemical residues may occur as a result of chemical migration from food contact materials, of which food packaging materials are the most important example. The analysis of the food for these chemical residues uses basically the same chemical analytical methods that are in the food analysts' armoury. What makes the topic special is the added dimension of needing to analyze also the food packaging materials themselves (to indicate what chemical(s) may migrate) and the analysis of food simulants used to test materials for their suitability for contact with different types of foods.

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19 Growth Promoters

Milagro Reig and Fidel Toldrá

CONTENTS

19.1 INTRODUCTION

Growth promoters include a wide range of substances that are generally used in farm animals for therapeutic and prophylactic purposes. These substances can be administered in the feed or in the drinking water. In some cases, the residues may proceed from contaminated animal feedstuffs.[1] Anabolic promoters have been administered in the United States to meat-producing animals, where estradiol, progesterone, and testosterone are some of the allowed substances. The regulations in 21 Code of Federal Regulations Part 556 provide the acceptable concentrations of residues of approved new animal drugs that may remain in edible tissues of treated animals.[2] Other countries allowing the use of certain growth promoters are Canada, Mexico, Australia, and New Zealand. However, the growth promotion of farm animals through the use of substances having a hormonal action (17β-estradiol, testosterone, progesterone, zeranol, trenbolone acetate, and melengestrol acetate) was prohibited through Directive 81/602/EEC while the use of growth promoters including the administration of synthetic and/or natural hormones was officially banned in the European Union through Directive 88/146/EC due to concerns about harmful effects on consumers.[3,4]

Growth promoters increase growth rate and improve efficiency of feed utilization and thus contribute to the increase in protein deposition that is usually linked to fat utilization, which means a reduction in the fat content in the carcass and an increase in meat leanness.[5–8] In addition, some fraudulent practices consist in the use of low amounts of several substances like β-agonists (clenbuterol) and corticosteroids (dexamethasone) and/or anabolic steroids, mixtures known as "cocktails," that have a synergistic effect and exert growth promotion[9] but making its analytical detection more difficult.

The presence of residues of growth promoters or its metabolites in meat and edible by-products as well as its associated harmful health effects on humans makes necessary the continuous

improvement of analytical methodologies in order to guarantee consumer protection. The use of growth promoters and veterinary drugs in food animal species is strictly regulated in the European Union, and, in fact, only some of them can be permitted for specific therapeutic purposes under strict control and administration by a veterinarian.[10]

Sanitary authorities in different countries are concerned about the presence of residues of growth promoters and veterinary drugs or its metabolites in edible by-products because they may exert some adverse toxic effects on consumers' health. The European Food Safety Authority has recently issued an opinion about substances with hormonal activity, specifically testosterone and progesterone, as well as trenbolone acetate, zeranol, and melengestrol acetate. The exposure to residues of the hormones used as growth promoters could not be quantified. Although epidemiological data in the literature provided evidence for an association between some forms of hormone-dependent cancers and red meat consumption, the contribution of residues of hormones in meat could not be assessed.[11] Other substances like β-agonists have shown adverse effects on consumers. This was the case of intoxications in Italy, with symptoms described as gross tremors of the extremities, tachycardia, nausea, headaches, and dizziness, after consumption of lamb and bovine meat containing residues of clenbuterol.[12] A recent study was performed with 20 heifers that were each administered 3–4 implants containing trenbolone acetate and then slaughtered 30 days after the implant. The analysis showed that residual content of 17 α-trenbolone in liver was around 4 ng/g, which was 10 times higher than residues levels of 17 β-trenbolone found in muscle.[13] The knowledge of the metabolism of an illegal substance is very important to understand its degradation pathways, its accumulation in certain tissues and thus to optimize the analytical methodology.[14] It is quite often that parent substance cannot be analyzed because of a rapid metabolism in other derived substances. This depends on the matrix to be analyzed.[15]

Liver and other by-products must be monitored for the presence of veterinary drug residues. Control strategies also include sampling at farm that helps to prevent on time before animals reach the slaughterhouse. This means samples of hair and urine as well as feed and water. This chapter reports the main strategies for the control of growth promoters as part of the wide range of veterinary drugs residues in edible animal by-products. The analysis of growth promoters in meat and processed meats has been recently reviewed[16–18] while the analysis of antibiotic residues will not be discussed in this chapter as it is the object of the following chapter.

19.2 MAIN GROWTH PROMOTERS

Steroid hormones and other substances having hormonal action. (Testosterone, progesterone, trenbolone acetate, zeranol, and melengestrol acetate). Substances that exert estrogenic (except 17β-estradiol and ester-like derivatives), androgenic, or gestagenic action. They may be used for growth promoting.

One of the misused steroids, sometimes considered as prohormone, is 1-testosterone (17β-hydroxy-5α-androst-1-en-3-one) has been reported to bind selectively to androgen receptor and has shown a high androgenic and anabolic potency in vivo with an increase in liver weight.[19] The use of precursor steroids (prohormones), which are potent androgen, may also have relevant anabolic properties but also have side toxic effects.

Synthetic hormones appear to bind to steroid receptors with equal or higher affinity than the natural hormones.[20,21] So, trenbolone mainly binds to the androgen receptor, zeranol to the estrogen receptor, and melengestrol that resembles natural progestins.[11] Maximum residue limit (MRL) have been established by national authorities and by the Codex Alimentarius. When analyzing these substances in meat, it is important to differentiate between the endogenous production and those residues present as a result from exogenous administration.[22]

Stilbenes (Diethylestilbestrol, dienestrol, and hexestrol). Substances consisting of synthetic non-esteroiedal estrogens that exert estrogenic activity (growth and development of female sexual

organs) and produce an increase of somatotropin secretion. Diethylestilbestrol, which was used as a feed additive or as an implant till the 1970s exerted a growth promoting effect and increased feed efficiency in cattle and sheep, was related to cancer and thus it was banned.[23]

Antithyroideal agents (thiouracyls). Substances able to interfere directly or indirectly on the synthesis, release, or effect of the thyroideal hormones (triiodothyronine T3 and thyroxine T4). These agents cause hypothyroidism by decreasing the basal metabolism rate, with water retention and weight increase, but their presence represents a risk due to their teratogenic and carcinogenic properties.[15]

β-*Agonists* (Clenbuterol, mabuterol, cimaterol, salbutamol, etc.). Substances used as therapeutic agents for respiratory disorders even though they have been illegally used as growth promoters because of their capacity to bind to β receptors of various tissues and change the carcass composition. In addition, these substances contribute to increased meat toughness because they reduce the proteolysis and tend to increase protein synthesis and lipolysis.[5]

Sedatives (carazolol, chlorpromazine, azarperone, and xylazine). Substances used for controlling the stress in farm animals as the main purpose. However, these substances can also induce some growth promotion by the redistribution of fat to muscle tissue after several weeks.

Corticoids (Corticoids dexamethasone, betamethasone, flumethasone, prednisolone, cortisone, desoxymethasone, and hydrocortisone). Substances primarily used as anti-inflammatory agent for therapeutic purposes but may also exert some growth promotion when used in combination with other hormones or β-agonists. They are hormones of the adrenal cortex, but synthetic corticoids also include derivatives of prednisolone.[22]

19.3 CONTROL OF GROWTH PROMOTERS

The monitoring of residues of substances having hormonal or thyreostatic action as well as β-agonists is regulated in the European Union through the Council Directive 96/23/EC[24] on measures to monitor certain substances and residues in live animals and animal products. The European Union Member States have set up national monitoring programmes and sampling procedures following this directive.

Main veterinary drugs and substances with anabolic effect are listed in Table 19.1, where group A includes unauthorized substances having anabolic effect and group B includes veterinary drugs some of them having established maximum residue limits (MRLs). Commission Decisions 93/256/EEC[25] and 93/257/EEC[26] gave criteria for the analytical methodology regarding the screening, identification, and confirmation of these residues. Council Directive 96/23/EC[24] was implemented by the Commission Decision 2002/657/EC,[27] which is in force since September 1, 2002. This directive provides rules for the analytical methods to be used in testing of official samples and specific common criteria for the interpretation of analytical results of official control laboratories for such samples. This means that, when using mass spectrometric detection, substances in group A would require 4 identification points (IPs) while those in group B would only require a minimum of 3. The number of IP depends on the technique. So, the triple quadrupole (QqQ) or ion trap can acquire 1 IP for the precursor or 1.5 IP for each product ion. The relative retention of the analyte must correspond to that of the calibration solution at a tolerance of ±0.5% for gas chromatography (GC) and ±2.5% for layer chromatography (LC). The guidelines given in this new directive also imply new concepts like the decision limit (CCα) or the detection capability (CCβ) that are briefly defined in Table 19.2. Both limits permit the daily control of the performance of a specific method qualified when used with a specific instrument and under specific laboratory conditions and thus contribute to the determination of the level of confidence in the routine analytical result. Each method used for the analysis of each specific analyte must be validated to demonstrate fitness for purpose and accomplish the regulations' requirements.[28]

TABLE 19.1
Lists of Substances Having Anabolic Effect Belonging to Groups A and B according to Council Directive 96/23/EC

GROUP A: Substances Having Anabolic Effect

1. Stilbenes
2. Antithyroid agents
3. Steroids
   - Androgens
   - Gestagens
   - Estrogens
4. Resorcycilic acid lactones
5. Beta-agonists
6. Other compounds

GROUP B: Veterinary Drugs

1. Antibacterial substances
   - Sulfonamides and quinolones
2. Other veterinary drugs
   - a. Antihelmintics
   - b. Anticoccidials, including nitroimidazoles
   - c. Carbamates and pyrethroids
   - d. Sedatives
   - e. Nonsteroideal anti-inflammatory drugs
   - f. Other pharmacologically active substances

Source: EC. Council Directive 96/23/EEC of 29 April 1996 on measures to monitor certain substances and residues thereof in live animals and animal products. *Off. J. Eur. Community L* 125: 10, 1996.

19.4 SAMPLING AND SAMPLE EXTRACTION

Preparation procedures and handling of samples are very important to improve the sensitivity of the screening tests.[29] Typical procedures include cutting, blending, and solid–liquid extraction procedures usually based on the homogenization of the product in an appropriate buffer. Extraction is mainly performed to remove interfering substances while retaining most of the analyte. Sample extraction must be performed very carefully and with optimized conditions in order to be sure of the correct extraction of the target analyte. Extraction solvents must be carefully chosen for each analyte as determined by pH, polarity, and solubility in different solvents. For instance, polar extraction methods for the determination of anabolic steroids are used because they avoid some cleanup problems when following nonpolar extraction, but they are insufficient. It has been reported that polar extraction followed by nonpolar extraction gives better results.[30] The homogenate is extracted with an organic solvent usually followed by a solid-phase extraction (SPE) for sample cleanup and concentration.

Previous to extraction, those residues that are present in the conjugated forms (i.e., as sulphates or glucuronides) need further cleavage by treatment with the juice of the snail *helix pomatia*, which has a mixture of sulfatase and β-glucuronidase, to release the free analytes. Other authors prefer enzymatic digestion with subtilisin to release steroids since they state that using enzymatic hydrolysis with *helix pomatia* may not reflect the conjugated fraction of steroids.[31] Enzymatic treatment assures a milder treatment than acid or alkaline hydrolysis. Those residues that are bound through weak interactions may need dialysis, proteolysis, or protein denaturation (heat or acid treatments) previous to extraction.[32]

TABLE 19.2
Definitions of Main Performance Criteria and Other Requirements for Analytical Methods

Term	Definition
Decision limit (CCα)	It is defined as the limit at and above which it can be concluded with an error probability of α that a sample is noncompliant
Detection capability (CCβ)	It is the smallest content of the substance that may be detected, identified, and/or quantified in a sample with an error probability of β
Minimum required performance limit (MRPL)	It means the minimum content of an analyte in a sample, which at least has to be detected and confirmed
Precision	The closeness of agreement between independent test results obtained under stipulated conditions
Recovery	The percentage of the true concentration of a substance recovered during the analytical procedure
Reproducibility	Conditions where test results are obtained within the same method on identical test items in different laboratories with different operators using different equipment
Specificity	Ability of a method to distinguish between the analyte being measured and other substances
Ruggedness	Susceptibility of an analytical method to changes in experimental conditions which can be expressed as a list of the sample materials, analytes, storage conditions, environmental, and/or sample preparation conditions under which the method can be applied as presented or with specified minor conditions
Interlaboratory study	Organization, performance and evaluation of tests on the same sample by two or more laboratories in accordance with predetermined conditions to determine testing performance
Within-laboratory reproducibility	Precision obtained in the same laboratory under stipulated conditions

Source: Friedel, A. et al., *Toxicol. Lett*., 165, 149, 2006.

The analyte extraction may be enhanced by using advanced extraction techniques like the accelerated solvent extraction or pressurized liquid extraction where the extraction is performed at high pressure and temperature, supercritical fluid extraction that uses a supercritical fluid as solvent, microwave assisted extraction where sample is rapidly heated, and thus its extraction efficiency is increased and solid-phase microextraction where the analyte may be retained by adsorption on the surface of a fiber.[4,33]

19.5 METHODS FOR CLEANUP OF GROWTH PROMOTERS AND THEIR RESIDUES

19.5.1 Solid-Phase Extraction Procedures

Due to the complexity of the samples, further cleanup and/or purification is usually required. There are several possibilities. Liquid extraction followed by solid-phase extraction (SPE) is extensively used for the isolation of a group or class of analytes. The type of extractant and cartridge depends on the target analyte.[34] Cartridges (C18, C8, and NH_2) are commercially available at reasonable prices (small cartridges) and have low affinity and specificity, but have high capacity. Furthermore, they can be performed in parallel, and, thus, they allow the simultaneous extraction of a large number of samples. Matrix solid-phase dispersion consists in the mechanical blending of the sample with a solid sorbent that progressively retains the analyte by hydrophobic and hydrophilic interactions. The

solid matrix is then packed into a column and eluted with an adequate solvent. This makes it valuable for a wide variety of analytes and matrices [35]. The QuEChERS (quick, easy, cheap, effective, rugged, and safe) technique has been recently introduced for the extraction of residues with similar polarity. The high-moisture sample is extracted with organic solvent in the presence of salts, providing high recovery and low waste.[32,35]

19.5.2 Immunoafinity Chromatography

This type of chromatography is based on the interaction antigen antibody, which is very specific for a particular residue. The columns are packaged with a specific antibody, which is bound to the solid matrix, usually a gel. When the extract is injected, the analyte (antigen) is retained. These chromatographic columns are highly specific and are only limited by potential interferences (i.e., substances that may cross-react with the antibody) that must be checked. These columns are rather expensive and can only be reused a certain number of times. In any case, due to the nature of the specific antibody when preparing the immunosorbent material, an in-depth assessment is necessary before considering its use in a routine analytical method.[36]

19.5.3 Molecular Recognition

There are several methods based on molecular recognition mechanisms for cleanup. Molecular imprinted polymers (MIPs) have shown promising results for the isolation of low amounts of residues as those found in meat. These are cross-linked polymers prepared in the presence of a template molecule that can be a β-agonist. When this template is removed, the polymer offers a binding site complementary to the template structure. MIPs have better stability than antibodies because they can support high temperatures, larger pH ranges, and a wide variety of organic solvents. The choice of the appropriate molecule as template is the critical factor for a reliable analysis.[37] The extracted residues are then analyzed by LC-MS and have shown good quantitative results for cimaterol, ractopamine, clenproperol, clenbuterol, brombuterol, mabuterol, mapenterol, and isoxsuprine but not for salbutamol and terbutaline.[38]

19.6 SCREENING METHODS

The wide variety of veterinary drugs and residues potentially present in a sample of liver or any other organ or by-product makes necessary to use screening procedures for routine monitoring. Screening methods are used to detect the presence of the suspect analyte in the sample at the level of interest. If the searched residue has a MRL, then the screening method must be capable to detect the residue below this limit. These controls are based on the screening of a large number of samples and thus must have a large throughput, low cost, and enough sensitivity to detect the analyte with a minimum of false negatives.[39,40] Compliant samples are accepted while those suspected noncompliant samples have to be further analyzed using confirmatory methods. According to the Commission Decision 2002/657/EC,[27] the screening methods must be validated and have a detection capability (CCβ) with an error probability (β) lower than 5%.

19.6.1 Immunological Techniques

Immunological methods are very specific for a given residue, because they are based on the interaction antigen antibody. The most well-known and extensively used technique is the enzyme-linked immunosorbent assay (ELISA). A wide variety of assay kits with measurement based on color

development are commercially available. The possibility of interferences by cross-reactions with other substances must be taken into account. Competitive ELISA can be divided into homologous format, where the same hapten is used for immunization and assay or heterologous format where the immunizing hapten and the competitor hapten differ in the molecular structures. Recently, a heterologous ELISA method was developed for the analysis of acetylgestagen residues in animal fat.[41]

Other immunological techniques like radioimmunoassay are based on the measurement of the radioactivity of the immunological complex[42]; dipsticks, based on membrane strips with the receptor ligands and measurement of the developed color[43] or the use of luminescence or fluorescence detectors.[44]

19.6.2 Biosensors

The need to screen a large number of meat samples in relatively short time has prompted the development of biosensors, which are based on an immobilized antibody that interacts with the analyte in the sample and the optical or electronical detection of the resulting signal.[45,46] Biosensors can detect simultaneously multiple veterinary drugs residues in a sample at a time[47,48] with no need for sample cleanup.[49] There are different types of biosensors like the surface plasmon resonance that measures variations in the refractive index of the solution close to the sensor[50] and has been successfully applied to the detection of different veterinary drugs residues[51,52] or the biosensors based on the use of biochip arrays that are specific for a certain number of residues[53] and are also applied to residues detection.[54]

19.6.3 Chromatographic Techniques

High-performance thin layer chromatography (HPTLC) has been successfully used for multi-residue screening purposes in meat and edible by-products. Samples are injected onto the plates and the residues eluted through the plate with the appropriate eluent. Once eluted, residues can be viewed under UV or fluorescence lights or visualized by spraying with a chromogenic reagent. HPTLC has been applied to meat to screen different residues in edible by-products like agonists,[55,56] nitroimidazol,[57] and thyreostatic drugs.[58,59]

GC and HPLC are powerful separation techniques able to separate the analyte from most of the interfering substances by varying the type of column and elution conditions.[40] In some cases, the analyte can be detected after appropriate derivatization.[60] In addition, these techniques can be used for multi-residue screening. The recent development of ultraperformance liquid chromatography systems and new types of columns with packagings of reduced size offers valuable improvements for residues detection as a considerable reduction in elution times and the possibility of a larger number of samples per day.[40,61] This procedure has been applied to meat for the detection of a wide variety of veterinary drugs residues,[62–66] anabolic steroids,[67,68] quinolone residues,[69] and corticosteroids.[70–73] Additional advantages of GC and HPLC are automation and the possibility to couple the chromatograph to mass spectrometry (MS) detectors for further confirmatory analysis.

19.7 CONFIRMATORY ANALYTICAL METHODS

Confirmatory methods are preferentially based on MS because they provide direct information on the molecular structure of the suspect compound and thus an unambiguous identification and confirmation of the residue. However, these methods are costly in time, equipments, and chemicals. When the target analyte is clearly identified and quantified above the decision limit for a forbidden substance (i.e., substances of group A) or exceeding the MRL in the case of substances

having a MRL, the sample is considered as noncompliant (unfit for human consumption). A suitable internal standard must be added to the test portion at the beginning of the extraction procedure. If no suitable internal standard is available, the identification of the analyte can be done by co-chromatography. This consists in dividing the sample extract into two parts. The first part is injected in the chromatograph as such. The second part is mixed with the standard analyte to be detected and injected into the chromatograph. The amount of added standard analyte must be similar to the estimated amount of the analyte in the extract. Identification is easier for a limited number of target analytes and matrices of constant composition.[74] An example of a full procedure for the screening and confirmatory analysis of a growth promoter in an edible tissue is shown in Figure 19.1.

GC with MS detection has been used for many years even though derivatization (i.e., silyl or boronate derivatives) was required for some nonvolatile residues like agonists. This is used for the analysis of residues of estrogens, gestagens, and androgens in kidney fat, which is a good tissue of choice for control at the slaughterhouse.[75] But derivatization constitutes a serious limitation that adds some extra time and cost to the analysis.

In recent years, the rapid development of MS coupled to liquid chromatography has expanded its applications in this field, especially for nonvolatile or thermolabile compounds. The use of full-scan MS methods offers an important advantage for multiresidue analysis since hundreds of compounds can be analyzed in one single injection. This can be performed for groups of residues with similar extraction solvents and chemical properties. MS-MS has shown high selectivity

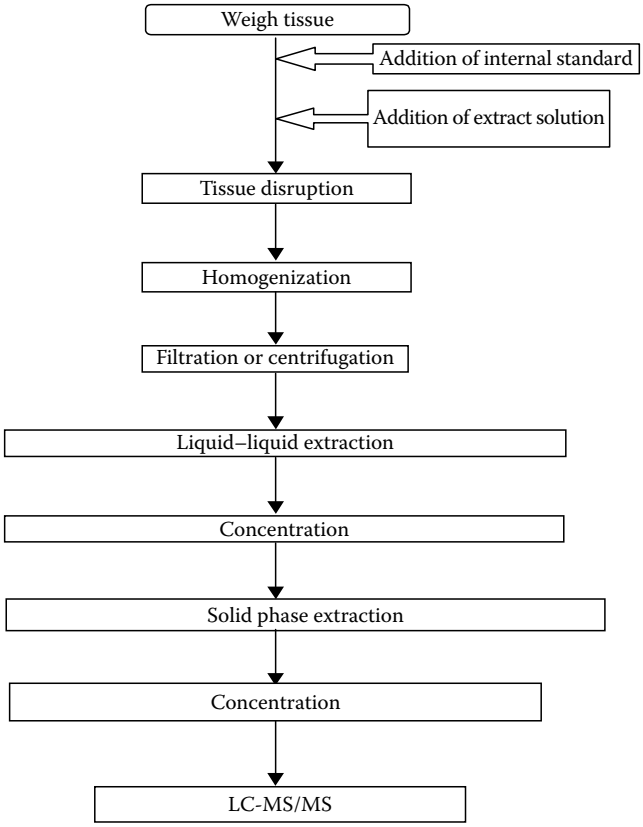

FIGURE 19.1 Flow chart summarizing an example of the full procedure for the screening and confirmatory analysis of the presence of a growth promoter in an edible animal by-product.

and sensitivity and thus allows the analysis of more complex matrices, like liver, with easier sample preparation procedures. LC-MS-MS allows the selection of a precursor *m/z*, which is performed first. This contributes to eliminate any uncertainty on the origin of the observed fragment ions, eliminate potential interferences from the sample or from the mobile phase, and reduce the chemical noise and increase the sensitivity.[76] Triple QqQ is the most used mass analyzer for measurement and quantification of residues while ion trap detectors provide sensitive full scan mass spectra but also MS^n that can help in characterizing the structure and confirm the identity of the residue or its metabolite.[23]

The interface technology has been rapidly developed. Electrospray ionization (ESI) and atmospheric pressure chemical ionization (APCI) interfaces are the sources of choice depending on the polarity and molecular mass of analytes.[76] ESI ionization technique facilitates the analysis of small to relatively large and hydrophobic to hydrophilic molecules.[60,77,78] An important limitation of LC-MS-MS quantitative analysis is its susceptibility to matrix effect that is dependent on the ionization type, type of sample, and sample preparation. APCI ionization has been reported to be less sensible than ESI to matrix effects.[79–82] ESI is the source to be chosen for the MS analysis of nonsteroideal anti-inflammatory drugs due to their polar nature; however, some interfering substances of the matrix like fat may give ion suppression problems.[83] The extraction of the analyte must be more selective and further purified and cleaned up.

A rapid qualitative method using online column-switching liquid chromatography/tandem mass spectrometry (LC/MS/MS) has been developed and validated for screening 13 target veterinary drugs in different animal organs.[84] This system may reduce the cost and time for confirmatory analysis.

The ion suppression phenomenon in LC-MS must be taken into account. This is due to matrix effects problems and the presence of interfering compounds that affect the analyte detection. A wide review about ion suppression phenomenon and its consequences for residue analysis has been recently published.[85] The main mechanism for ion suppression corresponds to the presence of matrix interfering compounds that appear to reduce the evaporation efficiency. The consequences are a reduced detection capability and repeatability. The ion ratios, linearity, and quantification are also affected. It could even lead to the lack of detection of an analyte or the underestimation of its concentration or the nonfulfilment of the identification criteria.[85] The prevention of this phenomenon includes an improved purification and cleanup of the sample as well as the use of an appropriate internal standard. Another strategy is to modify the elution conditions for the analytes to elute in an area nonaffected by ion suppression.[85]

According to the Commission Decision 2002/657/EC,[27] a system of IPs is used for confirmatory purposes with a minimum of 4 points required for the substances of group A and a minimum of 3 for group B substances. So, 1 IP can be earned for the precursor ion with a triple QqQ spectrometer and 1.5 points for each product ion. A high-resolution mass spectrometer acquires 2 IPs for the precursor ion and 2.5 for each product ion. Variable window ranges for MS peak abundances are also established in the new decision.[27] So, the relative ion intensities must be >50%, >20%–50%, >10%–20%, and ≤10%. In the case of GC-MS with electron ionization, the maximum permitted tolerances are ±10%, ±15%, ±20%, and ±50%, respectively, while in the case of GC-MS with chemical ionization, $GC\text{-}MS^n$, LC-MS, and $LC\text{-}MS^n$ are ±20%, ±25%, ±30%, and ±50%, respectively.

A list of recent performance reports for the analysis of growth promoters residues in edible byproducts is shown in Table 19.3. Typical current methods include liquid chromatography techniques coupled to either triple QqQ or time-of-flight detectors, LC-MS-QqQ-MS, and LC-Tof-MS, respectively, which have excellent sensitivity and selectivity. These methods are increasing in use because they have the advantage that no assumptions for the presence of any specific drug are required.[86] In general, the performance is increasing very rapidly due to the availability of more new powerful mass analyzers.

TABLE 19.3
Examples of Recent Methods of Analysis of Growth Promoters in Animal Edible By-Products

Analyte	Matrix	Extraction	Column	Separation Conditions	Detector	Ccα (ng/g)[a]	Ccβ (ng/g)	Recovery (%)	Reference
Clenbuterol	Bovine liver	Liquid extraction	Synergi MAX-RP 80 A (150 mm × 2 mm) 4 µm	T = 40°C	LC-MS/MS	0.08	0.27	—	[87]
Ractopamine		Enzymatic hydrolysis		A gradient of 0.1% acetic acid in water and 0.1% acetic acid in a mixture of acetonitrile/water (90:10)	ESI^+	0.15	0.32		
Cimaterol		SPE C18		Flow rate = 0.4 mL/min		0.13	0.52		
Clenbuterol	Bovine liver	Liquid extraction	A-chrom C18 (250 mm × 3 mm) 5 µm	A gradient using acetonitrile–ammonium acetate–water (pH 7.0), and acetonitrile–ammonium acetate	LC-MS/MS	LOD = 0.05	—	—	[88]
Salbutamol		Hydrolysis with helix pomatia		Flow rate = 0.5 mL/min	$APCI^+$	LOD = 0.05			
Cimaterol		SCX				LOD = 0.05			
Mabuterol						LOD = 0.05			
Terbutalina						LOD = 0.1			
Terbutaline	Bovine liver	Liquid extraction	Inertsil C8 (150 mm × 2.1 mm) 5 µm	T = 28°C	LC-MS/MS	0.3	0.5	41	[89]
Ritodrine		Protease hydrolysis		Gradient of ammonium formate buffer (pH 3.2) and acetonitrile	$APCI^+$	0.2	0.4	67	
Hydroxymethylclenbuterol		SPH Oasis HLB		Flow rate = 0.3 mL/min		0.1	0.2	74	

Tulobuterol						0.1	0.2	41	
Clenpeterol						0.1	0.2	57	
Isoxsuprine						0.3	0.5	28	
Salbutamol						0.3	0.5	65	
Cimaterol						0.1	0.2	71	
Ractopamine						0.2	0.3	52	
Clenbuterol						0.1	0.2	61	
Brombuterol						0.2	0.3	53	
Mabuterol						0.1	0.2	64	
Salbutamol	Bovine liver	Liquid extraction Enzymatic hydrolysis	Betamax Base analytical column (100 mm × 2 mm) 5 µm	Gradient of 0.1% formic acid (aq) and acetonitrile	LC-MS/MS	0.3	—	—	[90]
Zilpaterol		SPE HCX		Flow rate = 0.3 mL/min	ESI^+	0.2			
Terbutaline						0.1			
Cimaterol						0.08			
Fenoterol						0.2			
Clenbuterol						0.25			
Ractopamine						0.1			
Brombuterol						0.5			
Mabuterol						0.15			
Albendazole sulphoxide	Benzimidazoles (antihelmintics) in liver	Liquid extraction	XTerra C18, (150 mm × 3 mm), packed 3.5 µm	T = 40°C	HPLC-DAD	1303	1556	50–73	[91]
Albendazole sulphone		SPE C18		A gradient using ammonium dihydrogen phosphate buffer (pH 6.8), methanol and acetonitrile. Flow rate = 0.5 mL/min		1164	1343	20–27	

(*continued*)

TABLE 19.3 (continued)
Examples of Recent Methods of Analysis of Growth Promoters in Animal Edible By-Products

Analyte	Matrix	Extraction	Column	Separation Conditions	Detector	Ccα (ng/g)[a]	Ccβ (ng/g)	Recovery (%)	Reference
Thiabendazole						116	132	55–96	
Oxfendazole/fenbendazole						561	627	57–85	
Hydroxy–mebendazole						481	544	60–103	
Fenbendazole sulphone						587	670	55–87	
Oxibendazole						242	281	61–120	
Mebendazole						467	520	59–100	
Flubendazole						493	558	53–85	
Albendazole						1216	1397	36–63	
Halofuginone	Poultry liver	Enzymatic (trypsin) hydrolysis Liquid–liquid extraction SPE-oasis	Prodigy C18 (250 mm × 4.6 mm) 5 µm	Methanol–water–glacial acetic acid (40:59.5:0.5) Flow rate = 1 mL/min	LC-MS/MS ESI^+	35.4	43.6	—	[92]
Halofuginone	Chicken liver	Liquid extraction Enzymatic (trypsin) hydrolysis Liquid–liquid extraction SPE C18	Lichrosorb RP18 (250 mm × 4 mm 5 µm	T = 40°C acetonitrile:0.25 M ammonium acetate (pH 4.3):water (5:3:12 by vol) Flow rate = 1.2 mL/min	HPLC-UV	LOD: 50	—	78.8–82.2	[93]
Trenbolone	Bovine liver	Liquid extraction	Capcell pak phenyl (250 mm × 2 mm) 5 µm	T = 40°C	LC-MS	LOD = 1		62–69	[94]

		SPE silica		A gradient of methanol and water Flow rate = 0.2 mL/min	ESI^+				
Zeranol	Rabbit liver	Liquid extraction Hydrolysis with β-glucuronidase + arylsulfatase	Waters XTettraä C18 column (50 × 2.1 mm) 3.5 µm	Gradient elution with acetonitrile and 20 mM ammonium acetate	LC-MS/MS	LOD =1	—	—	[95]
		SPE C18			ESI^-				
Zeranol	Bovine liver	Liquid extraction deproteinization	Zorbax XDB-C18 column (150 × 2.1 mm) 5 µm	T = 40°C	LC-MS/MS	LOD = 0.5	—	78.9	[96]
		SPE OAS/SHLB		0.005% acetic acid–acetonitrile (60:40, v/v) Flow-rate = 0.2 mL/min	ESI^-				
α-Trenbolone	Bovine liver	Liquid extraction deproteinization	Zorbax XDB-C18 column (150 × 2.1 mm) 5 µm	T = 40°C	LC β-trenbolone MS/MS	LOD = 0.5	—	76.3	[96]
β-Trenbolone		SPE OAS/SHLB		0.005% acetic acid β-trenbolone acetonitrile (60:40, v/v) Flow-rate = 0.2 mL/min	ESI^+			79.1	
Melengestrol acetate	Kidney fat	Gel permeation chromatography	Hypersil ODS (150 mm × 4.6 mm) 5 µm	A gradient of water and methanol	LC-MS/MS	0.20	0.33	—	[97]
Megestrol acetate				Flow rate = 0.7 mL/min	$APCI^+$	0.22	0.38		
Medroxyprogesterone acetate						0.22	0.37		

(*continued*)

TABLE 19.3 (continued)
Examples of Recent Methods of Analysis of Growth Promoters in Animal Edible By-Products

Analyte	Matrix	Extraction	Column	Separation Conditions	Detector	Ccα (ng/g)[a]	Ccβ (ng/g)	Recovery (%)	Reference
Melengestrol acetate	Kidney fat	Liquid–liquid extraction	Waters Sunfire C18 (100 mm × 2.1 mm) 3.5 µm	Gradient elution with acetonitrile and 10 mM ammonium formate	LC-MS/MS	0.15	0.19	—	[98]
Megestrol acetate		Defatting procedure		Flow-rate = 0.25 mL/min	ESI^+	0.15	0.19		
Chlormadinone acetate		SPE silica				0.37	0.47		
Medroxyprogesterone acetate						0.24	0.32		
Methyl testosterone	Bovine and sheep liver	Liquid extraction	Ultra-1 methylsilicone (18 m × 0.2 mm)	Derivatization with MSTFA	GC-MS	LOD = 0.04	0.08	92.5–93.2	[99]
		SPE silica and HLB	Film thickness = 0.1 µm	Splitless model EI 70ev					
Multi-residue (50 anabolic hormones)	Liver	Liquid extraction	Acquity UPLC BEH C18 (100 mm × 2.1 mm) 1.7 µm	Water + methanol containing 0.1% formic acid	LC-MS/MS	LOD range = 0.04–2.0		76.9–121.3	[100]

		Hydrolysis with β-glucuronidase + arylsulfatase SPE NH_2		Flow rate = 0.3 mL/min	ESI^+				
Diethylestilbestrol	Veal liver	Liquid extraction Hydrolysis with β-glucuronidase deproteinization	DB5-MS (30 m × 0.25 mm)	Derivatization at inlet with BSTFA/TMSI	GC-MS	0.10	0.17	29–67	[101]
Dienestrol		SPE XTRX	Film thickness = 0.25 µm	Splitless mode		0.15	0.25		
Zeranol				EI 70ev		0.27	0.45		
Taleranol						0.32	0.55		
Zearalanone						0.26	0.45		
Zearalanone						0.33	0.57		
α-Zearalenol						0.33	0.56		
β-Zearalenol						0.31	0.53		

Abbreviations: ECD, electrochemical detector; APCI, atmospheric pressure chemical ionization; ESI, electrospray ionization.

[a] LOD given when CCα is nonavailable.

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20 Antibiotics

Houda Berrada and Guillermina Font

CONTENTS

20.1 INTRODUCTION

Antibiotics have revolutionized the treatment of infectious diseases, contributing significantly to improve the health of the population. Antibacterials, anti-infectives, antimicrobials, or chemotherapeutics comprise synthetic and natural compounds. Antibiotics are "natural antimicrobial substances produced at low concentrations by certain microorganisms, fungi, and bacteria for inhibiting the growth of other microorganisms." Strictly, antibiotics should include only five classes—penicillins, tetracyclines, macrolides, aminoglycosides, and amphenicols [1,2].

The more general term "antibacterials," also categorized as anti-infectives, antimicrobials, or chemotherapeutics, includes both synthetic and natural compounds. Semisynthetic antibiotics are antimicrobial agents of natural origin, but modified synthetically to improve their pharmacological properties. Synthetic drugs are obtained by chemical synthesis, (quinolones or nitrofurans) and also include substances of high molecular weight as peptide antibiotics. The term "antibiotic" refers to any substance with antibacterial, antifungal activity of natural, semisynthetic, or synthetic origin [3].

Antibiotics are the most detected veterinary drug residues in food. They were used in veterinary medicine and in aquaculture for the prevention and treatment of microbial infections. They were also added to animal feeds at subtherapeutic levels to improve the growth rate and efficiency of feed utilization [4]. In this sense, since January 1, 2006, the European Union (EU) has banned the use of these drugs as growth promoters in animal feed. One of the main drawbacks associated with antibiotic usage is the development of antimicrobial resistance. The appearance and evolution of microbial resistance is a serious clinical and public health problem on a global basis [5–7].

The transfer of resistance from food-producing animals to humans via the food chain, the development of multiresistance and the ability to transfer this resistance to other organisms have given rise to widespread international debate. As a consequence, there is an increasing interest to reduce antibiotic use [8].

This chapter presents an overview on the state of the art of antibiotic analysis in animal by-products. It covers some important aspects related to current analytical methods applied to antimicrobial analysis, legislation, and selected applications. Emphasis is given in sample preparation regarding isolation and purification, chromatographic conditions, and real samples analyzed concerning the multi-residue as well as the multi-class analysis from animal by-products like entrails and internal organs of butchered animals.

20.2 EUROPEAN UNION CRITERIA

The EU has strictly regulated controls on the use of veterinary drugs, particularly growth-promoting agents, in food-animal species, by several regulations and directives. Since 1998, antibiotics used in human medicine were prohibited from being added to feed [9]. The EU decided to phase out the use of antibiotics as growth promoters in feed and the total ban on the use of antibiotics as growth promoters in animal feed entered into effect on January 1, 2006. On that date, the following four substances were removed from the EU Register of permitted feed additives:

- Monensin sodium used for cattle for fattening
- Salinomycin sodium used for piglets and pigs fattening
- Avilamycin used for piglets, pigs for fattening, chickens for fattening and turkeys
- Flavophospholipol used for rabbits laying hens, chickens for fattening, turkeys, piglets, pigs, calves and cattle for fattening

This measure was in line with the commission's overall strategy to combat the threat to human, animal, and plant health posed by antimicrobial resistance. The ban is the final step in the phasing out of antibiotics used for non-medicinal purposes. Markos Kyprianou, Commissioner for Health and Consumer Protection, said: "This ban on antibiotics as growth promoters is of great importance, not only as part of the EU's food safety strategy, but also when considering public health. We need to greatly reduce the nonessential use of antibiotics if we are to effectively address the problem of microorganisms becoming resistant to treatments that we have relied on for years. Animal feed is the first step in the food chain, and so a good place to take action in trying to meet this objective."

EU Council Regulation 2377/90/ EC regulated the use of veterinary drugs through the procedure for establishing maximum residue limits (MRLs) for veterinary medicinal products in foodstuffs of animal origin in four annexes [10]. Authorized substances at annexes I, II, or III and forbidden substances (substances for which no MRL can be fixed) were listed in the fourth annex like chloramphenicol and nitrofurans. The prohibition of the use of growth-promoting agents (e.g., hormones and beta-agonists) was laid down in Council Directive 96/22/EC [11]. Council Directive 96/23/EC contains guidelines for controlling veterinary drug residues in animals and their products with detailed procedures for EU member states to set up national monitoring plans, including details on sampling procedures [12]. Groups of residues and substances (group A; commonly referred as unauthorized, illegal, banned, or prohibited substances having anabolic effects and group B veterinary drugs and contaminants) at the 96/23/ first annex. For reasons of ease of use, all pharmacologically active substances should be listed in alphabetical order in one Annex according to the Commission regulation (EU) 37/2010, in two separate tables one for allowed substances, and one for prohibited substances [13].

20.3 CLASSIFICATION

Although there are several classification schemes for antibiotics, based on bacterial spectrum, route of administration and type of activity, the most useful is based on chemical structure. Antibiotics within a structural class will generally show similar patterns of effectiveness, toxicity, and allergic potential. The main antibiotic classes are briefly presented.

Macrolide antibiotics are derived from *Streptomyces* bacteria, and got their name due to their macrocyclic lactone chemical structure. Erythromycin, the prototype of this class, has a spectrum and use similar to penicillin. Newer members of the group, azithromycin and clarithyromycin, are particularly useful for their high level of lung penetration. Clarithromycin has been widely used to treat *Helicobacter pylori* infections, the cause of stomach ulcers. For people who are allergic to penicillin, erythromycin is a good alternative [14].

Aminoglycosides are particularly useful for their effectiveness in treating *Pseudomonas aeruginosa* infections, and the lincosamides, clindamycin, and lincomycin, which are highly active against anaerobic pathogens. Aminoglycosides and macrolides are groups of antibacterial compounds that are widely used in medical and veterinary practices. They are broad-spectrum antibiotics with bactericidal activity against some Gram-positive and many Gram-negative organisms. These antibiotics are among the most used chemotherapeutics in veterinary practice. They are available in various formulations for the treatment of a wide range of infections because they are cheap and have a wide spectrum of activity to prevent or treat acute and chronic bacterial infections [15–17].

Nitrofuran antibiotics include furazolidone, furaltadone, nitrofurantoine, and nitrofurazone. All uses of nitrofuran drugs in food-producing animals were prohibited because they pose a public health risk as result of evidence that these drugs may induce carcinogenic residues in animal tissues. Since 1991, nitrofurans have been banned for systemic use in poultry and swine but topical uses have been allowed thinking that this application did not reach edible tissues. Recent evidence, however, showed that these drugs reached milk and tissues, including muscle, kidney, and liver in cattle treated with ophthalmic preparations so the Food and Drug Administration (FDA) decided to disallow even topical use [18,19].

β-*Lactams*: Penicillins are the oldest class of antibiotics, belonging to the same group of β-lactams with the cephalosporins as they have a common chemical structure. Each generation has a broader spectrum of activity than the one before.

Fluoroquinolones are synthetic antibacterial agents, and are not derived from bacteria. An earlier, related class of antibacterial agents, quinolones, were not well absorbed, and could be used only to treat urinary tract infections. Fluoroquinolones are broad-spectrum bactericidal drugs.

Tetracyclines got their name because they share a chemical structure having four rings. They are derived from a species of *Streptomyces* bacteria. Broad-spectrum bacteriostatic agents, the tetracyclines may be effective against a wide variety of microorganisms, including *rickettsia* and amoebic parasites.

Sulphonamides exhibit a bacteriostatic rather than bactericidal effect. The sulphonamide functional is a sulfone group connected to an amine group. Sulfadiazine, sulfamethizole, sulfamethoxazole, sulfasalazine, and sulfisoxazole are included as the sulphonamide family. When sulfonamides are combined with 2,4-diaminopyrimidines, such as trimethoprim, a synergistic antimicrobial effect is often obtained. Although the enhancement in antimicrobial activity is mutual, the 2,4-diaminopyrimidines are often referred to potentiate sulphonamide. This synergistic antimicrobial activity is very effective against Gram-positive and Gram-negative bacterial pathogens.

Amphenicols (e.g., thiamphenicol, florfenicol, and chloramphenicol) are synthetic antibiotics with a similar broad spectrum of activity. These antibiotics proved to be beneficial for the treatment of bacterial infections and were administered to animals for disease prevention. They are broad-spectrum antibiotics, but not usually effective against *Pseudomonas*. Their use in food-producing animals is illegal in most countries.

Figure 20.1 illustrates the chemical structures of the main antibiotic classes.

20.4 ANALYSIS OF ANTIBIOTICS

There are several problems related with the determination of antibiotic residues in animal by-products. The matrix is very complex, rich in proteins, and some antibacterials bind easily to them. Different antibiotic classes possess different chemical properties making their simultaneous

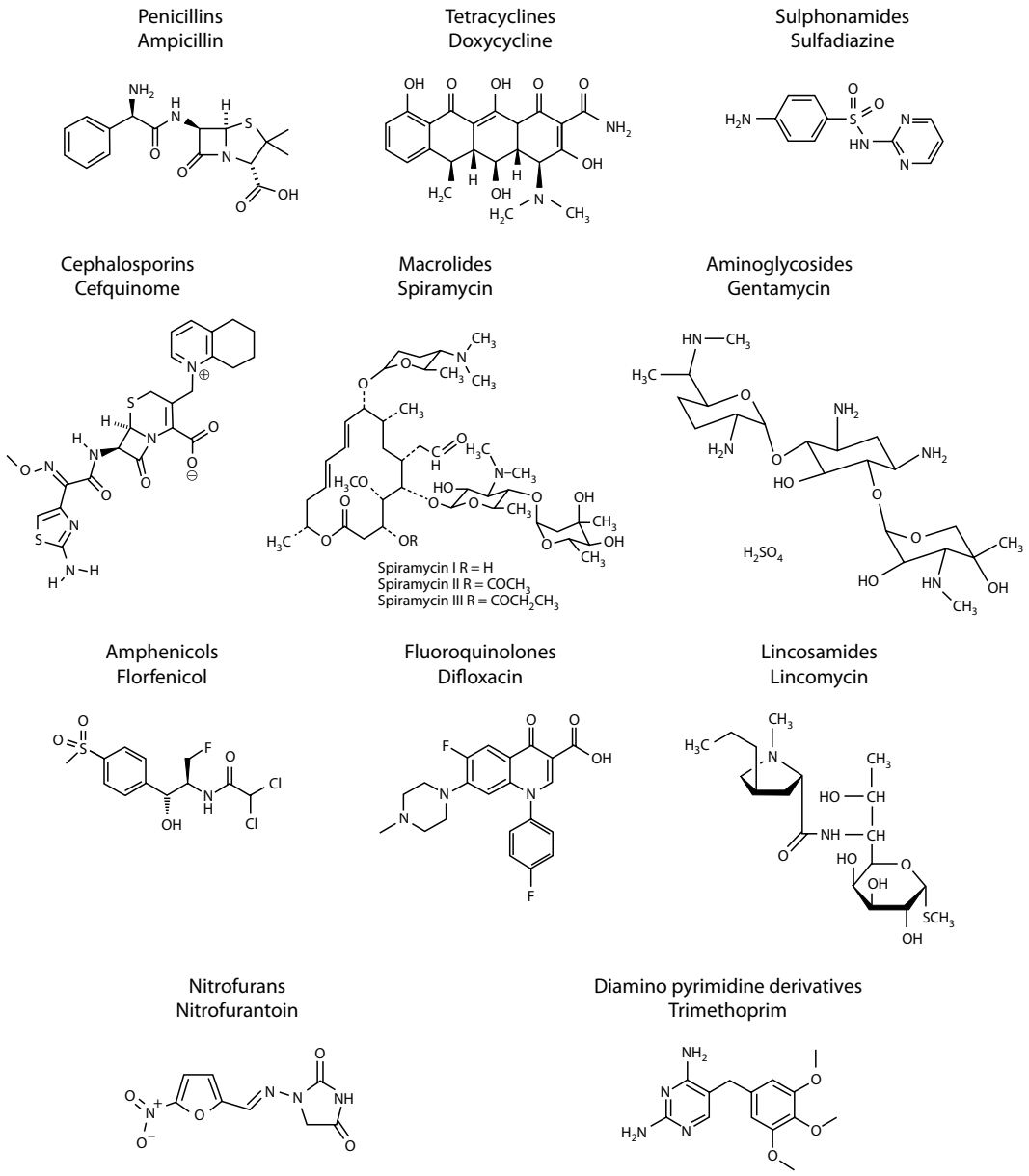

FIGURE 20.1 Chemical structures of the main antibiotic classes.

determination at very low concentrations not an easy task. Therefore, many analytical methods have appeared in the literature for the determination of residues of different antibiotic classes in animal by-products. Only a limited number focused on multi-class residue analysis, a simultaneous analysis of antibiotic members of different classes. Some of them are multi-residue methods, which analyze simultaneously more than one antibiotic from the same class.

Criteria for analytical methods for the determination of residues (e.g., detection level, selectivity, and specificity) for residue control in the framework of Directive 96/23/EC were described in Commission Decision 2002/657/EC [20] amended by Commission decision 2003/181/EC [21]. Besides the general performance requirements, additional requirements were described for

confirmatory methods by the criteria of performance, system of identification points (IPs), and defining criteria for ion intensities. For the confirmation of the identity of Group A substances, a minimum of four IPs is required. For confirmation of the identity of Group B substances, a minimum of three IPs is required. The number of IPs collected depends on the technique used. These techniques have to be based on mass spectrometric (MS) detection even the commission decision still allow diode array detection (DAD) and fluorometric detection (FLD) as possible confirmatory techniques for specified antibacterials. Some new definitions, e.g., decision limit (CCα) and detection capability (CCβ), have been introduced in Commission Decision 2002/657/EC. CCα and CCβ are intended to replace the following method characteristics: limit of detection (LOD) and limit of quantification (LOQ). CCα is defined as "the concentration at and above which it can be decided with a statistical certainty of 1-α that the permitted limit has been truly exceeded (positive)." CCβ is defined as "the smallest content of the substance that may be detected, identified, and/or quantified in a sample with an error probability of β."

The first selection that has to be made when setting up a monitoring program is the type of sample material. For monitoring drugs having an MRL, animal tissues (e.g., muscle, liver, kidney, and fat) are the most frequently selected ones. Since the drug concentration in the consumable parts of an animal has to be below the MRL, these matrices are of interest.

In this chapter, recently published papers are reviewed to present the state of the art in analytical strategies concerning the multi-class and the multi-residue analysis of antibiotics in animal by-products (Table 20.1). Special focus has been placed on extraction and cleanup, the impact of Commission Decision 2002/657/EC and its application in detection of antibacterial residues on this complex matrix.

20.4.1 Macrolides

Analytical methods for the detection of macrolide residues in animal by-products have been described [22–24]. All these methods started with a liquid–liquid extraction (LLE) at basic pH. The sample cleanup was by solid-phase extraction (SPE) on a polymer column (Oasis, Stratra-X) or strong cation-exchange column. The final analysis was by LC C18 columns. A confirmatory method for three macrolides (tylosin, tilmicosin, and erythromycin) in bovine liver and kidney by micro-liquid chromatography–mass spectrometry/mass spectrometry (LC-MS/MS) using an atmospheric pressure ionization source and an ion spray interface has been reported [24]. This developed method complied with the criteria proposed by the Commission Decision 93/256/EEC.

A sensitive and specific method was described for the quantitative analysis of tylosin A in various swine tissue samples by reversed-phase liquid chromatography–electrospray ionization–mass spectrometry/mass spectrometry (LC-ESI-MS/MS) [23]. A liquid extraction and a further solid-phase cleanup on strong cation exchange (SCX) column have been found to provide a sufficient sample cleanup. Internal standardization using spiramycin enhanced the reliability of the method. The method has been validated according to the requirements of the EC at the maximum residue limit (MRL), half the MRL and double the MRL levels. Limits of detection were inferior at 1 μg/kg for the various tissues (muscle, skin, fat, kidney, and liver). The method was successfully used for a residue depletion study of tylosin in swine medicated with tylosin by intramuscular injection and was found to be highly specific for tylosin A, which is the marker residue of interest for EC guidelines.

Seven macrolides (erythromycin, josamycin, roxythromycin, spiramycin, tilmicosin, troleandomycin, and tylosin) could be detected in a single run using LC-DAD by C18-bonded phase and mass spectrometry confirmation [22].The corresponding DAD and SIM chromatograms are shown in Figures 20.2 and 20.3, respectively. This method was fully validated according to the EU criteria, and the CCα and CCβ are listed in Table 20.1.

TABLE 20.1
Chromatographic Methods for Determining Antibiotics in Animal By-Products

Compound	Extraction	Separation	Detection	Recovery (%)	LOD, LOQ, CCα and CCβ (μg/kg)	Samples	Reference
Macrolides							
Tylosin Tilmicosin Erythromycin A	Protein precipitation with 10 mM KH_2PO_4 and chloroform. Cleanup on Bond elut diol column	Column: LC-ABZ Supelcosil C_{18}. Mobile phase acetonitrile (A), methanol (B) 1% trifluoroacetic acid (C)	Micro-LC-API-QqQ	>70	LOQ = 40 LOQ = 150 LOQ = 80	Liver and kidney	[24]
Tylosin	LLE with methanol, SPE cleanup on strong cation-exchange column	Column: Nucleosil C_{18}. Mobile phase 0.01 M ammonium acetate in water (A), acetonitrile (B)	LC-ESI(+)-QqQ	97 74	LOD = 0.4 LOD = 1	Kidney Liver	[23]
Erythromycin A Josamycin Roxithromycin Spiramycin Tilmicosin Troleandomycin Tylosin	20 mL EDTA-McIlvaine were added to the sample, SPE cleanup on Oasis HLB cartridges	Column: Kromasil 100 C_{18}. Mobile phase water with phosphate/phosphoric acid (pH 3.5) (A), acetonitrile (B)	LC-DAD	60–88	CCα = 222, CCβ = 243 CCα = 60, CCβ = 70 CCα = 120, CCβ = 142 CCα = 408, CCβ = 416 CCα = 1005, CCβ = 1010 CCα = 80, CCβ = 96 CCα = 116, CCβ = 132 Calculated at liver beefs	Liver	[22]
Aminoglycosides							
Dihydrostreptomycin	Protein precipitation with 10 mM KH_2PO_4 with 2% (w/v)TCA. Cleanup on CBA column (weak cation exchanger)	Column: Nucleosil C18; Mobile phase (A) water and (B) water/acetonitrile (50/50), each 20 mmol/L PFPA	LC-ESI(+)–ion trap	86 for liver, 82 for muscle, 78 for fat and, 75 for kidney	LOD = 1.9 LOD = 2.2 LOD = 2.7 LOD = 4.2	Fat Liver Muscle Kidney	[25]

Gentamycin	Samples homogenized with a Polytron probe, centrifuged and filtered. Cleanup on Bond-Elut C_{18}	Waters ODS-AM C18. Mobile phase 0.11 MTFA aqueous solution (A) and (B) acetonitrile	LC-ESI(+)-QqQ	96–104	LOQ = 26	Kidney	[26]
Spectinomycin	Protein precipitation with TCA. Cleanup on Oasis HLB cartridges	Column: Zorbax extended C18; Mobile phase (A) water and (B) acetonitrile, each 20 mmol/L HFBA	LC-ESI(+)-QqQ	69–77	LOD = 30	Liver	[27]
Streptomycin					LOD = 15		
Dihydrostreptomycin					LOD = 10		
Amikacin					LOD = 40		
KanamycinB					LOD = 20		
Paromomycin					LOD = 30		
Apramycin					LOD = 40		
Tobramycin					LOD = 15		
Sisomycin					LOD = 25		
Neomycin B					LOD = 20		
Genramycin C1					LOD = 30		
Streptomycin	Protein precipitation with TCA. Cleanup on Oasis HLB cartridges	Column: UG-120 C18, Mobile phase (A) water and (B) acetonitrile, each 20 mmol/L HFBA	LC-ESI(+)-QqQ	65–109	CCα = 1131, CCβ = 1313	Liver	[16]
Dihydrostreptomycin					CCα = 1080, CCβ = 1160		
Neomycin B					CCα = 5278, CCβ = 5538		
Paromomycin					CCα = 1595, CCβ = 1674		
Kanamycin B					CCα = 49, CCβ = 59		
Amikacin					CCα = 19, CCβ = 25		
Tobramycin					CCα = 22, CCβ = 28		
Spectinomycin					CCα = 577, CCβ = 657		
Apramycin					CCα = 121, CCβ = 143		
Gentamycin					CCα = 223, CCβ = 235		
Hygromycin					CCα = 27, CCβ = 37		
					Calculated at liver		

(*continued*)

TABLE 20.1 (continued)
Chromatographic Methods for Determining Antibiotics in Animal By-Products

Compound	Extraction	Separation	Detection	Recovery (%)	LOD, LOQ, CCα and CCβ (μg/kg)	Samples	Reference
Streptomycin	Protein precipitation with TCA. Cleanup on Oasis HLB cartridges	Column: UG-120 C18, Mobile phase (A) water and (B) acetonitrile, each 20 mmol/L HFBA	LC-ESI(+)-QqQ	69–101	CCα = 578, CCβ = 650	Kidney	[16]
Dihydrostreptomycin					CCα = 566, CCβ = 631		
Neomycin B					CCα = 596, CCβ = 680		
Paromomycin					CCα = 1642, CCβ = 1747		
Kanamycin B					CCα = 49, CCβ = 58		
Amikacin					CCα = 21, CCβ = 28		
Tobramycin					CCα = 26, CCβ = 36		
Spectinomycin					CCα = 115, CCβ = 132		
Apramycin					CCα = 122, CCβ = 148		
Gentamycin					CCα = 117, CCβ = 138		
Hygromycin					CCα = 29, CCβ = 38		
Dihydrostreptomycin	Protein precipitation with 10 mM KH_2PO_4 with 0.4 mM EDTA and 2% (w/v)TCA. Cleanup on C_{18} column	Column: Nucleosil C18, Mobile phase (A) water and (B) acetonitrile, each 20 mmol/L HFBA	LC-ESI(+)-QqQ	82	LOD = 0.1	Kidney	[28]
Gentamycin				71	LOD = 0.1		
Neomycin B				69	LOD = 0.1		
Streptomycin				85			
Tobramycin				80			
Dihydrostreptomycin	Protein precipitation with 10 mM KH_2PO_4, 0.4 mM EDTA and 2% (w/v) TCA. Cleanup on C_{18} column	Column: Nucleosil C18, Mobile phase (A) water and (B) acetonitrile, each 20 mmol/L HFBA	LC-ESI(+)-QqQ	96	LOD = 0.2	Liver	[28]
Gentamycin				81	LOD = 0.2		
Neomycin B				65	LOD = 0.5		
Streptomycin				85			
Tobramycin				82			
Nitrofuran							
Furazolidone	Tissues minced, mixed with methanol, centrifuged and liquid–liquid extraction with ethyl acetate	Column: Waters Symmetry C_{18}; Mobile phase (A) water and (B) acetonitrile both at 1% acetic acid	LC-ESI(+)-QqQ	59–74	LOD = 0.2	Liver and kidney	[29]
Furaltadone					LOD = 0.2		
Nitrofurantoin					LOD = 2		
Nitrofurazone					LOD = 1		

β-*Lactams*							
Amoxicillin	MSPD with sand and aqueous extraction with water at 65°C	Column: Altima C18; Mobile phase (A) methanol and (B) water	LC-ESI(+)-QqQ	74–95	LOD = 2.1, LOQ = 3.1	Liver and kidney	[30]
Ampicillin					LOD = 0.5, LOQ = 0.8		
Amoxicillin	LE using 0.01 M potassium dihydrogen phosphate, SPE cleanup on C_{18} cartridges	Column: PLRP-S polymeric C18; Mobile phase (A) 0.1% formic acid in water and (B) acetonitrile	LC-ESI(+)-QqQ	>90	CCα = 52, CCβ = 57	Kidney	[31]
					CCα = 52, CCβ = 56	Liver	
Deacetylcephapirin	Protein precipitation with acetonitrile SPE cleanup on C_{18} cartridges	Column: YMC ODS-AQ C18; Mobile phase (A) water and (B) acetonitrile both at 0.1% formic acid	LC-ESI(+)-ITP	>80	LOQ = 10	Kidney	[32]
Amoxicillin					LOQ = 10		
Cephapirin					LOQ = 10		
Ampicillin					LOQ = 10		
Cefazolin					LOQ = 10		
Penicillin G					LOQ = 10		
Oxacillin					LOQ = 10		
Cloxacillin					LOQ = 10		
Naficillin					LOQ = 10		
Dicloxicillin					LOQ = 10		
Benzylpenicillin	Sample homogenized with 5% sodium tungstate aqueous solution and 0.17 M sulfuric acid and 2% NaCl	Column: TSKgel ODS-80Ts; Mobile phase (A) water and (B) acetonitrile both at 2 mM Di-n-butylammonium acetate	LC-ESI(+)-QqQ	66–95	LOQ = 3	Liver and kidney	[33]
PhenoxymethylP					LOQ = 10		
Oxacillin					LOQ = 10		
Cloxacillin					LOQ = 5		
Nafcillin					LOQ = 5		
Dicloxacillin					LOQ = 10		
Quinolones							
Cinoxacin	10 mL acetonitrile and 2.5 g were added to the sample acidified before SPE cleanup on SDB-RPS cartridges	Column: Nucleosil C18; Mobile phase (A) diluted formic (pH 2.5) acid water and (B) acetonitrile	LC-ESI(+)-QqQ	89–109	LOD = 20	Kidney	[38]
Ciprofloxacin					LOD = 15		
Danofloxacin					LOD = 10		
Enoxacin					LOD = 20		
Enrofloxacin					LOD = 10		
Flumequine					LOD = 10		
Marbofloxacin					LOD = 20		
Nalixidic acid					LOD = 10		
Norfloxacin					LOD = 10		
Ofloxacin					LOD = 10		
Oxolinic acid					LOD = 15		

(*continued*)

TABLE 20.1 (continued)
Chromatographic Methods for Determining Antibiotics in Animal By-Products

Compound	Extraction	Separation	Detection	Recovery (%)	LOD, LOQ, CCα and CCβ (μg/kg)	Samples	Reference
Cinoxacin	10 mL acetonitrile and 2.5 g were added to the sample acidified before SPE cleanup on SDB-RPS cartridges	Column: Nucleosil C18; Mobile phase (A) diluted formic (pH 2.5) acid water and (B) acetonitrile	LC-ESI(+)-QqQ	98	LOQ = 12	Kidney	[37]
Ciprofloxacin				99	LOQ = 0.1		
Danofloxacin				100	LOQ = 4		
Enoxacin				101	LOQ = 13		
Enrofloxacin				100	LOQ = 0.1		
Flumequine				98	LOQ = 19		
Marbofloxacin				100	LOQ = 4		
Nalixidic acid				98	LOQ = 10		
Norfloxacin				100	LOQ = 5		
Ofloxacin				100	LOQ = 2		
Oxolinic acid				98	LOQ = 13		
Enrofloxacin	Homogenized extract was centrifuged and SPE cleanup with C_{18} cartidges	Column: Lichrospher C_{18} Mobile phase (A) water, (B) acetonitrile and (C) Triethylamine	LC-DAD		13% samples (90) >LMR	Liver	[40]
Enrofloxacin	Homogenized extract was centrifuged and SPE cleanup with C_{18} cartidges	Column: Lichrospher C_{18} Mobile phase (A) water, (B) acetonitrile and (C) Triethylamine	LC-DAD		24% samples (90) >LMR	Kidney	[40]
Norfloxacin	Liver cooked in water and prepared in sodium phosphate/sulphite buffer	Column: Novapak C18; Mobile phase (A) methanol, (B) acetonitrile, (C) 0.4 M citric acid	LC-DAD		56% samples (714) >LOQ	Liver	[48]
Enrofloxacin	Homogenized extract with phosphate buffer was extracted with trichloromethane	Column: Novapak C18; Mobile phase (A) methanol, (B) aqueous buffer solution	LC-DAD	>90	LOQ = 8	Liver and kidney	[39]

Sarafloxacin	Extraction with a mixture of methanol and phosphate-buffered saline		Enzyme-linked immunosorbent assay (ELISA)		CCβ < 4	Kidney	[34]
Norfloxacin					CCβ < 10		
Difloxacin					CCβ < 10		
Ciprofloxacin					CCβ < 10		
Pefloxacin					CCβ < 10		
Ofloxacin					CCβ < 10		
Cinoxacin					CCβ < 200		
Danofloxacin					CCβ < 10		
Enrofloxacin					CCβ < 10		
Marbofloxacin					CCβ < 10		
Lomefloxacin					CCβ < 10		
Enoxacin					CCβ < 10		
Flumequine					CCβ < 100		
Oxolinic acid					CCβ < 25		
Nalidixic					CCβ < 10		
Enrofloxacin	Samples extracted with a mixture of acetonitrile, water and formic acid. SPE cleanup on Oasis HLB cartridges	Column: Chromolith Speed Rod, RP 18e	TFC-ESI(+)-QqQ	72–106	LOQ = 25	Kidney and liver	[35]
Ciprofloxacin					LOQ = 25		
Danofloxacin	Samples extracted with a mixture of acetonitrile and ammonium hydroxide	Column: Luna Phenylhexyl. Mobil phases: acetonitrile-methanol (A), 2% acetic acid with ammonium hydroxide (B)	LC-DAD FLUORE	>70	LOQ = 0.3	Liver	[36]
Ciprofloxacin					LOQ = 3		
Norfloxacin					LOQ = 0.8		
Enrofloxacin					LOQ = 0.3		
Ciprofloxain					LOQ = 3		
Sarafloxacin					LOQ = 5		
Ciprofloxacin	Samples extracted with a mixture of acetonitrile and ammonium hydroxide	Column: Zorbax Eclipse XDB-Phenyl. Mobil phases: acetonitrile (A), 1% formic acid with ammonium hydroxide (B)	LC-ESI-ITP	60–93	LOQ = 0.3	Liver	[49]
Norfloxacin					LOQ = 1.2		
Danofloxacin					LOQ = 0.2		
Enrofloxacin					LOQ = 3		
Sarafloxacin					LOQ = 2		
Orbifloxacin					LOQ = 1.5		
Difloxacin					LOQ = 0.3		

(*continued*)

TABLE 20.1 (continued)
Chromatographic Methods for Determining Antibiotics in Animal By-Products

Compound	Extraction	Separation	Detection	Recovery (%)	LOD, LOQ, CCα and CCβ (μg/kg)	Samples	Reference
Tetracyclines							
Oxytetracycline	Na_2EDTA McIlvaine buffer solution and SPE cleanup with C_{18} cartidges	Column: Lichrospher C_{18}; Mobile phase (A) methanol, (B) acetonitrile and (C) Methanolic oxalic acid	LC-DAD		95% samples (90) >LMR	Liver	[41]
Oxytetracycline	Na_2EDTA McIlvaine buffer solution and SPE cleanup with C_{18} cartidges	Column: Lichrospher C_{18}; Mobile phase (A) methanol, (B) acetonitrile and (C) Methanolic oxalic acid	LC-DAD		17% samples (90) >LMR	Kidney	[41]
Tetracycline Oxytetracycline Chlortetracycline	Na_2 EDTA McIlvaine buffer solution and SPE cleanup with Oasis HLB cartridges	Inertsil ODS-2, Mobile phase 0.01 M oxalic acid (A), acetonitrile (B)	LC-FLD	>77 muscle, >65 kidney	LOQ < 100	Kidney	[44]
Tetracycline Chlortetracycline Oxytetracycline Doxycycline	LE with sodium succinate solution (pH 4.0) followed by protein removal with trichloroacetic acid. SPE cleanup on an HLB column	Column: PLRP-S polymeric reversed phase. Mobile phase 0.001 M oxalic acid, 0.5% (v/v) formic acid and 3% (v/v) tetrahydrofuran (THF) in water (A), tetrahydrofuran (B)	LC-ESI (+)-QqQ	72–105 at half MRL	LOD < 5	Liver and kidney	[42]
Oxytetracycline	LLE with sodium succinate solution, protein removal with TCA and SPE cleanup with Oasis HLB cartridges	Column: PLRP-S C18 Mobil phase 0.5% (v/v) formic acid in water (A), tetrahydrofuran (B)	LC-ESI (+)-QqQ	47–56	CCα = 311, CCβ = 361 CCα = 648, CCβ = 798	Liver Kidney	[50]

Minocycline Tetracycline Oxytetracycline Methacycline Dmeclocycline Chlortetracycline Doxycycline	Extracted with 20% TFA and 0.4 M oxalate buffer. SPE cleanup with Lichrolut SPE cartridges	Column: Chromasil ODS-3 Mobil phase 0.01 M oxalic acid (A), acetonitrile (B)	LC-DAD	93–125	23,69,312,329[a] 24,74,323,331[a] 46,139,311,324[a] 10,31, 318, 334[a] 44, 133, 321, 335 [a] 36,108, 318, 342 [a] 44, 133, 318, 332 [a]	Liver	[43]
Minocycline Tetracycline Oxytetracycline Methacycline Dmeclocycline Chlortetracycline Doxycycline	Extracted with 20% TFA and 0.4 M oxalate buffer. SPE cleanup with Lichrolut SPE cartridges	Column: Chromasil ODS-3 Mobil phase 0.01M oxalic acid (A), acetonitrile (B)	LC-DAD	81–120	54,163,312,329[a] 24,74,323,331[a] 46,139,311,324[a] 41,124,621,649[a] 44,133, 619,647[a] 36, 108, 646,690[a] 44, 133, 612,637[a]	Kidney	[43]
Oxytetracycline Tetracycline Chlortetracycline	Extraction with EDTA-McIlvaine. SPE cleanup with Oasis HLB cartridges	Column: Chromasil ODS-3 Mobil phase 0.01M oxalic acid (A), acetonitrile (B)	LC-API (+)-MS	71–83	100	Kidney	[44]
Multiresidue							
Quinolones [15] Lincomycine [5] Macrolide [11] Nitroimidazole [6] Penicilline [12] Sulphonamide [23] Tetracycline [6]	Protein precipitation with acetonitrile, SPE cleanup with Oasis HLB cartridges	Column: HSS T3; Mobile phase (A) actonitrile and (B) water, each 1 mmol/L formic acid	LC-MS-TOF	76–98 66–107 20–91 79–101 49–107 54–408 29–100	CCα and CCβ data are calculated for each antibiotics (100) at liver and kidney samples	Liver and kidney	[45]
β-lactamics [3] Tetracyclines [3]	Samples homogenized with water followed by centrifugal ultrafiltration	Column: TSK-Guardegel ODS; Mobile phase (A) water and (B) methanol, each 0.05% formic acid	LC-APII (+)-QqQ	70–115	LOQ = 0.2	Liver and Kidney	[46]

(*continued*)

TABLE 20.1 (continued)
Chromatographic Methods for Determining Antibiotics in Animal By-Products

Compound	Extraction	Separation	Detection	Recovery (%)	LOD, LOQ, CCα and CCβ (μg/kg)	Samples	Reference
Tetracyclines [4]	Samples homogenized with 0.1 MEDTA and extracted by methanol	Column: Genesis C_{18}; Mobile phase (A) acetonitrile and (B) 0.2% formic acid at 0.0 mM oxalic acid	LC-ESI (+)-QqQ	26–39	S/N > 25 at MRL	Kidney	[47]
Sulfonamides [4]				74–88			
Quinolones [4]				49–66			
β-Lactamics [4]				77–107			
Macrolides [3]				44–68			

Note: KH_2PO_4, Potassium dihydrogen phosphate; NH_4A_C, Ammonium acetate; EDTA, Ethylenediaminetetraacetic acid; MSPD, Matrix solid-phase dispersion; MIP, Molecularly imprinted polymer.

[a] Data corresponding at LOD, LOQ, CCα, and CCβ.

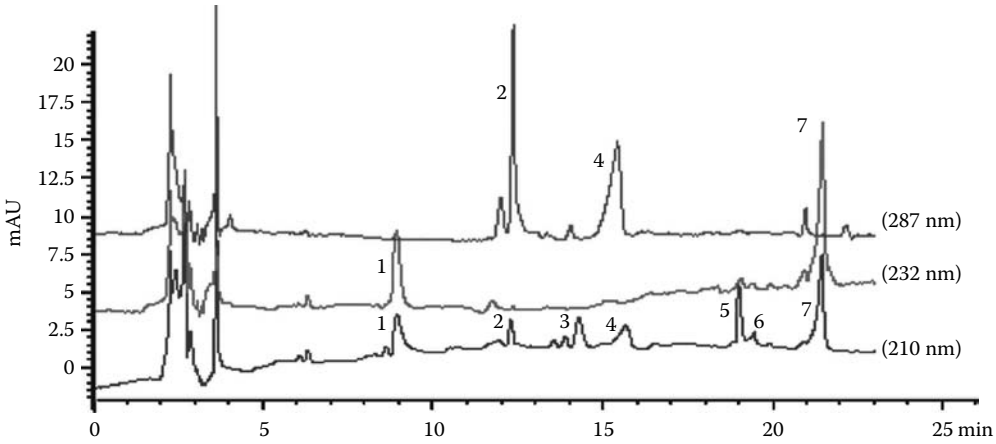

FIGURE 20.2 LC-DAD chromatograms at 210 (a), 232 (b), and 287 nm (c) of an extract of spiked kidney with 200 µg/kg of each macrolide. Peaks: (1) spiramycin, (2) tilmicosin, (3) erythromycin, (4) tylosin, (5) roxithromycin, (6) troleandomycin, and (7) josamycin.

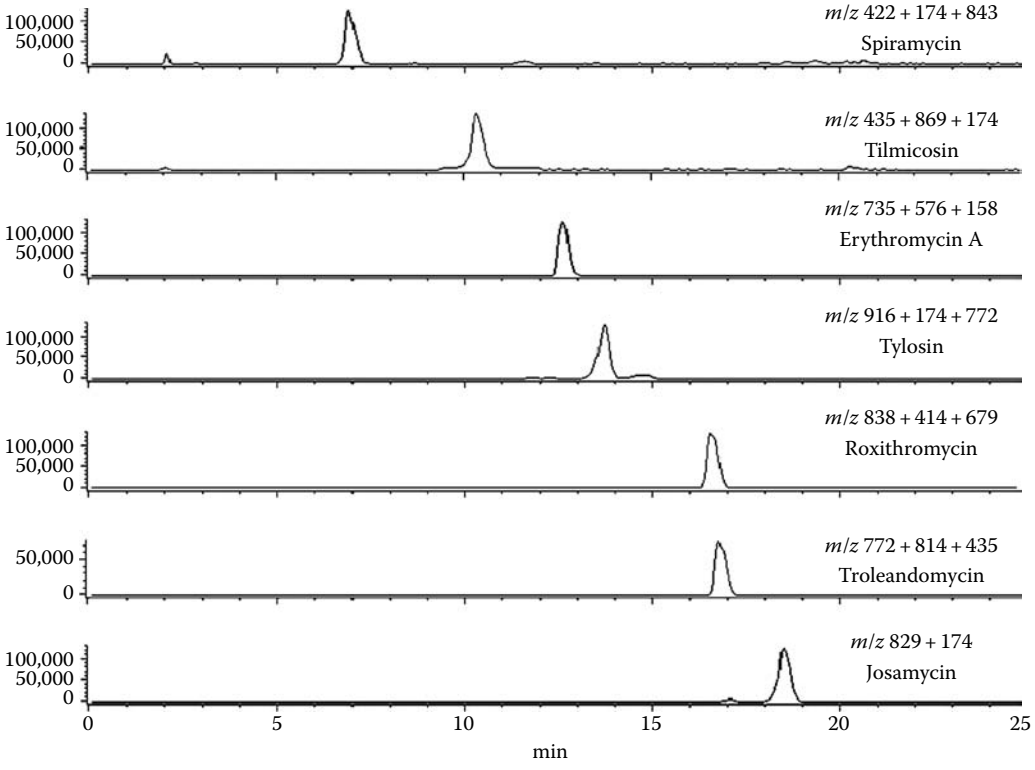

FIGURE 20.3 SIM chromatograms corresponding to an extract of rabbit liver sample where tilmicosin (1) was found at 250 µg/kg and erythromycin (2) at 168 µg/kg.

20.4.2 Aminoglycosides

Various analytical methods for the detection of residues of aminoglycosides have been described [16,25–28]. Most of these methods started with a protein precipitation with trichloroacetic acid (TCA). The sample cleanup was by SPE on a polymer column or weak cation exchanger or with hydrophilic–lipophilic balance cartridges (oasis HLB). The final analysis was by LC-MS/MS triple quadrupole. This demonstrates that there is a need for sensitive, quick, and accurate analytical methods to monitor the use of aminoglycosides. Extraction of dihydrostreptomycin (DHS) from animal by-products was performed using a liquid extraction with a 10 mM phosphate buffer containing 2% (w/v) TCA, followed by a SPE cleanup procedure on a carboxypropyl weak cation exchange column [25]. This method was based on an ion-pair chromatography, using a mixture of 20 mM pentafluoropropionic acid (PFPA) in water and acetonitrile in the mobile phase for the reversed-phase C18 column. The components were detected and quantified by ESI—ion trap. The method was validated according to EC requirements at one-half of the MRL and at one and a half times the MRL and was used for the monitoring of DHS residues in incurred cattle medicated with DHS. Gentamycin was analyzed in several bovine tissues using trifluoroacetic acid (TFA) in the mobile phase and the positive-ion pneumatically assisted electrospray detector by selected reaction monitoring (SRM) [26]. The extraction technique with aqueous TCA was for both, to precipitate proteins and to liberate gentamicin from the matrix. Matrix-matched tissue standards were required for this developed and validated method to compensate the matrix effects on ionization. The authors concluded that tobramycin was not usable as internal standard due to differences in apparent recovery between gentamicin and tobramycin in kidney. The LC-MS/MS method was also reported for determining DHS, gentamicin C1, and neomycin in veal kidney and liver [28]. The extracts are purified online on a reversed-phase column, with the help of TCA. Four secondary ions were quantified for every analyte and recoveries in kidney were 76%, 57%, and 51% for dihydrostreptomycin, gentamicin C1, and neomycin, respectively. The authors reported that this method was applied for the analysis of more than 1000 veal samples over a 1 year period.

In addition, LC-QqQ-MS method for the determination of 11 aminoglycosides at beef and kidney, respectively, were fully validated according to the EU criteria (Kaufmann and Butcher 2008, [27,45]). The developed methods used a low-pH extraction with TCA to ensure a complete extraction considering the significantly different pKa values of the studied analytes. The extracts were purified on a SPE column. Good performance characteristics were obtained for recovery, precision, decision limits, and decision capabilities. For the MRL substances—with MRLs of 100–5000 µg/kg—CCα and CCβ values were in the ranges of 121–5278 and 143–5538 µg/kg, respectively, and for nonauthorized compounds like tobramycin, CCα, and CCβ, values were 22 and 28 µg/kg, respectively.

20.4.3 Nitrofurans

Four nitrofurans were determined using LC-MS/MS on a C18 column [29]. The authors described the furazolidone, furaltadone, nitrofurantoin, and nitrofurazone analyses and emphasized the benefits of monitoring 3-amino-2-oxazolidinone, 3-amino-5-morpholinomethyl-2-oxazolidinone, 1-aminohydantoin, and semicarbazide, their stable metabolite. Various groups of pigs were given feed medicated with nitrofuran drugs at a therapeutic concentration for 10 days. Animals were slaughtered at intervals and tissue samples were analyzed both for parent nitrofuran and metabolites. The parent drugs were detectable only sporadically and only in pigs subjected to no withdrawal period. In contrast, metabolites were accumulated to high concentrations in tissues (ppm levels). The authors concluded that while all four major nitrofuran antibiotics are undetectable in edible pig tissues within 1 week after cessation of treatment, their metabolites are detectable at high concentrations at least 6 weeks after cessation.

20.4.4 β-Lactams

The application of matrix solid-phase dispersion (MSPD) as a generic extraction technique was applied by [30] with hot water as extractant followed by LC-MS/MS to determine two penicillins (amoxicillin and ampicillin) in bovine liver and kidney. The SRM acquisition mode with two fragmentation reactions for each analyte was adopted and the authors said that the most important condition to be satisfied for ascertaining the presence of a selected compound is that at least two signals produced by the decomposition reactions were distinguishable from the background ion current. As can be seen in the table, amoxicillin and ampicillin could be detected in kidney and liver at levels of <3 μg/kg.

In addition, LLE with potassium dihydrogen phosphate was also used to extract amoxicillin, followed by SPE cleanup on C18 column using liquid chromatography combined with positive electrospray ionization tandem mass spectrometry. The method was validated according to the EU criteria and CCα and CCβ were calculated at kidney and liver separately [31]. The method was tried out on incurred kidney, liver, fat and skin samples that were taken from broiler chickens slaughtered at different times. Amoxicillin concentrations above the MRL level were only determined in samples taken within an hour after the last drug administration.

Multi-residue methods within the β-lactamic class were reported [32]. Eleven β-lactam antibiotics were analyzed in fortified and incurred beef kidney tissue using LC-ESI/SRM ion trap tandem mass spectrometry (MS^n). The presented method was also tested on incurred kidney tissue that had previously been analyzed using a microbial assay. Good correspondence was found between the results from this new method and the bioassay. Authors found that SRM-MS^n mode provided rapid and unambiguous identification of analytes in unknown incurred tissue, and amoxicillin and ampicillin could be quantified in bovine tissues down to 3.1 and 0.8 μg/kg levels.

A multi-residue analytical method was developed for the quantification of 6 β-lactams in bovine tissues using the deuterated benzylpenicillin and nafcillin as internal standard by LC- ESI tandem mass spectrometry with a multiple reaction–monitoring technique [33]. An ion-exchange cartridge cleanup and ion-pair LC were combined to remove the influence of sample matrix on the MS response. The average recoveries from liver and kidney at the tolerance levels of (50 μg/kg) ranged from 77% to 101% with the coefficients of variation below 5%. The use of deuterated compounds greatly contributed to the improvement of repeatability and accuracy of the quantification.

20.4.5 Quinolones

Most of the analytical methods to detect residues of quinolones in liver and kidney are based on LC using C18-bonded phase. The extraction is mostly based on LLE with acetonitrile or methanol followed by SPE cleanup [34–38]. The multi-residue method allowing the determination of 11 fluoroquinolones in swine kidney was developed using liquid chromatography-tandem mass spectrometry and limits of quantification were below 50 μg/kg. These studies were carried out in order to support the EU policy on consumer health protection [37,38]. A simple high-performance liquid chromatography (HPLC) method for the simultaneous analysis of enrofloxacin and its primary metabolite ciprofloxacin has been developed. Tissue sample preparations were carried out by phosphate buffer and extraction with trichloromethane, followed by separation on a reversed-phase column using an internal standard and fluorescence detection. The accuracy, precision, and detection limits obtained allow this procedure to be used in MRL residue studies [39]. Turbulent flow chromatography-tandem mass spectrometry was also applied [35] for this determination. Tissue samples were extracted with a mixture of acetonitrile, water, and formic acid and the matrix components contained in the injected sample were separated from the retained analytes on a column suited for pretreatment of samples at high flow rates. Mean recoveries rates were in the range from 72% to 105% and a run time of only 4 min enabled a high sample throughput.

Enrofloxacin was also determined in liver and kidney samples from Tehran slaughterhouses [40,41] using HPLC-DAD. This study confirmed the widespread misuses of these antibiotics in local farms and the lack of implementation of recommended withdrawal times.

The immunochemical approach was also explored and an enzyme-linked immunosorbent assay (ELISA) was developed to detect 15 different FQs in kidneys [34]. The estimated detection capabilities CCβs were in the range 4–200 μg/kg. This alternative offered support for the generation of rapid screening techniques as well as simple and cost-effective sample cleanup methods.

20.4.6 Tetracyclines

After LLE with McIlvaine buffer followed by SPE in C18 columns, oxytetracycline was determined by LC-DAD in liver and kidney samples from 90 broiler farms in Tehran province of Iran [41]. Quantitative data of recovery, limits of quantification were not presented; however, the monitoring data showed a high level of samples contamination and the authors emphasized on harder regulations for the use on antimicrobial drugs in poultry industry.

Multi-residue method to determine tetracycline (TC) residues as well as their respective 4-epimers in edible tissues of pig was also developed and validated according to the requirements of the EC at the MRL for kidney, fat, skin, and liver [42]. Limits of quantification were obtained for the analysis of the TCs and the 4-epiTCs in muscle, skin, fat, liver, and kidney tissues of pig. This is important because the EC has set an MRL for the sum of the TCs and their 4-epimers in edible tissues of all food-producing animals.

Method with diode array detection, at 355 nm, was also proposed and validated for the determination of seven TC in liver and kidney muscle tissues [43]. The examined TCs were extracted from both tissues with 20% TFA and 0.4 M oxalate buffer (pH 4) and purified with LiChrolut SPE cartridges. Overall recoveries ranged from 93.2% to 125.5% and 81.3%–120.4% for liver and kidney tissues, respectively, and the new criteria of CCα and CCβ specified by EU were also calculated. The extraction procedure developed for the isolation of TCs from kidney using common buffers and similar SPE protocols was effective and LOD values were below the MRL set by the EU [44].

20.4.7 Multi-Residue

A quantitative multi-residue method including more than 100 drugs (70 antibotics) belonging to different drug families, has been developed [45]. The authors used ultra-performance liquid chromatography coupled to time of flight mass spectrometry. The proposed bipolarity liquid–liquid–solid extraction technique recovered a large range of analytes regardless of its polarity and pKa value. The method was validated according to the Commission Decision 2002/657/EEC for muscle, liver, and kidney.

A rapid analysis method for oxytertracycline, tetracycline, chlortetracycline, penicillin G, ampicillin, and nafcillin in liver and kidney has been developed by using electrospray ionization tandem mass spectrometry after addition of internal standards (demeclocycline, penicillin G-d5, ampicillin-d5, and nafcillin-d6) [46]. Analysis time, including sample preparation and determination, was minimal. The overall recoveries from animal (bovine and swine) kidney and liver fortified at the levels of 0.05 and 0.1 mg/kg ranged from 70% to 115% with satisfactory detection limits.

Nineteen analytes from five classes of antibiotics, i.e., tetracyclines, sulfonamides, quinolones, β-lactams, and macrolides in kidney were screened by LC-MS/MS [47]. Acetylisovaleryltylosin and its metabolite 3-*ortho*-acetyltylosin are also included. The accuracy of the method is sufficiently good for screening antibiotics at the MRLs in kidney from pig, cattle, sheep, deer, horse, and reindeer. More than 60 samples could be analyzed and evaluated in 24 h. Validation was performed according to Commission Decision 2002/657/EC.

20.5 GENERAL CONSIDERATIONS

In the last decade, an important scientific effort has been done to improve the existing HPLC methods, paying special attention to the development of new, simple, reliable, and sensitive HPLC methods for the determination and confirmation of antibiotics in animal by-products.

Some antibiotics are permitted with corresponding MRL values, whereas others are prohibited. These MRL values are not the same for all the organs and animals, so different validation approaches are required. Significant improvements in sensitivity have been achieved to comply with the strict regulatory demands.

The cost-effectiveness of analytical procedures is an important issue for all laboratories involved in residue analysis. An efficient way to improve cost-effectiveness is to maximize the number of analytes that may be determined by a single procedure.

There is increasing interest in analytical methods for the simultaneous analysis of various classes of veterinary drugs. LC-QqQ-MS utilizing selected MRM transitions was the starting point for multi-residue method development. Full-scan MS technologies also offer the advantage of retrospective analysis without reanalysis and a clear trend toward full accurate MS techniques (e.g., ToF-MS) in multi-residue methods can be observed. Today, in theory, all compounds can be measured by full-scan MS. The starting point now is no longer the detection conditions used, but the sample material. Starting with different matrices, most of the veterinary drugs can be detected, and the most important part of method development is the extraction of the compounds of interest from the different matrices. This increases the interest in a variety of modern sample pretreatment techniques.

We can conclude from the published methods that LC-QqQ-MS is the preferred method for residue analysis of antibiotics in animal by-product samples. The sample pretreatment techniques most frequently used in combination with LC-QqQ-MS are LLE and/or SPE. A specific combination of LLE and SPE can be very selective for a specific class of veterinary drugs.

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21 Environmental Contaminants: Pesticides

Pablo Vazquez-Roig and Yolanda Picó

CONTENTS

21.1 INTRODUCTION

There are numerous chemical pesticides used to control insects and other pests in livestock facilities. Industrial farms apply these chemicals directly to the skin, fur, and feathers of livestock such as cattle, pigs, and poultry in order to kill off flies, mites, spiders, cockroaches, ticks, and other pests [1,2]. Furthermore, accidentally, pesticide sprays can be blown onto livestock by wind or livestock can be fed with plants that have been sprayed [1,3]. Domestic animals can also accumulate such substances from contaminated feed and water [4]. As a consequence, these pesticide residues accumulate in the animals' fatty tissues, such as fatty meat, and by-products (including organs, fat or lard, skin, feet, abdominal and intestinal contents, bone, and blood) [5]. Thus, animal-derived products are considered to be an indirect source of pesticides, which is supported by various surveys, indicating that the most persistently and highly contaminated foodstuffs are animal products, followed by leafy vegetables and garden fruits [6–9].

Analytical determination of pesticide residues in animal by-products is very important in order to ensure that these products are not a risk for the human health and can be utilized for the required purposes [10–12]. The use of contaminated by-products can be an entry of these compounds in the food chain [9,11].

Animal by-products are challenging matrices for the determination of residues and contaminants [13–16]. The variability of matrices is enormous. It ranges from relatively simple matrices like serum, blood, or intestines to all kinds of by-products from agro and food industry. The composition of the matrix of an animal by-product can often be not known to the laboratory conducting the analysis [17].

The number of publications on the determination of chemical residues and contaminants in vegetables, fruits, and other foodstuffs is extensive [18–30]. However, the number of papers dedicated to animal by-product analysis is relatively limited and mainly restricted to organochlorine (OC)

pesticides such as DDT, BHC, dieldrin, and lindane [10,27]. This chapter covers the analysis of major classes of pesticides in edible animal by-products. It briefly provides some basic information on food sample preparation, extraction, cleanup, pesticides separation and detection methods, as well as their advantages and disadvantages followed by applications in food analysis.

21.2 LEGISLATION

The use of pesticides has, as possible consequence, the presence of their residues in the organs of animals. To avoid any adverse impact on public health, these residues should not be found in food at levels presenting a risk to humans.

All around the world, the levels of pesticide residues in any food, including any edible animal by-products, are controlled in terms of maximum residue limits (MRLs) or tolerances (both are more or less synonyms). An MRL is the maximum concentration of pesticide residue (expressed as mg/kg) to be legally permitted in or on food commodities and animal feeds. Foods derived from commodities that comply with MRLs are intended to be toxicologically acceptable and safe for human consumption. These limits are based on good agricultural practices and are designed to ensure that the amount of pesticide residues in foods is maintained as small as practically possible. During the approval process, the potential exposure of consumers to these residues in food is carefully assessed and MRLs are established. Although these MRL are not directly associated with safety criteria, are considered to be toxicologically acceptable with respect to the general population and to result in theoretical or measured exposures compatible with the acceptable daily intake (ADI). The ADI estimates of the amount of a substance in food or drinking water, expressed on a body mass basis (usually mg/kg body weight), which can be ingested daily over a lifetime by humans without appreciable health risk, on the basis of all of the facts known at the time. However, there are many legislative authorities that regulate the legislation on pesticide residues—Table 21.1 provides a short overview of the international and governmental bodies in charge of announcing MRLs or tolerance in each different country, including the most essential Web sites where information is available. As a consequence, the legislation is not harmonized through the different countries, even though they agree upon procedures for the evaluation of pesticide residues in terms of edible animal by-products safety [31–33]. The Codex Committee on Pesticide Residues provides recommendations to the *Codex Alimentarius Commission* about MRLs for pesticide residues for specific food items or food groups [34]. Codex MRLs apply in international trade and are derived from evaluations conducted by the joint meeting on pesticide residues.

The new regulation on animal by-products adopted by the European Parliament and the Council applies since May 1, 2003. It aims to integrate the animal by-products sector into the "farm to table" approach for food safety as set out in the white paper on food safety adopted in January 2000. It introduces stringent conditions throughout the food and feed chains requiring safe collection, transport, storage, handling, processing, uses, and disposal of animal by-products. In this way, MRLs are also set by the European Commission for all food and feed products [35]. From September 1, 2008, Regulation (EC) No 396/2005 of the European Parliament and of the Council on MRLs of pesticides in products of plant and animal origin defines a new fully harmonized set of rules for pesticide residues [36]. This regulation simplifies the existing legislation by harmonizing pesticide MRLs and making them directly applicable. Legislation may include relevant metabolites or degradation products of the pesticides. Strict monitoring is, therefore, an important activity to guarantee food safety.

A database order by name of pesticide, active substance, or animal by-product can be found in the Web site of the European Union (EU) [37]. The EU pesticide residues database provides for a search tool for all the EU-MRLs set in Reg. (EC) No 396/2005. The animal products (including edible animal by-products) considered in the legislation are meat, preparations of meat, offal, liver, kidney, blood, animal fats; fresh chilled or frozen, salted, in brine, dried or smoked or processed as flours or meals; other processed products such as sausages and food preparations based on them.

TABLE 21.1
Characteristics of the Current Pesticide Residues MRLs Legislation and Recommendation of the International Organizations and Governmental Bodies

	Legislative Bodies and Issues
International Organizations	
World Health Organization (WHO) (http://www.who.int/about/en/)	
Food and Agriculture Organization (FAO) of the United Nations (UN) (http://www.fao.org/)	Since it was founding in 1945, FAO has focused special attention on developing rural areas, home to 70% of the world's poor and hungry people. FAO is also a source of knowledge and information. It helps developing countries and countries in transition modernize and improve agriculture, forestry, and fisheries practices and ensure good nutrition for all
Codex Alimentarius Commission (http://www.codexalimentarius.net/web/index_en.jsp)	*Codex Alimentarius Commission* was created in 1963 by FAO and WHO to develop food standards, guidelines, and related texts such as codes of practice under the Joint FAO/WHO Food Standards Program. About 170 countries are members of the Codex
World Trade Organization (WTO) (http://www.wto.org/)	WTO is the only global international organization dealing with the rules of trade between nation
Governmental Bodies	
USA (http://www.fda.gov/)	U.S. Food and drug administration (FDA) is the main agency besides 15 other agencies like USDA[a] and FSIS[b]
European Union (EU) (http://europa.eu/)	The *White Paper on Food Safety* of January 12, 2000 sets out the plans for a proactive new food policy. The white paper established as strategic priorities (1) to create a EFSA,[c] (2) to implement a farm to table approach in food legislation and (3) to establish the principle that feed and food operators have primary responsibility for food safety
United Kingdom (http://www.food.gov.uk/)	Food Standard Agency controls the food safety using food safety Act 1990
Australia and New Zealand (http://www.foodstandards.gov.au/)	ARMCANZ[d] developed Australian standards and ANZFA[e] developed national food standards
Canada (http://www.hc-sc.gc.ca/)	The Bureau of Chemical Safety in Health Canada's Food Directorate is responsible for policy, standard setting, risk assessment, surveillance, research, and evaluation activities with regard to chemicals in foods sold in Canada
Japan (http://www.ffcr.or.jp/zaidan/FFCRHOME.nsf/pages/eng.h-page)	The Food Safety Commission is responsible for the control of food in Japan
China (http://eng.sfda.gov.cn/eng/)	State Food and Drug Administration (2003) is responsible for the pollution-free food in China
India (http://foodsafetyindia.nic.in/)	Monitoring System for Food Safety for India (Under Food Safety and Standards Act—2005) is done by Ministry of Health

[a] USDA: U.S. Department of Agriculture.
[b] FSIS: Food safety and inspection service.
[c] EFSA: European food safety authority.
[d] ARMCANZ: Agriculture and Resource management Council of Australia and New Zealand.
[e] ANZFA: Australia-New Zealand food authority.

Currently, MRLs for pesticides in edible animal products range from 0.01 mg/kg (chlorobenzilate, dinocap, lactofen, etc.) to 1 mg/kg (DDT, ion fluoride, furfural, etc).

Farmers, traders, and importers are responsible for food safety, which includes compliance with MRLs. Member state authorities are responsible for control and enforcement of the MRLs. These checks will entail taking samples, analyzing them and identifying the pesticides and respective

pesticide levels present therein. To ensure that it is done in an adequate and uniform way, the Commission has three instruments:

1. The coordinated EU multi-annual control program sets out for each member state the main pesticide–crop combinations to monitor and the minimum numbers of samples to take. Member states have to report the results, which are published in an annual report.
2. Community reference laboratories coordinate, train staff, develop methods of analysis, and organize tests to evaluate the skills of the different national control laboratories.
3. The Food and Veterinary Office of the Commission carries out inspections in the member states to assess and audit their control activities.

If pesticide residues are found at a level of concern for consumers, the rapid alert system for food and feed circulates the information and measures are taken to protect the consumer.

21.3 GENERAL CONSIDERATIONS

21.3.1 Pesticides

Pesticides are a large group of substances effective to control various insects, pests, and diseases. They belong to different chemical groups, show different structures and, consequently, present significant differences in their metabolism, biotransformation, and elimination in animals [38].

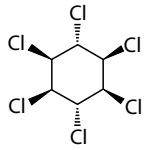
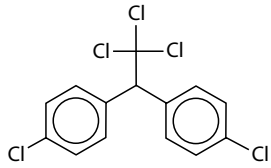
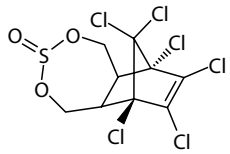
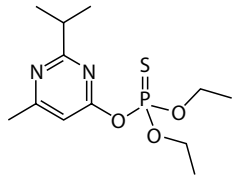
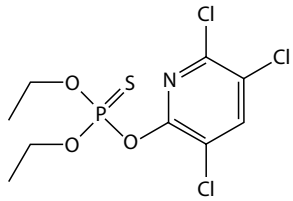
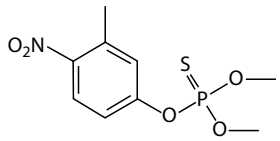
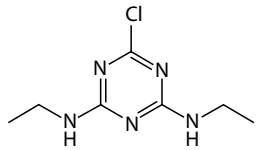
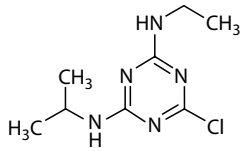
Pesticides used nowadays are mostly organic compounds characterized by such a variety of chemical structures and functional groups to make their chemical classification quite complex [39,40]. The most commonly applied pesticides are insecticides (to kill insects), herbicides (to control weeds), rodenticides (to kill rodents), and fungicides (to kill or inhibit fungi or fungal spores). However, these classes of pesticides are not so well-defined as be desired, and their members could be discussed under several classes. For example, most herbicides have some bactericidal or fungicidal activity. Representative structures of each chemical group of pesticides are shown in Table 21.2. The major chemical groups of the most classical categories of pesticides are as follows:

1. Organochlorines (OCs), which are a group of hydrocarbons with one or more chlorine atoms. DDT, lindane and mirex are examples.
2. Organophosphates (OPs) integrated by esters of phosphoric, phosphonic, thiophosphoric, or related acids. Some of the more common examples in this group are diazinon and malathion.
3. Triazines consist of a number of substituted 1,3,5-triazines, which are classical herbicides, like simazine.
4. Carbamates formed by salts or esters of carbamic acid, such as carbofuran and furathiocarb.
5. Others, like substances derived from 1,3-indandione, dicarboxamides, etc.

The spectrum of toxicity ranges from compounds that are acutely toxic, such as some organophosphate and carbamate insecticides, to compounds that are virtually nontoxic to mammals, such as many growth regulators and “biological” insecticides, including those compounds that are not acutely toxic but that possess a significant potential for chronic toxicity, such as many fungicides [41–51].

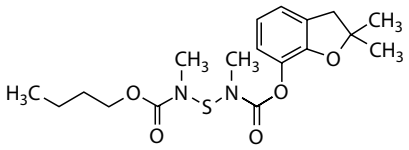
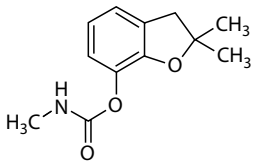
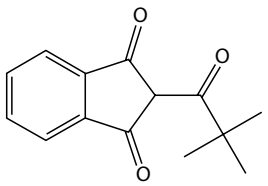
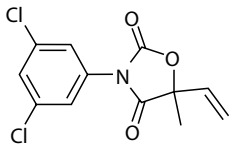
The majority of studies published in the determination of pesticides in animal by-products have focused their attention on the analysis of OCs, mainly DDT and its metabolites (DDD, DDE), followed by organophosporus. OC pesticides have been of great concern due to their persistent nature and chronic adverse effects on wildlife and humans. Some studies have suggested that these compounds may affect the normal function of the endocrine system and have also been linked to human breast, liver, and testicular cancers as well as to low sperm counts in humans [40,45]. Exposure to

TABLE 21.2
Chemical Structures of the Major Classes of Pesticides Used in Livestock

Class	Typical Pesticide	Structure
Organochlorines	Lindane	
	DDT	
	Endosulfan	
Organophosphates	Diazinon	
	Chlorpyrifos	
	Fenitrothion	
Triazines	Simazine	
	Atrazine	

(*continued*)

TABLE 21.2 (continued)
Chemical Structures of the Major Classes of Pesticides Used in Livestock

Class	Typical Pesticide	Structure
Carbamates	Furathiocarb	
	Carbofuran	
Indandiones	Pindone	
Dicarboxamides	Vinclozoline	

these compounds has been monitored, and regulatory limits are currently mandated. Monitoring indicates that 99.5% of the population has an average of 1.8–12.6 pg/μL of DDT in their serum from nutritional and environmental exposure [52].

Despite organophosphorus (OP) are less persistent than OCs, they can also reach the food chain, and the EU has set MRLs in fatty matrices of animal origin [41,43–44].

Nowadays, the number of pesticides that can be used in crops protection is decreasing yearly, and they are increasingly polar, hydrosoluble, and thermolabile. However, they could reach the food chain due to great stability of some of them and for an illegal use too. Because of this, pesticides analyses must be directed toward analyses of old commonly used as well as modern ones.

21.3.2 Edible Animal By-Products

Meat by-products are produced by slaughterhouses, meat processors, wholesalers, and rendering plants and include tissues such as diaphragm (skirt, cattle only), head meat (ox cheek, cattle only), and the heart, kidneys, liver, pancreas, tail meat, thymus and tongue to blood, blood plasma, feet, large intestines, small intestines, lungs, oesophagus meat, rectum, stomach (non-ruminant), first stomach (tripe, after cooking), second stomach (tripe, after cooking), fourth stomach, testicles, and udder. It also includes poultry parts such as gizzards and necks [53–59]. However, analyses have been mainly performed in liver, and, in minor proportion, in kidney, fat, heart, brain, etc. of the most frequently consumed animals like pork, lamb, chicken, fish, etc. Liver is one of the lipophilic tissues of the animal anatomy, in which pesticides can be found, especially OPs that are

metabolized in this organ [60]. This is due to the fact that main persistent pesticides, like OCs and OPs, are highly lipophilic and have a great stability, and, because of that, they tend to bioaccumulate in fatty organs of the animals.

Fish oil is a by-product of the fish meal manufacturing industry. Concentrated Omega-3 fatty acids can be found in fish oil, whose daily ingestion slows down the progression of coronary artery disease. However, as these chlorinated compounds accumulate in the lipid compartment of the fish, the oil extracted from the animal may be contaminated with them [61–63]. Therefore, it is of special interest to investigate the levels of DDT and its metabolites in fish oil, where these compounds tend to accumulate [52].

21.3.3 Analysis

The analyst has to check the current legislation about MRLs and to utilize those analytical techniques that achieve the limits of detection (LODs) required for the legislation and an unequivocal identification and quantification of analyzed compounds. Efficient sample preparation is a very important aspect of the analytical method. In order to avoid erroneous data, a special care is needed in ensuring that the sample is representative to the original composition of the raw material, since it can lead to erroneous conclusions. After sampling, it is necessary to prepare the sample for the determination of analytes through extraction, trace enrichment and, usually, removal of interferents. The type of matrix analyzed should be taken into consideration because the presence of fat in these matrices could interfere with analytes isolation and determination [64]. These procedures often take up most of the total analysis time, contributing highly to the total cost of analysis and can be the main source of error, and, because of that, an optimal sample preparation involves an important profit in time-consuming and money. Appropriate sample preparation can enhance sensitivity of the analytical method. Traditional methods are laborious and usually involve large amounts of organic solvents, which are expensive and generate considerable waste. Nowadays, analytical procedures tend to use a minimal quantity of organic solvents, be faster, and amenable to automation to increase the number of samples to be tested.

In the past decades, the determination of pesticides was performed by gas chromatography (GC) in combination with different detectors. Methods based on liquid chromatography (LC) with ultraviolet (UV) or diode array (DAD) and fluorescence detectors are often less sensitive than GC instruments, and thus they were applied more rarely in the past. In the last years, the availability of tandem mass spectrometry (MS/MS) with atmospheric pressure ionization (typically, electrospray, ESI), has made possible to do only one injection in the instrument to achieve the four identification points required by the EU for the correct quantification and confirmation of pesticides in positive samples.

A summary of the extraction methods and determination techniques used in the analysis of samples is presented in Tables 21.3 and 21.4.

A general diagram of the different stages of a generic analytical method can be seen in Figure 21.1. In the next sections of the chapter, we will examine the details of each stage of the analysis deeper.

21.4 EXTRACTION PROCEDURES

Extraction consists of the transfer of analytes from the original matrix to another, adequate for the analysis. Extraction means an isolation of the analytes removing interfering substances, and in the case of edible animal by-products, this usually entails transference from a solid matrix into a liquid phase. In some cases, extraction involves an enrichment of analytes concentration to a level that allows their analytical determination.

Conventional extraction of organic analytes from food samples usually begins with a homogenization step (with Ultra Turrax® or similar), followed by tedious liquid extraction with one or several

TABLE 21.3
Examples of Gas Chromatography Methods for Pesticides Residues in Edible Animal By-Products

Matrix	Pesticides	Extraction Technique	Recoveries (%)	Determination Technique	LOQ	LOD	Reference
Blood, liver, lung, kidney, brain, bone marrow and abdominal organs of rabbits	Diazinon and endosulfan	Blood samples: SPE C18 Diazinon: SE (hexane) Endosulfan: SE (ethyl acetate)	92–100	GC-MS	10–50 ng/mL	3–15 ng/mL	[79]
Fish oil	DDT and metabolites	CC (acidified silica)	88–115	GC-ECD	8–16 ng/mL	2–5 ng/mL	[52]
Fish liver	13 OCs	CC (silica gel)	90–99	GC-ECD/GC-MS		0.5–10 ng/g	[89]
Chicken, pork, and lamb livers	33 OCs and OPs plus vinclozoline	MSPD-GPC	62–115	GC-MS/MS	0.01–9 ng/g	0.08–23 ng/g	[60]
Boar liver	3 OPs	SE (acetone:acetonitrile) + SPE C18 (acetonitrile)	65–75	GC-NPD	5 ppb	1 ppb	[64]
Blubber, liver, and kidney of whale	14 OCs and 4 OPs	SE (hexane) + HPLC	57–105	GC-MS		1–10 ng/g	[68]
Hair and various pork tissues (abdominal fat, liver, lung, brain, spinal marrow, heart, kidney, and spleen)	8 OCs	SE Soxhlet (hexane:acetone)-CC (acidified silica)	72–80	GC-ECD		0.2 ng/g	[73]
Liver and kidney of camel, cattle, and sheep	9 OCs	LLE-CC (florisil)	86–109	GC-ECD			[77]
Fish oil	10 OCs	Online GPC	64–122	GC/TOF-MS			[93]
Fish oil	12 OCs	GPC-CC (acidified silica + florisil)	30–102	GC-MS	2.5–9.3 pg/g		[90]
Blood, plasma, urine, cerebrospinal fluid, liver, and kidney	4 OPs	HS-SPME		GC-NPD		2–55 ng/mL	[84]

Lard and chicken heart. Harbor seal liver, kidney, and blubber	8 OCs	ASE (hexane:methylene chloride)-CC (activated carbon/silica gel)	45–86	GC/ITMS	0.8–1.9 pg/g		[75]
Liver and perirenal fat of roe deer	12 OCs	SE (petroleum ether:acetone)-SPE C18	85–110	GC-ECD		0.5–1 ng/g	[72]
Fish oil	13 OCs	On line GPC		GCxGC/TOF-MS			[92]
Fish oil	7 OCs	SPE (acid and neutral silica + alumina)	72–80	GC-MS		0.2 ng/g	[70]
Bird liver	14 OCs	SE Soxhlet (hexane:acetone)-CC (aluminum oxide + silica gel)	75–110	GC-ECD		≤0.2 ng/g	[74]
Fish liver	14 OCs	SE (hexane)-HPLC	71–102	GC-MS/MS		0.1–1.5 ng/g	[71]
Cod liver oil	DDTs	SE (dichloromethane)-GPC		GC-AED	20 ng/g	6 ng/g	[67]
Heart, kidney, and liver of swine, and cattle fat	21 OCs	ASE (dichloromethane:acetone) -GPC-SPE (silica-gel)	41–122	GC-MS			[83]
Pork fat and cod liver oil	10 OCs	SE (hexane)-HPLC	80–100	GC-ECD		2–50 ng/g	[65]
Blubber and liver from harbor seal	3 OCs	SE (hexane)-CC (florisil)-HPLC	85–107	GC-ECD			[66]
Animal tissues	368 pesticides	SE (cyclohexane:ethyl acetate)-GPC	40–120	GC-MS		0.2–600 ng/g	[94]

Note: SE, solvent extraction; CC, column chromatography; HS, headspace; SPME, solid-phase microextraction.

TABLE 21.4
Examples of HPLC and Other Methods for Pesticides Residues in Edible Animal By-Products

Matrix	Pesticides	Extraction Technique	Recoveries (%)	Determination Technique	LOQ	LOD	Reference
Bovine liver	5 OPs	MSPD C18-CC (silica gel)	>94% and 55% chlorfenvinphos	HPLC-DAD Confir. GC-MS	150–300 ng/g	50–100 ng/g	[82]
Sausages	Alachlor	SE (acetonitrile)	100	FPIA		8 ng/mL	[76]
Animal liver	4 indandiones	SE-SPE (HLB)	83–105	RFIC-MS	0.2–1 ng/g		[78]
Sheep liver	4 triazines	MAE (methanol)-CC (aluminum oxide)	91–102	HPLC-DAD	22–160 ng/mL	14–88 ng/mL	[80]
Rat liver and kidney	Furathiocarb and 3 metabolites	SE (acetone)-CC (silica gel)	50–100	HPLC-Fluorescence		100–200 ng/mL	[69]
Beef tallow, lard, and chicken fat	DDTs	MSPD	58–93	HPLC-DAD	180 ng/g		[81]
Animal tissues	69 pesticides	SE (cyclohexane:ethyl acetate)-GPC	40–120	HPLC-MS/MS		0.2–600 ng/g	[94]

Note: SE, solvent extraction; CC, column chromatography; HS, headspace; SPME, solid-phase microextraction; DSI, direct sample introduction.

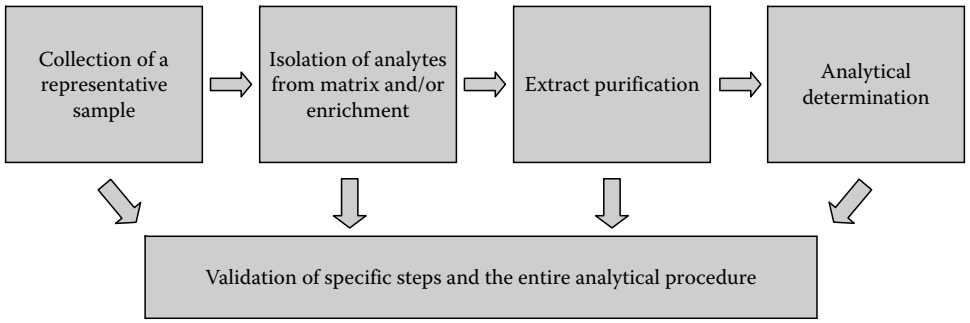


FIGURE 21.1 Main steps of analytical procedures used for determining pesticides in edible animal by-products.

cleanup steps to remove co-extractants. Extraction procedures for pesticides from edible animal by-products are carried out by means of solvent extraction (SE) [62–79], microwave assisted extraction (MAE) [80], matrix solid phase dispersion (MSPD) [81,82], pressurized liquid extraction (PLE) [75,83], and head-space solid phase microextraction (HS-SPME) [84].

Organic SE is probably the most widely used technique and can be performed using different devices. Soxhlet apparatus, consisting of a glass reservoir that sits between a lower flask at the bottom and a condenser at the top, the Soxhlet allows to extract chemicals from a solid sample into a organic solvent, leaving behind insoluble impurities. Aulakh et al. [85] developed a method to extract nine OC pesticide residues in poultry feed, chicken muscle, and eggs. The samples were extracted for 8 h in 200 mL hexane–acetone (1:1, v/v) mixture. The cleanup of the samples was performed by silica-gel column chromatography (CC) and analysis was done on a GC-ECD (electron capture detection). Main disadvantage of Soxhlet is that it is time-consuming and requires high amounts of organic solvents. SE aided by ultrasonication, refluxing, or shaking achieves a more efficient extraction of the analytes from a solid sample than if sample is simply put in direct contact with a suitable solvent, and is more rapid than Soxhlet apparatus. In some cases, the SE is followed by liquid-liquid extraction (partitioning), which usually involves consumption of a great quantity of organic solvent. Liu et al. [69] determined simultaneously the amount of furathiocarb and its metabolites in biological tissues such as liver and kidney. The method consists of extraction of samples with acetone, filtration, partition with water, and purification of target analytes through a silica gel column. Reasonable recoveries for routine analysis were obtained (50%–100%), and the LODs with fluorescence detection were 0.2, 0.1, 0.1, and 0.2 mg/L for furathiocarb, carbofuran, 3-hydroxycarbofuran, and 3-ketocarbofuran, respectively, with a signal-to-noise ratio of 3. Ueno et al. [86] developed a multiresidue analysis of 78 pesticides in animal and fishery products. The crude sample extract obtained by acetone–hexane extraction for solid samples or acetonitrile extraction for liquid samples was cleaned up with a gel permeation chromatography (GPC)/solid-phase extraction (SPE) system. The first GPC pesticide fraction containing lipids and pigments was selectively collected, and loaded directly onto a graphitized carbon/PSA 2-layered column. After the second GPC pesticide fraction was collected, the 2-layered column was eluted with acetone–hexane (3:7). Pagliuca et al. [64] analyzed chlorpyriphos and dimethoate in liver of a wild boar by liquid partition with acetone–acetonitrile (1:4) followed by cleanup with solid phase cartridge (SPE C_{18}). The recovery, investigated by analyzing samples spiked at 5, 10, and 50 ppb, ranged from 60% to 81%. The limit of quantification (LOQ) and the LOD were, respectively, 5 and 1 ppb for each compound, and allowed quantifying the residues below the MRLs. Jin et al. [78] developed a method to analyze five indandione anticoagulant rodenticides, which were extracted from homogenized animal liver tissues with methanol–acetonitrile (10:90, v/v), and then, the extracts were subjected to SPE process using Oasis® HLB cartridges. The relative average recoveries of compounds spiked in animal liver tissues were between 83.4% and 104.9%. The LOQs were 0.2–1.0 ng/g for them. Within-day and day-to-day relative standard deviations (RSDs) were less than 10.4% and 13.3%, respectively.

MSPD is also widely applied in fatty matrices. This technique is based on blending the sample with a dispersant agent in a mortar, causing a rupture of sample architecture to provide a new mixed phase being easier isolating analytes from various sample matrices. Dispersion sorbent is typically C_{18}-bonded silica because its nonpolar character provides the best affinity for the studied compounds, and because it causes the complete disruption and distribution of the lipophilic entities commonly existing in animal tissues such as liver. De Llasera and Reyes-Reyes [82] developed a reproducible analytical MSPD-LC method for determining residues of OP pesticides in bovine samples. The MSPD extraction procedure using C_{18} sorbent followed by silica cleanup was optimized to maximize recovery of the pesticides contained in bovine samples while eliminating most of the interfering matrix components. Acceptable recoveries for pesticides ≥94% were obtained, except for chlorfenvinphos in liver, for which the recovery seems to be a function of the analyzed tissue (recovery is 55% in liver and 98% in muscle). The results demonstrate that the accuracy, precision, and selectivity are satisfactory for the analysis of the OPs examined. LODs were between 25 and 100 ng/g, allowing the procedure application for detection below the levels imposed by existing regulations. However, Frenich et al. [60] compared SE using ethyl acetate and subsequent cleanup by GPC and MSPD extraction with C_{18} sorbent, Florisil cleanup and analyte elution with ethyl acetate. MSPD allowed reaching the acceptable recovery of a lower number of pesticides (25 compounds) than SE (34 compounds). Wang et al. [87] presented a simple and rapid method to determine simultaneously five types of fluoroquinolones, OPs, and *N*-methyl carbamates in porcine tissue by MSPD using C_{18} as dispersant, and *n*-hexane–acetonitrile as eluent. The results showed a recovery ratio of 60.1%–107.7% with satisfactory RSDs. The LODs in porcine tissue are between 9 and 22 μg/kg. The added value of this study is that it reports, for the first time, the simultaneous determination of the veterinary drugs and pesticide residues in animal tissues. Some researchers suggested that the cocktail effect and combined effects of veterinary drugs, pesticides, and medicines to human health should be focused upon for its chronic, potential, and accumulated toxicity. Furusawa [81] proposed MSPD dispersing with Toyobo-KF®, an activated carbon fiber, as a new MSPD sorbent. KF® has an extremely large surface area ranging from 1000 to 1600 m^2/g. This sorbent is readily mixed with samples, like meat or animal fats, which can then be packed into columns. The average recoveries of the study (spiking levels: 0.2, 0.5, and 1.0 μg/g) ranged from 58% to 93%, with RSDs of <7%. The LOQs were 0.18 μg/g.

Other alternative techniques, to reduce both solvent consumption and time effort, are MAE and PLE. MAE utilizes microwave energy to heat the solvent and the sample to increase the mass transfer rate of the solutes from the sample matrix into the solvent. Cheng et al. [80] determined four triazine herbicides, simazine, atrazine, propazine, and promethryne, in sheep liver by MAE technique using methanol as extractant. The operational parameters of the technique, the solvent type and volume, extraction temperature and time, were optimized in detail with respect to extraction efficiency of the target compounds from sheep liver. The recoveries of the method at two different spiked levels were assessed by analyzing spiked liver samples, and were in the range from 90% to 102% with good precision (<11%).

PLE, also known as accelerated SE (ASE®), is a patented technique for the extraction of solid and semisolid sample matrices using common solvents at elevated temperatures and pressures. PLE is a fully automated system, requiring smaller quantities of solvent than traditional methods, and reduced time of analysis. Wang et al. [75] analyzed OCs in harbor seal tissue samples from Alaska. Tissue samples were homogenized, lyophilized, and then extracted by PLE with a mixture of hexane and methylene chloride (1:1, v/v). Recent studies showed that the PLE method using an equal volume mixture of hexane and methylene chloride, under the conditions adapted in this study, efficiently co-extracts OCs and lipids. Lipids were removed with 40% sulfuric acid-modified silica gel columns. Recoveries averaged 45%–86% for OCs with RSDs of 2%–14%, 52%–137% for polychlorinated biphenyls (PCBs) with RSDs of 3%–29% and 36%–152% for polychlorinated naphthalenes (PCNs) with RSDs of 7%–29% from lard and chicken heart samples, which were used as alternative matrices to harbor seal samples in recovery studies. Saito et al. [83] developed a new

analytical method for the quantification of 59 different persistent organohalogen compounds, such as polybrominated diphenyl ethers (PBDEs), PCNs, PCBs, PCB metabolites, and OC pesticides in biological organ tissues. The optimum extraction and cleanup procedures were examined using PLE, automated GPC on Biobeads S-X3, and automated SPE on silica gel. The target compounds were divided into two fractions, nonpolar compounds and more polar compounds, which in the latter fraction were subsequently methylated using diazomethane.

Within the current trend to use miniaturized procedures, headspace solid phase micro extraction (HS-SPME) has advantages as high purity of the extract, avoidance of organic solvents, and simple technical manipulation. The unique example in literature is focused on the analysis of various tissues for the determination of four OPs: parathion, methyl parathion, malathion, and diazinon. The experimental methodology carried out by Tsoukali et al. [84] was based on HS-SPME and GC-NPD (nitrogen–phosphorus detector). HS-SPME was optimized by studying the effect of several experimental parameters: extraction temperature, salt addition, sample pH value, preheating, and extraction time. The proposed methodology renders an efficient, cost-effective, and simple sample preparation process for the determination of OPs in various biological specimens (liver, kidney, cerebrospinal fluid, whole blood, and blood plasma). The technique overcomes limitations and obstacles of conventional methods, such as the use of expensive and toxic organic solvents and the application of tedious and cumbersome procedures. However, it is evident that systematic studies are required for each different biological specimen in order to suppress matrix effects and enhance extraction efficiency.

During the extraction of edible by-product samples, not only analytes are co-extracted but also other matrix constituents that can interfere into the analysis, mainly fats, and others like carbohydrates, proteins, water, etc. Therefore, usually the extract purification is needed. Cleanup stage most commonly performs CC [52,64,66,69,77,82,88–90] using silica gel (alone or impregnated with sulfuric acid) and Florisil as stationary phases. Alternatively, SPE cartridges [70,78,79,83,91], gel GPC [60,67,83,90,92,93] and HPLC fractionation of the extracts [62,63,65,66,68,71] are frequently utilized too. The combination of several clean-up techniques results in a powerful tool [66,69].

CC is a method to purify individual chemical compounds from mixtures of compounds. The classical preparative CC is a glass tube filled with stationary phase, where the analytes are eluted by the addition of mobile phase. In addition to separate lipids from OCs using a column filled with acidic silica gel and hexane or dichloromethane as eluent [52,64,66,77,88–90], CC can also separate different persistent organochlorine pollutants into several fractions. For example, Wang et al. [75], after PLE extraction and conventional CC cleanup, used an activated carbon/silica gel column to separate OCs and noncoplanar PCBs into the first fraction, and PCNs and coplanar PCBs into the second fraction.

SPE is an isolation process for compounds dissolved or suspended in a liquid. For that, sample is passed through a solid (stationary phase), in which the analytes of interest are retained on the stationary phase, and then, eluted with an appropriate solvent [64,66,69,74,78,79,82,83,88,90]. Berzas Nevado et al. [52] proposed and validated an analytical methodology to be applied to the determination of p,p′-DDT and its metabolites, p,p′-DDE and p,p′-DDD, in fish oil. The analytical procedure presented involves a single-step cleanup through a mixed silica column, prior to the analysis by GC-ECD. The LODs ranged from 2.6 to 4.7 pg/μL. The analytes were determined in three different fish oil pills sold in Spain as a supplementary vitamin support. The sum of p,p′-DDT and its metabolites was from 13.2 to 51.3 ng/g, p,p′-DDE being the dominant compound.

Lo Turco et al. [89] researched the levels of OC pesticides in samples of cultivated and wild *Dicentrarchus labrax* (European Seabass). Muscles and liver tissues sampled over 5 months, within the same year, were analyzed. Samples were ground with anhydrous sodium sulfate and were placed in a ultrasonication bath. Afterward, an aliquot of the tissue fat was introduced in a glass column packed with silica gel, and eluted with hexane. The quantitative determination of the OC compounds was performed by GC-ECD and confirmed with GC-MS. The results showed that the concentrations of DDTs in muscles and livers as such of reared sea bass were in the range 0.2–1.3 μg/kg and 9.6–48.4 μg/kg, respectively. In wild fish, the concentrations of DDTs were very much lower: 0.1 μg/kg in muscles, 5.1–9.0 μg/kg in livers.

GPC is a separation technique that separates analytes on the basis of size (size exclusion chromatography, SEC). Pang et al. [94] developed an extensive study for 600 pesticide residues in animal tissues by GPC coupled to GC-MS and LC-MS/MS. In the method, 10 g animal samples were mixed with 20 g sodium sulfate and extracted with 35 mL of cyclohexane + ethyl acetate (1 + 1) twice by blender homogenization, centrifugation, and filtration. Evaporation was conducted and an equivalent of 5 g sample was injected into a 400 mm × 25 mm S-X3 GPC column, with cyclohexane + ethyl acetate (1 + 1) as the mobile phase, at a flow rate of 5 mL/min. The 22–40 min fraction was collected for subsequent analysis. Recoveries at three fortification levels (low, medium, and high) were performed, giving values that fell within 40%–120%, among which 417 pesticides recoveries between 60% and 120%, accounting for 95%, 20 analytes between 40% and 60%, accounting for 5%. The RSD was below 28% for all 437 pesticides. The LODs for the method were 0.2–600 μg/kg, depending on each pesticide. Hoh et al. [93] optimized an analytical method using GPC, followed by direct sample introduction comprehensive two-dimensional (2D) GC, in which the GPC cleanup removes the bulk of the oil matrix. In other study, developed for the same authors [92], additional cleanup of the GPC extracts was done by silica adsorption and acidification, which helped to provide clues in the identification of untargeted compounds. But they concluded that in routine analysis, only GPC is needed for this analytical approach.

LC, used mainly in pesticide analysis like semi-preparative approach, removes fats present in the extracts and collects those fractions of interest (analytes) free of fats [62,63,66,68,71]. Van Der Hoff et al. [65] described a cleanup method for OC compounds in fatty samples based on normal-phase liquid chromatography (NPLC). To this end, an existing cleanup procedure, which uses column

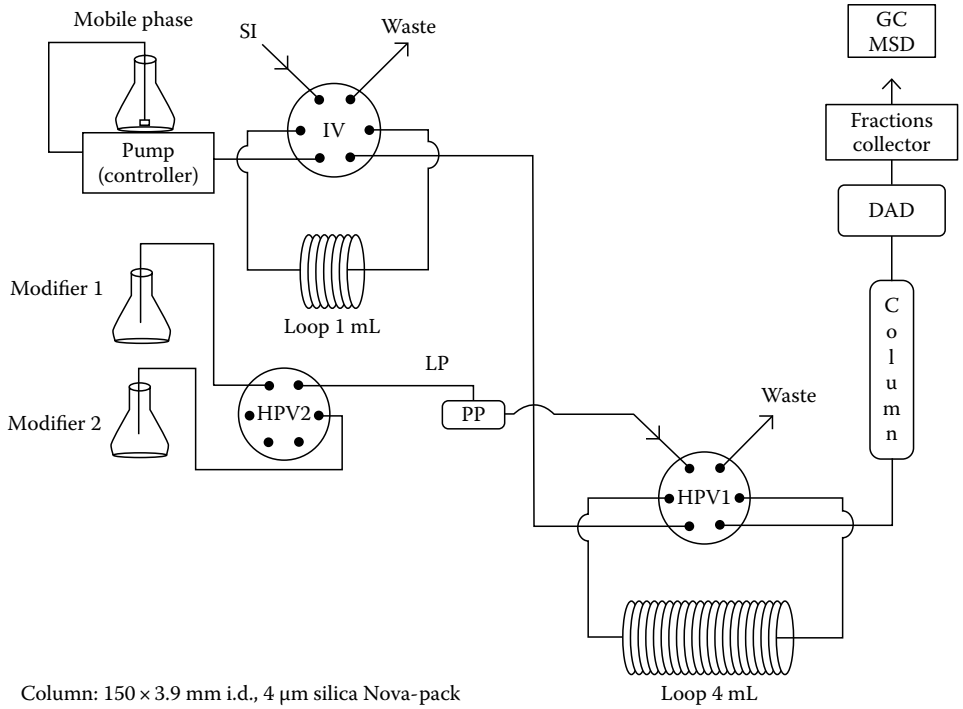


FIGURE 21.2 Scheme of the automated LC system used for the developing and optimization of analytical procedure. SI: sample injection; LP: low pressure line; IV: injection valve; HPV: 6-way high-pressure valve; DAD: diode array detector; PP: peristaltic pump. (From *Mar. Pollut. Bull.*, 40, Hernández, F., Serrano, R., Roig-Navarro, A.F., Martínez-Bravo, Y., and López, F.J., Persistent organochlorines and organophosphorus compounds and heavy elements in common whale (*Balaenoptera physalus*) from the Western Mediterranean Sea, 426–433, Copyright 2000, with permission from Elsevier.)

TABLE 21.5
Techniques of Sample Extraction and Purification

Technique	Extraction	Purification	Characteristics
LLE	++	–	Laborious, highly time-consuming, large solvent volume, purification step needed
Soxhlet extraction	++	–	Laborious, highly time-consuming, large solvent volume, no filtration required
MAE	++	–	Purification step needed, low solvent volume, but solvent must be able to absorb microwaves, evaporated temperature
ASE	++	–	Purification step needed, low solvent volume, evaporated temperature
SPE	++	+	Easily automated, low solvent volumes
SPME	++	–	Decreased or no use of solvents, easily automated, no sensitivity to suspended matter, problems with reproducibility and optimization
MSPD	++	+	Low solvent volume, not very suitable for dry samples or samples with high lipid content—adsorbent consumption is then relatively high and MSPD requires an additional cleanup step
GPC	–	++	Easily automated, long life span of columns, good recovery, highly time-consuming
Column chromatography	–	++	Laborious, highly time-consuming
LC	–	++	Easily automated, low solvent volumes

Source: From *Food Chem.*, 108, Beyer, A. and Biziuk, M., Applications of sample preparation techniques in the analysis of pesticides and PCBs in food, 669–680, Copyright 2008, with permission from Elsevier.

Note: ++, main application; +, secondary application; –, generally no application.

switching for the separation of OC pesticides and PCBs from the fat matrix, was simplified to a single silica LC column procedure. The use of an LC column packed with 3 μm silica enables complete fat/OC separation in a total fraction volume, and results in a fully automated cleanup procedure that takes only 32 min per sample. The method showed average recoveries of 80%–110% in the concentration range of 1–510 μg/kg, with RSDs of less than 10%. Limits of determination were in the range of 0.5–50 μg/kg.

Serrano et al. [68] determined OC and OP pesticides in whale tissues, extracting these compounds from blubber by fusing and dissolving the fat in *n*-hexane and from liver and kidney by reflux in *n*-hexane. Hexanic extracts were directly injected on the silica gel column of the automated LC cleanup system, using *n*-hexane as mobile phase. A schematic representation of the LC system used in this work for cleanup purposes is shown in Figure 21.2. The same research group [71] analyzed pesticides and PCBs in fish feed and fish tissues by means of automated off-line NPLC cleanup prior to GC–MS/MS.

Summarizing, Table 21.5 presents all the approaches as well as their main advantages and limitations.

21.5 ANALYTICAL DETERMINATION

For the identification, quantification, and/or confirmation of pesticide residues in edible animal by-products GC, LC, and immunoassays have been recently reported.

Immunoassays are chemical tests used to detect or quantify the analyte using an immunological reaction. Immunoassays are highly sensitive and specific but strongly suffer the influence of the matrix composition. There are several different methods used in immunoassay tests, such as immunoprecipitation, particle immunoassays, radioimmunoassay, enzyme immunoassay, fluorescent immunoassay, or chemiluminescent immunoassay. In the laboratory, immunoassays are rarely

carried out in edible animal by-products but if they are, then, the matrix effects on the immunological reaction must be considered. As a recent example, Nartova et al. [76] developed a procedure for the determination of Alachlor in liquid media using a fluorescence polarization immunoassay (FPIA). The effects of the structure and purification degree of a tracer (analyte with a fluorescent label) on the analytical signals, sensitivity, and selectivity in the determination of Alachlor were studied. The calibration graph was linear over the Alachlor concentration range 0.01–1.0 μg/mL; the detection limit was 8 ng/mL. The procedure was tested in the analysis of model solutions and soy-containing food products (sausages). The results are characterized by an appropriate reproducibility. The time taken to perform a single measurement was not longer than 10 min. The procedure can be recommended for the rapid semiquantitative testing of samples for Alachlor, or for the quantitative analysis of highly contaminated materials.

Chromatography techniques, mainly GC and LC are the most widely used chemical techniques for the separation of contaminants from edible animal by-products. Methods based on LC with UV or DAD [81–83,87] are often less selective and sensitive than GC ones, and they were applied rarely in the past. However, LC is required to analyze thermolabile, nonvolatile, or polar pesticides. Figure 21.3 shows a LC-UV chromatogram of (A) 5 μg/mL standard solution of triazines, (B) sheep liver blank sample, and (C) 10 μg/g spiked sheep liver sample with triazine. Most reports on triazines focus on the quantification of sorption and degradation processes in the soil or water, while quantification of residues to the animal tissue has attracted little attention. This chromatogram shows one of the few examples where liver was used as a representative animal matrix and chosen as the sample matrix. Fluorescence detectors have a limited use too, because natural fluorescent molecules are scarce. Post-column derivatization has been widely used in LC for the analysis of carbamates from various samples because fluorescence has unique advantages of enhancement of sensitivity and selectivity. Liu et al. [69] analyzed successfully furathiocarb and its three metabolites in tissues, such as liver and kidney, at ppb levels after post-column hydrolysis of the carbamates to methyl amine derivatization with *o*-phtaldehide and thiofluor.

LC-MS can provide more structural information on a molecule than any other of the analytical techniques. Nowadays, in the determination of pesticide residues in food, LC-MS (with the availability of MS/MS) has became the technique of choice displacing GC. It achieves, working in the single reaction monitoring mode, very low LODs, reducing chemical background noise produced from sample matrix. Even if matrix components co-eluted with analytes, they have different product ions that allow their distinction, providing a very good selectivity [95].

However, the pesticide residue determination in the edible animal by-products is not comparable to that of the other food items (Tables 21.3 and 21.4). One possible reason is suggested by Alder et al. [96], who individually compared for a large number of pesticides the applicability and sensitivity obtained with GC-EI-MS and LC-ESI-MS/MS. Only for one substance class, the OC pesticides that are the most widely determined pesticide class in edible animal products, GC-MS achieves better performance. There is only one example, reported by Pang et al. [94], who established a method for quantitative determination of 437 pesticides in some edible animal by-products using GC-MS and LC-MS/MS. GC-MS was used to determine 368 pesticides and LC-MS/MS for the determination of 69 nonvolatile and thermally unstable pesticides (mainly carbamates compounds).

GC systems with different detectors are preferred by researchers for target analysis of pesticides in edible animal by-products, coupled with MS [61,63,68,73–74,79,83,90,94], ECD [52,65,66,72, 73,85,87–98,91], atomic emission (AED) [67,101], and NPD [84]. This is due to some pesticides containing atoms of phosphorous, halogens, nitrogen, or sulfur, suitable to be specifically detected, even achieving a great sensibility. GC-ECD has been widely used that are for the analysis of OCs because of its high selectivity and low cost. However, GC-MS is clearly the preferred technique because it achieves enough sensitivity to check the compliance with current MRLs legislation and unequivocal confirmation of pesticide residue identity. In the case of GC-ECD and GC-NPD, confirmation of a positive sample requires a second injection in other gas chromatograph equipped with

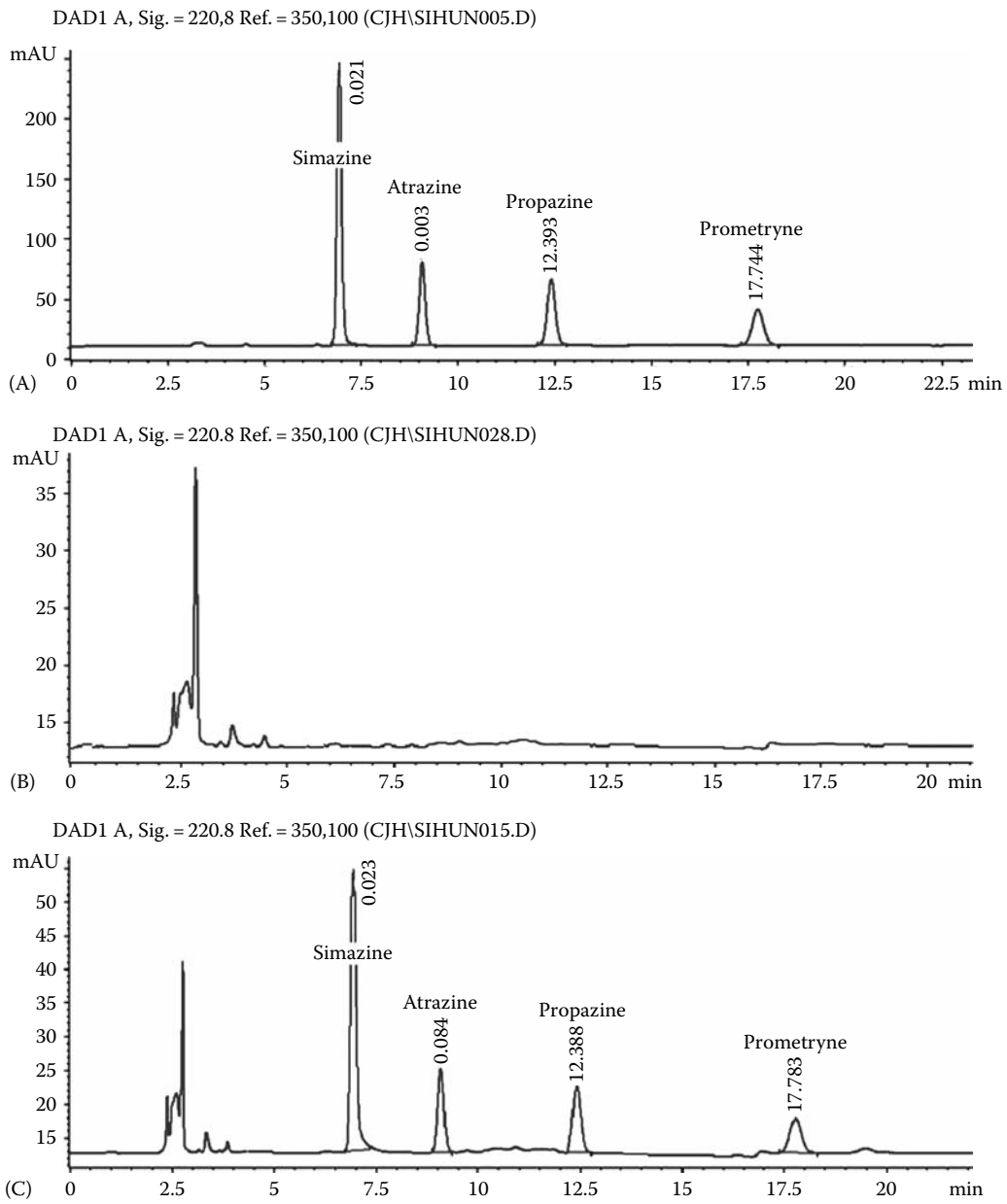


FIGURE 21.3 Chromatograms of triazines: (A) 5 µg/mL standard solution; (B) sheep liver blank sample; (C) 10 µg/g spiked sheep liver sample. (From Cheng, J.H. et al., *Anal. Chim. Acta*, 590, 34, 2007. With permission.)

a different type of column or detector, for example, with GC-MS [89], to obtain the four identification points as required in the Commission Decision 2002/657/EC [99] for the correct enforcement of legislation about MRLs and confirmation of banned substances.

GC-MS/MS has been also applied, either with triple quadrupole (QqQ) [60] or with ion trap (IT) [62,71,100]. Frenich et al. [60] determined OCs and OPs from animal liver by GC coupled to electron impact (EI)-MS/MS using a QqQ analyzer, achieving a high selectivity and sensitivity. Serrano et al. [71] developed a method to analyze OC pesticides in liver and muscle of fish using GC-MS/MS, after automated purification of sample extracts by NPLC. The analytical method applied showed excellent sensitivity and selectivity, as a consequence of the use of MS/MS. In Figure 21.4, the

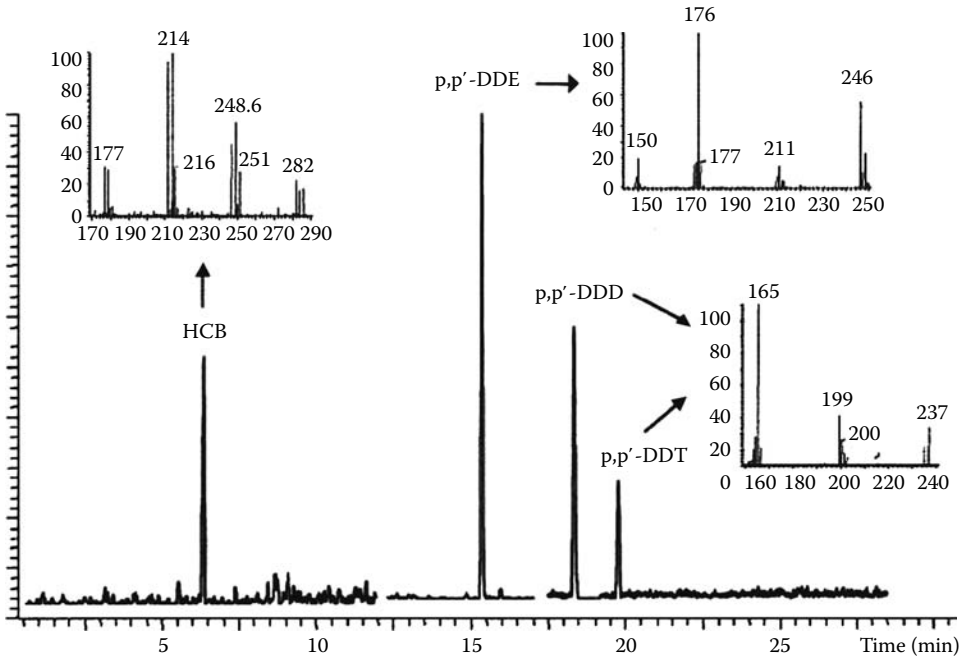


FIGURE 21.4 GC-MS/MS chromatograms of a fish food sample analyzed, and MS/MS spectra of the OC pesticides detected (extracted ion). (From Serrano Gallego, R., Barreda, M., Pitarch, E., and Hernández, F.: Determination of low concentrations of organochlorine pesticides and PCBs in fish feed and fish tissues from aquaculture activities by gas chromatography with tandem mass spectrometry, *J. Sep. Sci*., 2003, 26, 75–86. Copyright Wiley-VCH Verlag GmbH & Co. KGaA. With permission.)

absence of interferents in the analytical determination of HCB, DDT, and its metabolites using extracted ions is illustrated. As can be observed, the spectrum of each peak obtained by MS/MS confirms the identity of OCs detected in the samples.

Other interesting point within the pesticide residue determination in edible animal by-products is to develop analytical methods that can monitor both targeted and untargeted persistent organic pollutants and persistent organic pollutants-like compounds. There are still few examples of these applications, which mainly choose comprehensive 2D gas chromatography (GCxGC) coupled with time-of-flight mass spectrometry (TOF-MS) because of its superiority to conventional GC-MS. TOF-MS collects full mass spectra with better sensitivity than full-scan quadrupole-based MS, and GC × GC gives better separation power (especially, separation of analytes of interest from sample matrix by the second column) and sensitivity than GC allows. Hoh et al. reported the qualitative [92] and quantitative [93] capabilities of an analytical approach using direct sample introduction (DSI), GC × GC with (TOF-MS). This combined analytical approach efficiently increases the scope of organic chemicals monitored in the fish oils. DSI enables large-volume injection in a rugged manner. The greater selectivity of GC × GC affords cleaner mass spectra in complex extracts (fewer interferences), which helps in quantitation and qualitative identifications of targeted and nontargeted chemicals. Using DSI-GC × GC/TOF-MS, anthropogenic persistent organochlorine pollutants (PCBs, OCs, and PBDEs) in a dietary supplement of cod liver oil that was labeled "PCBheavy metal free" were identified. In the sample, several groups of halogenated natural products [1′-methyl-1,2′-bipyrroles (MBPs) 1,1′-dimethyl-2,2′-bipyrroles (DMBPs), methoxylated PBDEs (MeO-PBDEs), polybrominated hexahydroxanthene derivatives (PBHDs), polybromoindoles, and a halogenated monoterpene MHC-1] in addition to other organic contaminants (oxybenzone and octachlorostyrene) were identified and confirmed. As an excellent example of the power of GCxGC to separate very similar compounds, the Figure 21.5 shows a 2D chromatogram in which toxaphene

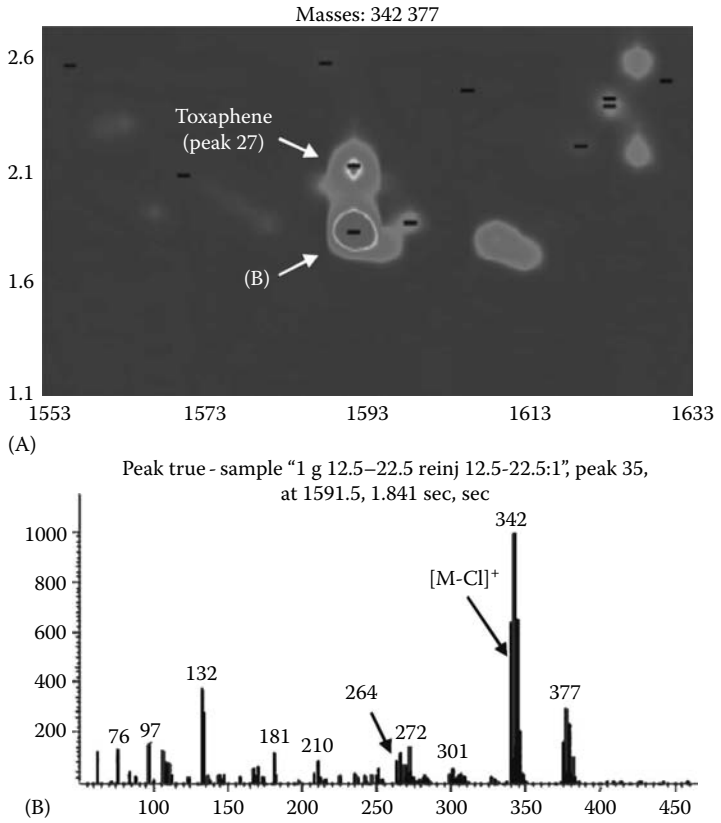


FIGURE 21.5 (A) GC × GC chromatogram (2D plot) of *m/z* 342 and *m/z* 377 indicating a toxaphene congener (peak 27) and an unknown compound (peak 63) in the cod liver oil, using the shorter 1D column configuration (see details in the Experimental Section). *x*- and *y*-axes represent 1D and 2D retention times in seconds; (B) EI mass spectrum of an unknown compound (peak 63) containing six chlorines. (With permission from Hoh, E., Lehotay, S.J., Mastovska, K., Ngo, H.L., Vetter, W., Pangallo, K.C., and Reddy, C.M., Capabilities of direct sample introduction—Comprehensive two-dimensional gas chromatography–time-of-flight mass spectrometry to analyze organic chemicals of interest in fish oils, *Environ. Sci. Technol*., 43, 3240–3247, 2009. Copyright 2009 American Chemists Society.)

has been separated of an unknown peak and the MS of this unknown peak. This peak could have six chlorines based on its isotope ratios of $[M^+]$ and $[M\text{-}Cl]^+$ in the MS. If *m/z* 375 is the M^+, then the compound structure should contain an odd number of nitrogen atoms according to the nitrogen rule. However, this requires further investigation for identification.

21.6 LEVELS OF PESTICIDES IN EDIBLE ANIMAL BY-PRODUCTS

Table 21.6 summarizes the levels of pesticide residues found in different edible animal by-products. Almost all the compiled studies are focused on OC compounds, the concentrations of which varied among the samples, being higher in fish oil followed by liver. These concentrations are in the range of ng/g level. Studies carried out in other edible animal by-products such as different fats, kidney, lung, brain, spinal marrow, heart, or spleen are scarce and disperse since it is difficult to achieve any conclusion on the frequency and incidence of pesticide residues.

As can be concluded for this table, the assessment of levels of pesticide residues in animal by-products is poor and it does not allow a picture of the general situation. Much more studies are needed in order to establish the potential contribution to the bioaccumulation of pesticides in edible animal by-products.

TABLE 21.6
Concentrations in Analyzed Samples of Edible Animal By-Products

Matrix	Pesticides	Found Concentration (ng/g)	Reference
Liver and perirenal fat of roe deer	8 OCs	0.4–629.3	[72]
Adipose tissue from bovines and swine	7 OCs	0.5–7.1	[97]
Hair and pork tissues (abdominal fat, liver, lung, brain, spinal marrow, heart, kidney, and spleen)	8 OCs	0.4–303.5	[88]
Liver and kidney of camel, cattle, and sheep	9 OCs	0.07–57.2	[77]
Duck and dog liver	4 indandiones	1.8–15.1	[78]
Fish oil	13 OCs	2.7–690	[92]
Fish oil	10 OCs	0.17–57	[93]
Fish oil	7 OCs	1.1–152.4	[70]
Fish oil	12 OCs	0.02–3310[a]	[90]
Liver of harbor porpoise	10 OCs	0.1–24.4	[73]
Bird liver	14 OCs	1.3–150[a]	[74]
Fish eggs	DDTs	1.5–81[a]	[98]
Fish liver	14 OCs	1.0–14.6	[71]
Cod liver oil	DDTs	489–714	[67]
Beef tallow, lard, and chicken fat	DDTs	Not detected	[81]
Blubber and liver from harbor seal	3 OCs	0.03–190	[66]
Fish liver and gills	12 OCs	78–154[a]	[63]
Fish liver	14 OCs	6.3–8.4[a]	[62]
Sausages	Alachlor	Not detected	[76]
Fish oil	DDTs	1.6–28.5	[52]
Fish liver	13 OCs	5.1–48.4	[89]
Chicken, pork, and lamb livers	33 OCs and OPs plus vinclozoline	<10	[60]
Sheep liver	4 triazines	Not detected	[80]
Lard and chicken heart. Harbor seal liver, kidney, and blubber	8 OCs	0.6–173	[75]
Blubber and liver from common whale	15 OCs	0.1–7.3	[61]

[a] Expressed as the sum of all concentrations of DDT isomers.

21.7 CONCLUSIONS AND FUTURE TRENDS

Monitoring of pesticide residues in animal by-products will remain an area of increasing concern and importance due to the possible impact to human health, and is one of the most important duties for public health agencies. However, the number of methods specifically devoted to the determination of edible animal by-products is still scarce. The analytical methods for drug residue monitoring should be accurate, simple, economical in time and cost, and capable of detecting residues below MRLs. This is a challenging task because the concentration of the analyte in the complex matrix is often very low. A particular characteristic of the pesticide residue determination in such products is that it is mostly focused on the determination of persistent OC compounds and despite extensive legislation at European level, which includes MRLs for a large number of pesticides, methods published for other types of pesticides can count on the fingers of one hand.

In this context, the current pesticide residues detection technologies are based on chromatographic methods. The instrumental methods, such as LC and GC, provide sensitive and specific techniques, but extraction and cleanup involve numerous different analytical steps that are time consuming and do not permit the monitoring of a large number of samples. However, state-of-the art methods strive to minimize organic solvent consumption and avoid environmental harm.

The development of new extraction techniques such as ASE, MAE, MSPD, SPE, and SPME has contributed to solvent reduction and, in some cases, to solvent-free extraction. The most universally applicable cleanup method is GPC, although new SPE methods are becoming increasingly popular. GC with selective detectors is the technique mostly used for pesticide analysis in edible animal by-products, and most detectors are able to adequately quantify 10 pg of analyte. GC-MS provides conclusive and defensible analytical information that is definitive. GC-MS instruments used for animal by-products analysis range from simple EI quadrupoles to TOF-MS with CI and positive/negative ion capabilities that can decrease the detection limits to parts per quadrillion (ptq), which will probably become routine in the near future.

The most innovative applications of GC relates to one of the most promising recent developments in chromatography, GC × GC. Until now, this approach has scarcely been used for animal by-products analysis, although it has been shown to be a very suitable technique for the full separation of complex mixtures, such as PCBs and OC, as well as solving difficult problems, such as identification of the other by-product' components.

New technologies in chromatography and MS are rapidly emerging. These, together with further developments in the automation of sample preparation, measurement, and data handling, will provide analysts with a unique opportunity for further innovation and improvements to meet the ever-expanding requirements in the field of analysis of pesticide residues in edible animal by-products.

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22 Environmental Contaminants: Heavy Metals

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CONTENTS

22.1 INTRODUCTION

Animals are at the interface between environment and human health because they are the final receptors of environmental contaminants and the providers of contaminants for humans through the consumption of their products. Edible animal by-products can concentrate heavy metals as a result of the environmental pollution of air, soil, and water and the contamination from agricultural practices and animal feeding. The heavy metals of particular concern are arsenic (As), cadmium (Cd), lead (Pb), and mercury (Hg), in relation to their harmful effects on human health and their known bioaccumulation property. The inorganic As (As(3) and As(5) forms) is more hazardous to humans than the organic As, and it is primarily of concern because of its cancer-causing properties. The chronic low-level As exposure gives skin, vascular, and nervous system disorders [1,2]. Cadmium causes renal and lung damages and negative effects on hemopoietic, cardiovascular, and skeletal systems [3,4]. Organic Hg compounds, usually methylmercury (MeHg), are more harmful than inorganic Hg, and they have been implicated in brain and liver damages [5]. Short-term Pb exposure adversely affects the brain and induces anemia and gastrointestinal symptoms [6].

Longer-term Pb exposure damages the kidneys and the reproductive and immune systems. The European Food Safety Authority (EFSA) has recently stated that the main concern of MeHg and Pb is that of their effects on brain and intellectual development in young children. Exposure during pregnancy is, therefore, considered to present particular risks.

Given the wide spectrum of effects of heavy metals on human health, supranational and international organizations set harmonized standards for food to improve the health of citizens of all nations and to facilitate food world trades. The European Union legislation has the form of maximum levels (MLs) for the tolerable concentration of heavy metals in food [7]. The General Standard for Contaminants and Toxins in Food (GSCTF) accepted in 1997 by the Codex Alimentarius Commission (CAC) has the philosophy that the MLs should be set as low as reasonably achievable—the ALARA principle [8]. The FAO/WHO Joint Expert Committee on Food Additives (JECFA) establishes the dietary intake of heavy metals and demands to the national authorities the responsibility to guarantee that food products do not contain heavy metals in concentration susceptible to affect the consumer's health status [9].

Most regulatory standards are established only after analytical procedures for enforcement have been fully validated and have been shown to comply with specified analytical criteria. Several legislations prescribed that the results must be expressed with their expanded uncertainty, so that the measurement data encompass three essential criteria, i.e., the utility, the reliability, and the transferability [10–13]. Analytical procedures for monitoring heavy metals in edible animal by-products have evolved to meet lower concentrations and to exploit improvements in analytical detection technology. In this light, there are usually two major steps to heavy metal quantification, the sample digestion and the detection method. The main methodologies for sample digestion are dry ashing of the sample and microwave digestion (MW) of the sample in strong acids. The detection methods most frequently used are atomic absorption spectrometry (AAS) and inductively coupled plasma (ICP) techniques. Atomic absorption spectrometry includes cold vapor AAS (CV-AAS) for Hg determination and hydride generation AAS (HG-AAS) for As quantification. The ICP techniques usually use mass spectrometry (ICP-MS) and optical emission spectrometry (ICP-OES).

In this chapter, a concise overview of the human exposure to heavy metals following the ingestion of animal by-products containing As, Cd, Hg, and Pb is provided also in the view of the currently proposed legislative limits or maximum tolerances in animal food and feed. Moreover, the recent developments and improvements in the analytical procedures used for preparing samples of food of animal origin and measuring the levels of As, Cd, Hg, and Pb are discussed together with a short guidance for quality control approaches that should be in place in a laboratory devoted to food control. Finally, the contamination level of As, Cd, Hg, and Pb in edible animal by-products as liver, kidney, lung, spleen, and heart is given based on the literature available so far.

22.2 SOURCES AND DIETARY INTAKE OF HEAVY METALS

22.2.1 Arsenic

The natural sources of inorganic As are rocks, soils and sediments, and water for both the inorganic and organic arsenical forms. Arsenic oxide is a common by-product of copper, lead, and nickel smelting considered as the major source of airborne anthropogenic As together with burning of fossil fuels as well as steel production and nonferrous alloying. In the past, arsenic insecticides, pesticides, fungicides, and rodenticides (mainly as lead arsenate) have been used worldwide in agricultural production. In wood preservation, the chromated copper arsenate is the most widely applied compound.

In general, food provides the main source of As exposure for man (with the exception of areas with an endemic high drinking water contamination). Daily As intakes for a number of countries are summarized in Table 22.1 [14–26]. A variety of studies from Canada, United States, Sweden, Croatia, Spain, France, Belgium, United Kingdom, Brazil, Chile, and Japan have established

TABLE 22.1
Average Daily As Intake (μg/Day) in Different Countries and Contribution (%) of Each Food Group

Country [Reference]	Year	Approach	μg/Day	Food Groups (%)
Canada [16]	1985–1988	TDS	47.3	N/A
Sweden [18]	1988	DMS	61 (women)	N/A
Croatia [14]	1988–1993	TDS	11.7	Fish and fishery products (75.8), cheese-dairy products (11.7), meat (2.2), offal (liver and kidney, 0.12)
United States [19]	1990	TDS	58.5 (men), 50.6 (women)	N/A
Spain [17]	1990–1991	TDS	286	Fish (97), Meat and alcoholic beverages (<0.4)
Japan [20]	1992	DMS	210	Fish, mollusks, crustacean (64.8), vegetable and seaweed (24.0), rice (8.8)
		TDS	280	
Belgium [21]	1992	DMS	<30	N/A
Brazil [22]	1992	DMS	12.4 (children), 6.9 (elderly)	N/A
United Kingdom [15]	1997	TDS	65	Fish (94), bread (1.5), cereals (1.5) and beverages (1.5), meat (0.15), offal (0.006)
France [23]	1998–1999	DMS	109 (total As), 11 (inorganic As)	N/A
France [24]	1998–1999	DMS	147	N/A
Chile [25]	2001–2005	TDS	77	Fish and shellfish (59), meat (8), spices (6), bread (5.8)
Spain [26]	2006	TDS	261 (total As); 26 (inorganic As)	Fish and shellfish (97), cereals (3)

DMS, duplicate meal study; TDS, total diet study.

intake estimates ranging between 11.7 and 286 μg/day. The highest As mean daily intakes are reported for Spain and Japan, while the lowest for Croatia and Brazil. A recent report (SCOOP) on exposure of the European population to heavy metals reported a mean As intake of the adult population between 0.5 μg/day (Greece) and 309 μg/day (Italy) [27]. It should be kept in mind that exposure values are reflective of different dietary habits (especially the presence or the absence of seafood in meals) and also mirror important variations in assumptions used to calculate them. In fact, data on the As content of the diet can be obtained by different methods and may involve either the collection of replicates of food eaten by individuals (duplicate meal study, DMS) or "total diet studies" (TDS) based on average food consumption statistics, which provide intake figures for the national "average person." The differences may also be partly related to the detection limits of the technique used for As quantification. Following the FAO/WHO JECFA recommendation, the cumulative exposure from different sources to inorganic As (the most toxic form) should not exceed the provisional total weekly intake (PTWI) of 15 μg/kg of body weight (bw), equivalent to 128.5 μg/day for a 60 kg person [28]. Considering that the diet studies reported in Table 22.1 refer to total As, a direct comparison with the JECFA tolerable intake is not possible. In this context, two alternative approaches are suggested (1) by assuming a weekly load for total As equal to 350 μg/kg/bw/week and (2) by assuming that ca. 10% of total As is in the inorganic form [28–31]. Applying both ways of calculation, the daily As intakes reported for the different countries never exceed the PTWI established. Table 22.1 also reports the food groups, which contribute most to the dietary As intake in different countries. Fish provides the main source

of dietary As, even if measurable concentrations may also occur in meat and meat products. In Croatia, fish and fishery products are followed by cheese-dairy products and then by bovine muscle, while liver and kidney of animals contribute for 0.12% [14]. In the United Kingdom, fish contributes for 94% of the total As intake, followed by bread and miscellaneous cereals and beverages. Meat products contributed for 0.15% and offal for 0.01% to the total As intake [15]. In Canada, it has been found that, after fish and shellfish and bakery food and cereals, meat and poultry are the next most important contributors to the dietary intake of As [16]. In Spain, fish (97%) is followed by meat and alcoholic beverages [17]. The SCOOP report showed that fish and crustacean contributed for 87.2%, followed by fruits and vegetables, cereals and bakery wares, beverages, while offal represented 1.4% of the total As intake [27]. It may be concluded that through foodstuffs of animal origin, we consume significantly higher amounts of As than through foodstuffs of vegetable origin.

22.2.2 Cadmium

Cadmium is a divalent transition metal and is usually found as a mineral in combination with other elements to form oxide, chloride, or sulfate [32]. Natural emissions of Cd to the environment can result from volcanic eruptions, forest fires, generation of sea salt aerosols, marine phosphates, or other natural phenomena. Cadmium can enter in the environment from zinc smelting and refining, coal combustion, mine wastes, and iron and steel production [33]. One model estimated that the relative importance of various Cd sources to human exposure is as follows [34]: phosphate fertilizers, 41.3%; fossil fuel combustion, 22.0%; iron and steel production, 16.7%; natural sources, 8.0%; nonferrous metals, 6.3%; cement production, 2.5%; cadmium products, 2.5%; and incineration, 1.0%. Of the anthropogenic sources of Cd, phosphate fertilizers, fossil fuel combustion, and some industrial activities contribute far more to human Cd exposure than production, use, and disposal of Cd products and incineration of all Cd-containing materials.

The major route of exposure to Cd for the nonsmoking general population is via food; the contribution from other pathways to total uptake is small. Table 22.2 reports the Cd daily intake, as estimated in different countries [15,17,19,20,24–26,35–44]. Data are reflective of different dietary habits, study designs, and quantification approaches used. The daily dietary intakes varied between 2.7 µg/day (Brazil) and 41.9 µg/day (China). High exposures are also reported in Japan and New Zealand. In Europe, the estimated intakes ranged between 3.6 µg/day (France) and 17.3 µg/day (Croatia). At the EU level, the SCOOP report showed that the mean Cd intake of the adult population was between 0.39 µg/day (Ireland) and 25.1 µg/day (the Netherlands) [27]. As regards the Cd intakes classified according to age, the highest intake corresponded to children (5.1 years), while the lowest was observed in elderly people (84.0 years) in Brazil [22]. The same was found in China, where the dietary intake is higher in children than in adults [35]. In relation to gender, similar intakes for men and women are reported in Germany, while, in Canada, males showed Cd intakes higher than females for all the age groups considered [36,37]. For the assessment of health risks of the above intakes, these can be compared to the current tolerable limits recommended for Cd. In this regard, the Scientific Panel on Contaminants in the Food Chain (CONTAM) recently established a TWI for Cd of 2.5 µg/kg/bw (equivalent to 21.4 µg/day for a subject of 60 kg) replacing the earlier PTWI of 7 µg/kg/bw (equivalent to 60 µg/day) [3,45]. According to the studies selected in Table 22.2, only in China, Japan, and New Zealand the tolerable Cd intakes are higher than the TWI and they can be of some concern for the healthy of consumers. The major proportion of total Cd taken in is consumed via cereals and cereals-based products, tubers, and vegetables (see Table 22.2). Contributions from meat and meat products ranged between 0.8% (China and Lebanon) and 18.2% (Spain). Some papers reported that the food items that contained high levels of Cd are kidneys and liver but they are seldom consumed and, on average, Cd intake via offal is negligible. For example, in the United Kingdom,

TABLE 22.2
Average Daily Cd Intake (μg/Day) in Different Countries and Contribution (%) of Each Food Group

Country [Reference]	Year	Approach	μg/Day	Food Groups (%)
Canada [37]	1986–1988	TDS	13	Vegetables (40.3), bakery goods and cereals (36.8), meat and poultry (7.9)
Germany [36]	1988–1991	DMS	14 (men), 11 (woman)	Bread, cake and pastries (37), potatoes (19), vegetables (14), meat, sausage and fish (10), offal (1.5)
Croatia [38]	1988–1993	TDS	17.3	Vegetables (37.8), fish (17.3), milk (11.5), poultry muscle (4.9), fruit (4.9), offal (4.1)
United States [19]	1990	TDS	19.3 (men), 18.5 (women)	N/A
Spain [17]	1990–1991	TDS	10	Fish (27.2), potatoes (18.2), bread (18.2), meat and meat products (18.2), cereals (9.1), vegetables (9.1)
India [39]	1991–1994	TDS	4.33	Cereals (73.5), vegetables (12.2), pulses (9.93), meat (4.61)
Japan [20]	1992	DMS TDS	27 35	Rice (53.7), vegetable and seaweed (13.8), fish, mollusk, crustacean (8.0)
Brazil [22]	1992	DMS	4.1 (children), 2.7 (elderly)	N/A
Denmark [40]	1993–1997	TDS	16	Cereals (50), vegetables (25), beverages (12.5), meat (2.1)
Egypt [41]	1995–1997	TDS	19.7	N/A
United Kingdom [15]	1997	TDS	14	Bread (25), potatoes (25), cereals (17), vegetables (8), meat products (4), offal (0.7)
New Zealand [42]	1997	TDS	24 (men)	N/A
France [24]	1998–1999	DMS	3.6	N/A
China [43]	2000	TDS	21.2	N/A
Chile [25]	2001–2005	TDS	20	Fish and shellfish (46), spices (15), cereals (7.0), meat group (5.5)
Lebanon [44]	2004	TDS	12.3	Cereals and cereal-based products (36), vegetables (22), drinking water (24.3), potatoes (4.9), meat and meat based products (0.8)
Spain [26]	2006	TDS	9.80	N/A
China [35]	2006	TDS	41.9 (adult), 20.2 (children)	Adult: vegetables (56.5), corn (18.8), sea products (18), fruit (4.43), bean (1.06), meat (0.76) Children: vegetables (52.5), corn (19.9), sea products (11.8), fruit (11.5), bean (2.5), meat (1.22)

DMS, duplicate meal study; TDS, total diet study.

Cd concentrations were the highest in offal (0.077 mg/kg) and nuts (0.059 mg/kg) but bread and potatoes made the greatest contributions (25%) to total dietary intake [15]. Similarly, in German diets, offal contained the highest Cd (0.083 mg/kg) and contributed for 1.5%, while about 40% of the total Cd intake was delivered by bread, cake, and pastries [36]. It has been noted that the inclusion in the diet of separate groups of food as offal (kidney and liver) and shellfish can

markedly increase the estimated Cd intake. In Croatia, offal contributed for 4.1% to the mean Cd intake [38]. In the SCOOP report, the contribution of offal in the Cd intake was 0.2% in Belgium, 1.1% in Finland, 2.2% in France, 0.11% in Germany, 2.24% in Greece, 11.0 in Norway, and <1% in the United Kingdom [27].

22.2.3 Lead

Lead in naturally present in all Earth's elements (e.g., plants, rocks, air, soils, water). Its large use in gasoline, paint, and construction material made Pb one of the most important environmental contaminants. Additional sources of this element have included Pb weights, small trinkets and toys, shots and bullets for weapons, pesticides, cartridge inks, etc., as well as contamination due to many industrial activities.

The major source of Pb for nonoccupationally exposed adults is food and drink. Children are exposed to additional Pb from dust and soil, and so Pb from foods and beverages may not be the predominant sources of Pb for all age groups. The proportion of total intake derived from food is dependent on the concentration of Pb in air, water, and other sources. In Table 22.3, data on the Pb dietary intake are available from several countries, including the United States, New Zealand, Egypt, China, Chile, and Lebanon [15,17,19,23–26,35,37–44]. The comparison between countries showed that in Egypt (242 µg/day) and Chile (206 µg/day) the Pb exposure estimate was higher than in other countries and it was near to the PTWI of 25 µg/kg/bw (equivalent to 214 µg/day for a 60 kg person) recommended by the JECFA [6]. In the other countries, even with the maximal estimate (100 µg/day in Croatia), there was no risk of exceeding the PTWI. The EU SCOOP report on the dietary Pb assessment in 13 countries showed the minimum Pb intake of 1.1 µg/day in Ireland and the maximum of 133 µg/day in Portugal [27]. These variations are partly due to the dietary composition, the processing technologies employed, whether the consumption of water and alcoholic beverages are included in the dietary estimates, and the number of samples taken and the quantification technique used. An overview of the foods contributing to the dietary Pb levels in different countries is shown in Table 22.3. In general, beverages followed by cereals and vegetables are the food categories that contributed the most. Offal contributed for 0.71% in Croatia and for 0.35% in the United Kingdom [15,38]. In the SCOOP report, the category of offal contributed for 0.1% in Germany up to a maximum of 3.5% in Italy [27].

22.2.4 Mercury

Mercury is an element naturally present in the environment. It exists in several forms, such as metallic, inorganic (i.e., sulfide, chloride, and oxide) and organic (i.e., MeHg, ethylHg, or phenylHg). About 80% of the Hg released in the environment is in the metallic form coming from fossil fuel combustion, mining, smelting, and from solid waste incineration [46]. Metallic Hg is also used in thermometers, barometers, blood pressure devices, batteries, dental fillings, electric switches, etc. Some of the inorganic Hg salts are used as fungicides, laxatives, antiseptics, deworming agents, and disinfectants.

As regards the diet, the large part of Hg is in the form of MeHg and the majority of that comes from eating fish. Food of animal origin may contain Hg because animals eat fish feed. The PTWI for total Hg recommended by the JECFA is 5 µg/kg/bw, which is equivalent to 42.8 µg/day for a 60 kg person [47]. Daily Hg intake of different countries (Table 22.4) is lower than the PTWI, ranging between 0.6 µg/day (Sweden) and 18.0 µg/day (Spain) [15,17–20,24–26,35,40,48]. Exception has to be made for Egypt, where the Hg intake via diet (78.5 µg/day) was very high and surpassed the PTWI [41]. Additional data are available from other European countries and the range of the mean Hg intake for the adult population resulted to be between 1.37 µg/day

TABLE 22.3
Average Daily Pb Intake (µg/Day) in Different Countries and Contribution (%) of Each Food Group

Country [Reference]	Year	Approach	µg/Day	Food Groups (%)
Canada [37]	1986–1988	TDS	24	Beverages (20.9), bakery goods and cereals (20.6), vegetables (16.8), fruit juices (13.9), meat and poultry (7.6)
Croatia [38]	1988–1993	TDS	100	Vegetables (25.8), milk (21.2),wine (7.4), fruit (6), fish (5.1), offal (0.71)
United States [19]	1990	TDS	14.8 (men), 14.9 (women)	N/A
Spain [17]	1990–1991	TDS	43	Alcoholic beverages (20.5), fruits (15.4), meat and meat products (15.4), bread (12.8), fish (10.2)
India [39]	1991–1994	TDS	25.1	Pulses (55.4), cereals (32.3), vegetables (8.4), meat (3.2)
Denmark [40]	1993–1997	TDS	18	Beverages (47), vegetables (14), cereals (12), meat (5.5)
Egypt [41]	1995–1997	TDS	242	N/A
United Kingdom [15]	1997	TDS	26	Beverages (54), bread (8), cereals (8), green vegetables (8), meat products (4), offal (0.35)
New Zealand [42]	1997	TDS	12 (men)	N/A
France [23]	1998–1999	DMS	52	N/A
France [24]	1998–1999	DMS	34	N/A
China [43]	2000	TDS	81.2	N/A
Chile [25]	2001–2005	TDS	206	Milk (17.9), fruits (17), bread (16.1), sugar (12.6), meat group (11.3)
Lebanon [44]	2004	TDS	18.5	Cereals and cereal-based products (45.3), drinking water (16.2), vegetables (15.6), fruits and fruit juices (9.9), cheese (3.2), meat and meat based products (2.7)
Spain [26]	2006	TDS	45.1	Cereals (18.5), vegetables (15.9), meat and meat products (15.1), milk (15), fruits (13.1), tubers (5.2)
China [35]	2006	TDS	81.5 (adult), 43.3 (children)	Adult: corn (53.6), vegetables (29.4), meat (6.1), sea products (3.1), egg (2.5), bean (2.2), fruit (2.1), milk (1) Children: corn (51.4), vegetables (24.8), meat (8.8), fruit (5), bean (4.6), egg (2.3), sea products (1.9), milk (1.2)

DMS, duplicate meal study; TDS, total diet study.

(Ireland) and 14.4 µg/day (Portugal) [27]. The contribution of fish varies from ca. 30% (Denmark, Chile, United Kingdom) to 100% (Spain) (see Table 22.4). Different is the case of China, where corn contributed more than sea products (47.6% vs. 41.8%). Mean Hg in the other foods is low; meat contributed less than 10% and offal contributed in a very scarce percentage (<1%, United Kingdom and Germany) [27].

TABLE 22.4
Average Daily Hg Intake (μg/Day) in Different Countries and Contribution (%) of Each Food Group

Country [Reference]	Year	Approach	μg/Day	Food Groups (%)
Sweden [18]	1988	DMS	0.6	N/A
United States [19]	1990	TDS	8.6 (men), 8.2 (women)	N/A
Spain [17]	1990–1991	TDS	18	Fish (68.4), meat (5.3), milk (5.3), bread (5.3), potatoes (5.3), vegetables (5.3), fruits (5.3)
Japan [20]	1992	DMS TDS	3.5 9.9	Fish, mollusk and crustacean (53.8), rice (21.4), meat and egg (11.6)
Denmark [40]	1993–1997	TDS	3.5	Fish (26.8), beverages (21.4), cereals (16), milk (9.7), fruit (8.6), vegetables (8), meat (5.7)
Egypt [41]	1995–1997	TDS	78.5	N/A
United Kingdom [15]	1997	TDS	3	Fish (33), cereals (13), beverages (12), bread (7), sugar and preserves (6), meat products (5), offal (0.33)
France [24]	1998–1999	DMS	9	N/A
Chile [25]	2001–2005	TDS	5	Fish and shellfish (31), bread (19.4), cereals (14.4), milk and milk products (7.8), spices (6.6), sugars (6.6), meat and meat products (4)
Spain [26]	2006	TDS	12.6	Fish and shellfish (100)
China [35]	2006	TDS	2.14 (adult), 0.971 (children)	Adult: corn (47.6), sea products (41.8), vegetables (4.2), fruit (2.39), meat (1.41), bean (1.17), egg (1.12), milk (0.28) Children: corn (53.3), sea products (29.1), fruit (6.6), vegetables (4.1), bean (2.9), meat (2.4), egg (1.2), milk (0.41)
Spain [48]	N/A	TDS	5.7	Fishery products (95.9), milk (1.41), cereals (0.88), sausages (0.7), viscera (0.018)

DMS, duplicate meal study; TDS, total diet study.

22.3 REGULATIONS, ADVISORY, AND GUIDELINES FOR HEAVY METALS

To reduce the risk of disease and disability arising from the exposure to heavy metals, both EU and U.S. policies define standards guidelines and recommendations to be adopted in the member states with regards to animal feed and food safety (see Tables 22.5 and 22.6). As regards feed legislation (Table 22.5), following the Directive 2002/32/EC and the Commission Directive 2005/87/EC, products intended for animal feed should not contain substances that can cause danger to animal health and, because of their presence in livestock products, to human health. As, Cd, Hg, and Pb are considered as undesirable substances in feed materials and their MLs should be As, 2 mg/kg; Cd, 2 mg/kg in animal and mineral feed and 1 mg/kg in vegetable feed; Hg, 0.1 mg/kg; and Pb, 10 mg/kg [49,50]. Also the EFSA Panel for contaminants, on the basis of the available data on feed-to-food transfer, emanated opinions on As, Cd, Hg, and Pb as undesirable substances in animal feed [51–54]. For food safety (see Table 22.5), the Commission Regulation 1881/2006 set the MLs for certain metals in foodstuffs. In particular, the EU established the ML of 0.5 mg/kg of Pb and 0.5 mg/kg of Cd in edible animal liver and the ML of 1.0 mg/kg for Cd in edible animal kidney [7]. The CAC reported the ML for a contaminant in a food or feed commodity, this level is intended as the ML

TABLE 22.5
EU Limits for Metals in Feeding Stuffs and Food of Animal Origin

	Directive 2002/32/EC and Commission Directive 2005/87/EC	ML, mg/kg
As	Feed material	2
	Complete feeding stuffs	2
	Complementary feeding stuffs	4
Cd	Feed materials of vegetable origin	1
	Feed materials of animal origin	2
	Feed materials of mineral origin	2
	Complementary feeding stuffs for pet animals	2
	Other complementary feeding stuffs	0.5
	Complete feeding stuffs for cattle, sheep and goats and for fish	1
Hg	Feed materials	0.1
	Complete feeding stuffs	0.1
	Complementary feeding stuffs	0.2
Pb	Feed materials	10
	Complementary feeding stuffs	10
	Complete feeding stuffs	5
	Commission Regulation 1881/2006	**ML, mg/kg w/w**
Cd	Meat (excluding offal) of bovine animals, sheep, pig and poultry	0.05
	Horsemeat (excluding offal)	0.2
	Liver of bovine, sheep, pig, poultry and horse	0.5
	Kidney of bovine, sheep, pig, poultry and horse	1.0
Pb	Meat (excluding offal) of bovine animals, sheep, pig and poultry	0.1
	Edible offal of bovine animals, sheep, pig and poultry	0.5
	CAC, Codex Stan 193-1995, Rev.2-2006, Amended 2009	**ML, mg/kg**
Pb	Meat of cattle, pigs, poultry and sheep	0.1
	Edible offal of cattle, pig and poultry	0.5

TABLE 22.6
Health Agency Standards

	USEPA	ATSDR	JECFA
	Oral RfD, µg/kg/Day	MRL, µg/kg/Day	PTWI, µg/kg/bw/Week
As	0.3	0.3	15 (inorganic)
Cd	1	0.1	2.5 as TWI (previous PTWI, 7)
Hg	N/A	N/A	5
Pb	N/A	N/A	25

of that substance to be legally permitted in that commodity moving in international trade. In this context, the CAC suggested the ML of 0.5 mg/kg of Pb in edible offals of cattle, pig, and poultry [8].

Other standards for heavy metal contaminants (see Table 22.6) have been defined by health agencies and expert committees such as the U.S. Environmental Protection Agency (EPA), the U.S. Agency for Toxic Substances and Disease Registry (ATSDR), and the JECFA. The U.S. EPA established the oral reference dose (RfD) for substances that are likely to be without risk of deleterious effects during a lifetime. The RfD has been settled at 0.3 µg/kg/day for As and at 1 µg/kg/day for Cd [55]. The US ATSDR chose to adopt an approach similar to that of the EPA's RfD for deriving

substance specific health guidance levels for nonneoplastic endpoints. It derived a minimal risk level (MRL) for chronic As intake of 0.3 μg/kg/day and for Cd of 0.1 μg/kg/day. No MRL has been derived for Pb till now [56]. As already mentioned in the text, the JECFA has established the PTWIs for chemicals, i.e., the amount of a contaminant that can be ingested over a lifetime without an appreciable risk. It should be reminded that the PTWI value for Cd takes into consideration the fact that infants and children tend to bioaccumulate Cd at faster rates than older age groups and provides suitable margins for 50 years of renal exposure. Also the PTWI established for Pb considered the higher risk for infants and children, while the PTWI for total Hg considered that pregnant and breast feeding women are likely to be at much greater risk due to the vulnerability of embryos and infants.

As regards the presence of Cd in phosphatic fertilizers, several EU member states have set limits to the Cd concentration, and proposals to have a progressive reduction in the maximum concentration, ultimately to 20 mg Cd/kg P_2O_5 by 2016 are under consideration [57].

22.4 ANALYTICAL METHODS FOR HEAVY METAL ANALYSIS OF EDIBLE ANIMAL BY-PRODUCTS

22.4.1 Contamination Control

All stages of the heavy metal analysis are potential sources of contamination, from collection and transportation to the laboratory through homogenization, mineralization, and measurement of the metal. Efforts must be made to minimize the potential for contamination throughout the entire analytical procedure. In general, minimum handling, closed system, clean containers, and high purity standards and reagents afford low metal contamination.

Ideally, the preparation of the samples for heavy metal analysis must be performed in a special clean room (e.g., the Class 100 clean room) or at least in an area with laminar flow clean air to minimize accidental contamination from particles in the laboratory air. Moreover, every surface in contact with the sample must be considered to be a potential source of contamination, including sample containers, utensils (as knives), homogenization equipment, and laboratory gloves. The potential for contamination is reduced or eliminated by using cleaned materials that have been found not to contaminate the metal of interest and by washing all the materials with mineral acids. Also the replacement of stainless steel components with components made of materials of less interest, such as Ti, W, Zr, or the coating of components with polytetrafluoroethylene can be adequate means to reduce contamination. The large contribution of impurity from the hands suggests that talc-free gloves should be worn by the analyst. Finally, special grades of ultrapure HCl, HNO_3, H_2SO_4, and $HClO_4$ are commercially available.

22.4.2 Sample Treatment

Table 22.7 describes the sample treatment procedures that are available for measuring As, Cd, Hg, and Pb in offal of animals as kidney, liver, lung, heart, and spleen. The basic modes of treatment for the determination of these metals are most often ashing the samples or wet digestion with acids aided or not by MWs. Advantages and disadvantages of dry ashing, wet digestion, and MW digestion as sample treatment approaches for heavy metal analysis are listed in Table 22.8.

Conventional dry ashing is based upon incineration at high temperature in a muffle furnace. Crucible selection depends upon the specific analysis; quartz crucibles are disposable, unbreakable, resistant to acids and to high temperatures, while platinum crucibles are very inert but far too expensive for routine use. The advantages of dry ashing are that it is a safe method, it requires no added reagents and a large number of crucibles can be handled at once; the disadvantages are the length of time required and the risk of losses of metals by volatilization. Dry ashing is a traditional method for As, Cd, and Pb analysis in offal [16,36,38,58–67]. Commonly, conditions are the followings: (1) the crucibles can be of platinum or quartz; (2) the temperature can vary between 450°C and

TABLE 22.7
Digestion Procedures and Quantification Techniques Used for Heavy Metal Analysis in Edible Animal By-Products

Organs	Brief Procedure Outline	Technique	[Reference], Year
Kidney Liver	As, Cd, Pb: sample is dry ashed Hg: sample is digested in a pressure decomposition device	As: HG-AAS Cd, Pb: ASV Hg: CV-AAS	[58], 1987
Kidney Liver	As: sample (0.3 g) is dry ashed with MgO and $Mg(NO_3)_2$. The metal is reduced by 2% KI Hg: sample (0.5 g) is added with 7 mL of HNO_3 and digested on a heating block at 105°C for 1.5 h. After cooling and filtration, the metal is reduced by 2% $SnCl_2$ in H_2SO_4	As: HG-AAS Hg: CV-AAS	[59], 1991
Kidney Liver	Cd, Pb: sample is dry ashed in a muffle furnace and dissolved in HNO_3. Hg: sample (2 g) is added with 10 mL of HNO_3:H_2SO_4 (1:1), digested on a heating block under reflux for 6 h and left at room temperature overnight. After additional 6 h under reflux, NaOH is added dropwise until solution is clear. The metal is reduced by 3% $NaBH_4$ and 1% NaOH mixture	Cd, Pb: GF-AAS Hg: CV-AAS	[60], 1991
Kidney Liver	Sample (2 g) is acid digested on a heating block	As: HG-AAS Hg: CV-AAS	[90], 1991
Kidney Liver	Sample (2.5 g) is MW digested with 5 mL HNO_3 with the following program: 5 min at 20% power, 5 min at 40%, 5 min at 50%, 5 min at 70%	Pb: ICP-MS	[79], 1992
Meat organs	Sample is dry ashed with MgO. Coprecipitation with ammonium pyrrolidine dithicarbamate complex with Cu and Fe carriers, and dissolution of the complex in HNO_3 containing modifier	As: GF-AAS	[16], 1993
Kidney Liver	Sample (2–10 g) is wet digested with HNO_3 in a all-glass apparatus. The digest is diluted with H_2O and the excess of the oxidant removed with $NH_2OH \cdot HCl$. The metal is reduced by 20% $SnCl_2$ in H_2SO_4	Hg: CV-AAS	[96], 1991
Meat organs	Sample is digested with HNO_3 and $HClO_4$. Coprecipitation with ammonium pyrrolidine dithicarbamate complex with Cu and Fe carriers, and dissolution of the complex in 25% HNO_3, 2.5% H_2O_2, 0.5% H_2SO_4, and 0.5% $(NH_4)H_2PO_4$ mixture	Cd, Pb: GF-AAS	[37], 1995
Kidney Liver	Sample (10 g) is dry ashed at 450°C in quartz vessels without an ashing aid. Ash is dissolved in 20 mL diluted HCl. The metals are determined as diethylammonium diethyldithiocarbamate complexes in methyl isobutyl ketone	Cd, Pb: F-AAS	[65], 1996
Kidney Liver	Sample (3–5 g) is added with 10 mL HNO_3 and heated at 150°C for 30–45 min. The digest is added with 5–10 mL $HClO_4$ and heated at 200°C	Cd, Pb: ICP-MS	[75], 1996
Kidney Liver	Sample is dry ashed in a muffle furnace at 450°C with MgO and $Mg(NO_3)_2$	As: HG-AAS	[14], 1996
Kidney Liver	Sample (3–5 g) is dry ashed in a muffle furnace at 480°C	Cd, Pb: F-AAS	[38], 1996

(*continued*)

TABLE 22.7 (continued)
Digestion Procedures and Quantification Techniques Used for Heavy Metal Analysis in Edible Animal By-Products

Organs	Brief Procedure Outline	Technique	[Reference], Year
Kidney	Sample is dry ashed at 450°C. Ash is dissolved in HNO_3	Cd: F-AAS	[61], 1997
Offal	Sample is dry ashed at 450°C. Ash is dissolved in 2% HCl	Cd: GF-AAS	[36], 1998
Kidney Liver	Sample (5–10 g) is dry ashed at 450°C. Ash is dissolved in 20 mL HNO_3 0.1 M	Cd, Pb: GF-AAS	[68], 1999
Kidney Liver	Sample (1 g) is MW digested with 5 mL HNO_3 and 2 mL H_2O_2	Cd: GF-AAS	[81], 2000
Kidney	As, Cd, Pb: sample (1 g) is predigested in 2 mL HNO_3 overnight and digested on a heating block at 120°C for 1 h. 2 mL of H_2O_2 are added during digestion to enhance oxidization	As: HG-AAS	[72], 2000
Liver	As: the digest is evaporated to dryness and resuspended in 5 mL 2% KI and 5% HCl mixture and left 24 h to reduce As	Cd, Pb: GF-AAS	
Kidney	Cd, Pb: sample (1 g) is predigested with 2 mL HNO_3 for 24 h, digested on a heating block at 120°C for 1 h. 2 mL H_2O_2 are added to complete the digestion	As: HG-AAS	[91], 2000
Liver	As: the digest is evaporated to dryness on a heating block at 120°C. The metals is redissolved in 5 mL of 2% KI and 5% HCl mixture and, after dilution with H_2O, left 24 h to reduce As	Cd, Pb: GF-AAS	
Offal	Sample (0.5 g) is MW digested with 5 mL HNO_3 in plastic pressure vessels	As, Cd, Pb: ICP-MS Hg: CV-ICP-MS	[15], 2000
Heart Kidney Liver Spleen	Sample (5–10 g) is dry ashed at 450°C in a muffle furnace overnight. Ash is dissolved in 0.5 mL HNO_3 and evaporated at 450°C overnight. Ash is dissolved in 0.5 mL HNO_3	Cd, Pb: GF-AAS	[64], 2001
Kidney	Sample is dried at 100°C for 2 h in a oven and incinerated at 500°C in a muffle furnace with $Mg(NO_3)_2$	Cd: F-AAS	[63], 2001
Kidney	Cd: sample (0.2 g) is digested under pressure with 3 mL HNO_3 and 2 mL H_2O_2 at 700 W for 3 min	Cd: GF-AAS	[86], 2001
Liver	Hg: the digest is diluted with a 5% $NaHCO_3$ solution. The metal is reduced by $NaBH_4$ and NaOH mixture	Hg. CV-AAS	
Kidney Liver	Sample (0.5–1 g) is MW digested with 3 mL HNO_3 and 0.5 mL H_2O_2 with the following programme: 5 min at 300 W, 10 min at 600 W	Cd: F-AAS	[85], 2001
Kidney Liver	Sample (10 g) is added with 2 mL 10% $Mg(NO_3)_2$, carbonized on a heating plate at 250°C and ashed in a muffle furnace at 470°C. Ash is dissolved in 10 mL HNO_3 3 M	Cd, Pb: F-AAS Hg: AMA	[62], 2001
Kidney Liver	Sample (0.5 g) is wet digested with 8–12 mL of HNO_3 at 120°C for 5 h in Teflon vessels	Cd: GF-AAS	[95], 2002

Kidney Liver	Sample is high pressure wet digested with HNO_3 in PTFE-lined steel bombs	Cd, Pb: GF-AAS Hg: CV-AAS	[40], 2002
Kidney Liver	Sample is digested in HNO_3	Pb: F-AAS	[93], 2002
Heart Kidney Liver Lung Spleen	Sample (1 g) is added with 1 mL H_2O_2 and 3 mL HNO_3 and digested at 180°C in fluoro-plastic vessels in sealed containers	Cd, Pb: AAS	[69], 2003
Kidney Liver	Sample (1 g) is predigested in 1.5–2 mL HNO_3 overnight and digested at 120°C. 2 mL of H_2O_2 are added during digestion to enhance oxidization. The metal is reduced by $SnCl_2$ in H_2SO_4	Hg: CV-AAS	[73], 2003 [82], 2003
Kidney Liver	Sample (0.1 g) is digested with 2 mL HNO_3 at 250°C for 4 h	Cd: GF-AAS	[70], 2003
Kidney Liver	Sample (10 g) is added with 20 mL H_2SO_4 and heated for 30 min. After flocculation, the digestion continued on high flame for 2 h. H_2O_2 is added dropwise until solution is clear	As, Cd, Hg, Pb: AAS	[77], 2004
Kidney Liver	Cd, Pb: sample (0.3 g) is MW digested with 6 mL HNO_3 and 1 mL H_2O_2 Hg: sample is directly analyzed without a chemical pretreatment	Cd, Pb: GF-AAS Hg: AMA	[84], 2005
Gizzard Intestine Liver	Sample (20 g) is dry ashed in a muffle furnace at 450°C. Ash is dissolved in 2 mL HNO_3, evaporated to dryness on a hot plate and ashed at 450°C. Ash is dissolved in 10 mL HCl 1M	Cd, Pb: AAS	[66], 2005
Kidney Liver	Sample (0.2 g) is added with 2 mL HNO_3 in glass tubes and digested at room temperature for 20 h. The digest is added with 0.5 mL $HClO_4$, heated at 100°C for 3 h and at 150°C–180°C for 4 h, and diluted to 3 mL with H_2O. An aliquot of this solution is evaporated to dryness at 30°C and redissolved in H_2O	Cd: GF-AAS	[76], 2006
Heart Kidney Liver Lung Spleen	Sample is MW digested with 5 mL HNO_3 and 1 mL H_2O_2 with the following programme: 10 min at 250 W, 5 min at 400 W, 5 min at 500 W, 5 min at 600 W	Cd, Pb: SF-ICP-MS	[87], 2007
Kidney Liver	Sample (2 g) is MW digested with 5 mL HNO_3 and 2 mL H_2O_2	As, Cd, Hg, Pb: ICP-MS	[83], 2007

(*continued*)

TABLE 22.7 (continued)
Digestion Procedures and Quantification Techniques Used for Heavy Metal Analysis in Edible Animal By-Products

Organs	Brief Procedure Outline	Technique	[Reference], Year
Kidney Liver	Sample (1 g) is water bath digested with 7 mL HNO_3 and 3 mL H_2SO_4 for 1–2 h. The digest is diluted with H_2O, HCl, KBr 0.2 M and $KBrO_3$ 0.2 M and left overnight	Hg: CV-AFS	[78], 2007
Kidney Liver	Cd, Pb: sample (2.5 g) is dry ashed in a muffle furnace at 450°C. Ash is redissolved in HNO_3 Hg: sample (1.5 g) is predigested with 2 mL HNO_3 at room temperature overnight and digested at 80°C for 5 h in closed tubes	Cd, Pb: GF-AAS Hg: AMA	[67], 2008
Viscera	Sample (1–2 g) is MW digested (60–150 psi) with 4 mL HNO_3 and 2 mL H_2SO_4 for 40 min. After cooling, sample is diluted with H_2O. The Hg is reduced by $SnCl_2$ in HCl	Hg: CV-AAS	[48], 2008
Kidney Liver	Sample is digested with HNO_3 and $HClO_4$	Cd, Pb: GF-AAS	[94], 2009
Liver	Sample (2 g) is digested with HNO_3:H_2O 2:1 (v/v) at 130°C for 2 h	Hg: CV-ICP-OES	[71], 2009
Kidney Liver	Sample is added with 3 mL HNO_3 and left for 20 min at room temperature. The solution is digested on a heating block at 70°C. After cooling, 2 mL of H_2O_2 are added and the solution is heated at 50°C for 30 min	As, Cd, Pb: ICP-MS	[74], 2009
Liver	Sample is MW pressure digested with HNO_3/H_2O_2 6:1 (v/v)	Cd, Pb: GF-AAS Hg: CV-AAS	[88], 2009
Kidney Liver	Sample is MW digested with HNO_3 in pressure vessels	As, Cd, Pb: ICP-MS	[80], 2009

TABLE 22.8
Characteristics of the Different Digestion Procedures

Procedure	Advantages	Disadvantages
Dry ashing	Safe and easy	The need of elevated temperature
	The need for little or no reagents	Loss of volatile metals (e.g., As, Hg, P, Se)
	The ability to decompose large sample sizes	Loss due to retention to the ashing containers
	Many samples are handled simultaneously	Contamination from the ashing containers (e.g., porcelain, SiO_2)
	It allows the total ash content determination	Contamination from the muffle furnace
	It allows the determination of various types of ash content (e.g., water soluble, water insoluble, and acid insoluble)	Difficulty in dissolving certain metal oxides
		Consistency (hygroscopicity, lightness and fluffiness) of ashes make difficult their handling
		Formation of toxic gases in poorly ventilated areas
Wet digestion	No need of elevated temperature	Large use of acids is required
	No high costs are involved	Attention is required when acids are handled
	The instrumentation is easy to be used	Contamination from reagents
	The oxidation process is obtained in short time	Formation of toxic gases and fumes in poorly ventilated areas
	The final liquid solution useful for further analytical procedure	Small number of samples processed simultaneously
	Little or no loss of volatile metals	The procedure required long time
Microwave digestion	Fast, simple and relatively safe to use	Initial high costs
	The need for little reagents	It allows to decompose small sample sizes
	Low contamination risk	Limited to the digestion of a small number of samples per run
	High digestion quality	
	Little or no loss of volatile metals	

500°C; (3) the ashing time can range from a few hours to all night; (4) dissolution of the residues is often obtained with HNO_3, but HCl has also been used. In some cases, the sample is dry ashed with an ashing aid consisting of MgO and $Mg(NO)_3$ on their own or as a mixture. The mixture is frequently used in the determination of As with the hydride generation atomic absorption spectrometry (HG-AAS) [16,59]. For this quantification technique, a rigorous digestion of the sample is required to completely oxidize stable As compounds or residual organic compounds. Ulrich et al. carbonized kidney and liver on a heating plate at 250°C with $Mg(NO)_3$, and then the samples were placed in a muffle furnace at 470°C to be sure that a complete mineralization of the organic matter occurred [62]. The quality of the ashing procedure is generally checked against certified reference materials (CRMs) based on matrices as similar as possible to those analyzed, such as bovine liver (NIST 1577), horse kidney (IAEA H-8) or mixed diet (IAEA H-9), or by participating to collaborative trials and proficiency testing [36,61,63,67,68]. In other cases, to check the trueness of the procedure, recoveries are calculated by adding known amounts of standard solutions to samples and the amount are selected so as to be close to the amounts normally present in liver and kidney [64,66].

Wet digestion with acids simultaneously dissolves minerals and oxidizes all the organic material. Wet oxidation conserves volatile metals even if it is limited to few samples, and corrosive reagents are necessary. Wet oxidation can be assisted or not by MWs, and it is a frequently used treatment procedure for multielemental analysis of offal. The types of wet digestion can be classified as follows: (1) the dissolution under temperature of the sample directly into acid and (2) the MW digestion of the sample in a strong acid. In most cases, the dissolution with acids occurs in hot plates, and a single acid does not give a complete and rapid oxidation, so a mixture of acids is often used. Depending on the acid concentration and the temperature involved, the end products can be variable. The most common acidic approach to digesting offal is represented by the HNO_3/

H_2O_2 mixture or by HNO_3 only. Being a powerful oxidizing agent, HNO_3 reacts with many organic materials, and then the addition of H_2O_2 increases the oxidation potential and it is able to avoid the formation of the nitrous oxides. Rarely, $HClO_4$ and H_2SO_4 are used. In this context, Liu [69] dissolved internal organs of sheep and horses with 3 mL of HNO_3 and 1 mL of H_2O_2. Samples were then processed at 180°C in fluoroplastic vessels in sealed containers to avoid losses of vapor and variation of volume and concentration [69]. The paper of Sedki et al. reported the use of HNO_3 only to digest liver and kidney of bovine through heating at 250°C for 4 h [70], whereas Chibunda et al. used water diluted HNO_3 to hot digest organs of cattle at 130°C for 2 h [71]. Some authors suggested that a predigestion is beneficial. For example, two Spanish studies used an overnight predigestion at 120°C of liver and kidney with HNO_3. The next day, H_2O_2 was added to enhance the oxidative power of the mixture [72,73]. Also Nriagu et al. made a short (20 min) predigestion of bovine liver and kidneys in linear polyethylene tubes; then, samples were heated at 70°C until dissolution, and added with H_2O_2 to favor the complete solubilization of the organic matter [74]. Other wet digestion procedures reported the use of the combination of HNO_3 and $HClO_4$. This mixture of acids allows for a controllable digestion of organics; the HNO_3 will attack the easily oxidizable matter at lower temperature while, as the temperature rises, the $HClO_4$ will completely digest matter undigested by the HNO_3. This approach was followed by Husain et al. and Włostowski et al., and in both cases a predigestion with HNO_3 was followed by the addition of an adequate amount of $HClO_4$ under heating [75,76]. Also Dabeka et al. choose this approach to digest animal organs, but, in addition, they precipitated metals as complex in presence of ammonium pyrrolidine dithiocarbamate with copper and iron as carriers. The precipitate was further redissolved in a solution containing HNO_3, H_2O_2, H_2SO_4, and $(NH_4)H_2PO_4$. The H_2SO_4 can be used as digesting agent for its capacity to destroy the organic matter [37]. For example, Mariam et al. digested offal with this mineral acid for 2 h in a high flame, and, drops of H_2O_2 were added until a clear solution was obtained [77]. Ullrich et al. used a mixture (10 mL) of HNO_3 and H_2SO_4 to digest bovine liver and kidney. The procedure was simply performed in a water bath for 1–2 h [78].

The high-pressure MW digestion with acids is able to obtain higher digestion temperatures for the complete oxidation of the organic compounds. Samples are generally processed in a shorter time than on a conventional hot plate, allowing laboratories to increase their sample throughput significantly. Moreover, the MW systems permit to select temperature and pressure in a step-by-step ramping and some systems also enable to change the method while the reaction is running. Laboratories can select between the MW digestion in open or closed vessels. Open vessels are made with Teflon, quartz, or Pyrex and are mostly used for larger sample sizes. The open vessel technique is inexpensive, is of inestimable value for routine analysis because it can be automated easily, but it is limited by a low maximum digestion temperature, which cannot exceed the ambient-pressure boiling point of the corresponding acid. In fact, in some cases the oxidizing power of the HNO_3 is insufficient at its boiling point. Other disadvantages can be related to the contamination by air and the large amount of required reagents. Closed vessels can contain higher pressures and they allow to use HNO_3 for samples that might normally require harsher acids such as $HClO_4$ and H_2SO_4. Vessel liners available are in Teflon, TFM fluoropolymer, and quartz. The advantages of closed vessel systems are the following: (1) the possibility of losing volatile elements is virtually eliminated, (2) less acid is used because no evaporation occurs, (3) the fumes produced during the closed digestion are contained within the vessel, and (4) the airborne contamination of samples is minimized. In the analysis of metals in edible animal by-products (see Table 22.7), some MW digestion procedures adopted HNO_3 only [15,79,80], but most often the HNO_3/H_2O_2 mixture is used [72,73,81–88]. In particular, for the determination of heavy metals with ICP-MS, the addition of H_2O_2 may be required to reduce potentially interfering carbon content. However, obtaining H_2O_2 with a sufficiently low concentration of impurity elements may be difficult, which emphasizes the need to analyze reagent blanks in order to assure accurate results. Optimum MW digestion parameters are determined case by case. For example, Reykdal et al. digested offal of lamb in Teflon bombs with 3 mL of HNO_3 and 2 mL of H_2O_2 solution. They used a household MW oven to digest 3 bombs simultaneously placed around

a plastic beaker with about 100 mL of cold tap water. This procedure was performed in 3 min at 700 W [86]. Forte et al. obtained the complete dissolution of edible offal with 5 mL of HNO_3 and 1 mL of H_2O_2 in a high-pressure MW closed vessel system in a total time of 25 min [87]. Rubio et al. used a mixture of HNO_3 and H_2SO_4, and this last acid acts as a dehydrating agent in presence of organic material. The same authors compared the open vessel MW procedure (40 min in total) with the wet digestion performed at 40°C for 10–18 h. Considering the duration of the wet digestion procedure, the MW approach was highly preferred [48].

Direct solid sampling is another sampling means of eliminating potential contamination and losses from contamination of offal. Caggiano et al. developed a method to analyze Hg in liver and kidney with the advanced mercury analyzer without any previous chemical treatments of the samples [84].

22.4.3 Heavy Metals Quantification

The analytical techniques mostly applied to quantify As, Cd, Hg, and Pb, their advantages and disadvantages are reported in Table 22.9. The techniques are dissimilar in terms of basic principle, limits of detection, sensitivity, specificity, mono- or multielement detection, ease of use, costs, etc. In this context, each laboratory can choose the instrumentation taking into account its own necessities such as internal budget, daily sample throughput, number of metals they quantify, and degree of analytical performances.

The most common analytical methods used for the determination of total As in edible animal by-products are based on atomic absorption spectrometry (AAS) and on plasma-based technique (see Table 22.7). In particular, As can be quantified by the either HG-AAS or by ICP-MS. Before HG-AAS analysis, As must be pre-reduced from As(5) to As(3) with potassium iodide (KI) in HCl to generate the hydride with sodium borohydride ($NaBH_4$). The hydrides are transported to a quartz tube or a graphite furnace and heated to produce atomization for atomic absorption. With ICP-MS technique, the analytical solution is nebulized, reaching the Ar plasma where the analyte is atomized. Thus, the ion beam is accelerated by the vacuum, the path is optimized by the ion optics, and the masses are filtered by a quadrupole or by a magnetic field and collected by the detector considering the *m/z* ratio. ICP-MS can generally provide lower detection limits than absorbance detection methods allowing to accurately quantify fractions of μg/kg of metal. Differently from the HG technique, where the As signal is free from interferences, the As determination by ICP-MS is made difficult by the formation of the ArCl interference that overlaps the signal of As, resulting in the overestimation of the effective content of the sample. This problem can be solved by means of different approaches: (1) the use of mathematical equations to correct the As signal; (2) the use of an instrumentation equipped with collisional or dynamic reaction cells where, in presence of a proper gas, the ArCl is transformed in another compound with the different molecular weight to make free the As signal; and (3) the use of an instrumentation to be able to work in high resolution (7500 m/Δm) (namely, the sector field (SF) ICP-MS instrument), where the interference signal is shifted out of the integration window of the As [74,80,89]. Notwithstanding the advantages of the ICP-MS technique (see Table 22.9), the costs involved are much higher with respect to those for HG-AAS in terms of either instrumental or in training the personnel. Most of the studies on As in edible animal by-products uses the HG-AAS [14,58,59,72,90–92]. For example, López Alonso et al. quantified As in cattle organs by HG-AAS; the approach gave a detection limit of 0.008 mg/kg in liver and 0.007 mg/kg in kidney. Accuracy was calculated on the IRMM CRM 186, pig kidney, and resulted to be the 88.3%. The overall method precision was ca. 6% [72]. Similar low detection limits (0.015–0.02 mg/kg) are found for As quantification in liver and kidney by HG-AAS [59,90]. ICP-MS is used less for As quantification in offal because of the aforementioned interferences on the metal signal. With an ICP-MS equipped with the collision cell, Nriagu et al. obtained detection limit of 0.01 mg/kg in bovine offal [74]. Waegeneers et al. reported a limit of quantification of 0.03 mg/kg in blank solution for As by a collision cell ICP-MS [80].

TABLE 22.9
Characteristics of the Different Analytical Techniques

Technique	Advantages	Disadvantages
AMA-254	Liquid and solid samples	One shot combustion boat
	High sensitivity for Hg	Relatively high maintaining cost
	No sample treatment	
ASV	Sensitive for some metals	Long time of analysis
	Low costs are involved	
CV-AAS	High sensitivity for Hg	The need for high amounts of sample and reagent
CV-AFS	Low costs are involved	
	Absence of interferences	
F-AAS	High specificity for some metals	Flame is a source of noise
	Low costs are involved	Residence time of atoms in optical path of flame is very small
	Simplicity of operation	
	Free of spectral interferences	Linear dynamic range is narrow compared to ICP-AES and ICP-MS
	Faster than GF-AAS method	
	Results are highly reproducible	Use of dangerous gases mixture (e.g., air-acetylene or nitrous oxide/acetylene)
GF-AAS	High specificity for some metals	More time-consuming than F-AAS
	Lower detection limits than F-AAS	Interferences from matrix
	The need for a small amount of sample	Linear dynamic range is narrow compared to ICP-AES and ICP-MS
	Handles solid and liquid samples	
	Low noise from the furnace	
HG-AAS	High specificity for volatile hydrides metal	The need for high amounts of sample and reagent
	No interferences	
	Low detection limit	
	Low costs are involved	
ICP-OES	Low detection limit	The presence of spectral interferences
	Wide linear dynamic range	Cannot analyze very small sample volumes
	Multielement technique	
	Semiquantitative analysis is possible	
	Low running cost	
ICP-MS	Sensitivity higher than ICP-OES	The presence of isobaric, molecular and doubly-charged ion interferences
	Detection limits lower than ICP-OES	
	Multielement technique	Strong dependence of signal on plasma parameters
	Semiquantitative analysis is possible	Cannot analyze very small sample volumes
	Wide linear dynamic range	High costs are involved
	Isotope ratio measurements are possible	Memory effects
	High sample throughput	
NAA	High sensitivities for many metals	High cost for the nuclear reactor
	Reference method	Long time of analysis
	Non destructive technique	

As regards the quantification of Cd and Pb in edible animal by-products, the most used techniques are flame (F)-AAS, graphite furnace(GF)-AAS, and ICP-MS (see Table 22.7) [15,36–38,40,60–65,67–70,72,74–76,79–81,83–88,91,93–95]. In all cases, the samples must be acid digested because the instrumentation analyzes liquid solutions. Using the F-AAS, the analytical solution is aspirated into a flame that is usually air-acetylene or nitrous oxide-acetylene, producing the atomization of the analyte, which is detected by the UV absorbance. If the concentration of Cd and Pb in the dissolved sample is below the detection limit, preconcentration techniques may be employed.

Different strategies are available in order to concentrate the metals. The F-AAS sensitivity can be enhanced by the following: (1) atom trapping where analyte atoms are concentrated within the flame before measurement; this approach is simple, is carried out at great speed, has low cost, and offers less risk of contamination; (2) flow-injection online preconcentration; this method is performed in minicolumns by means of ion exchange or sorbent extractions technique. The analyte is eluted with an appropriate reagent into the nebulizer burner of AAS. It offers low consumption of reagents, sample and time, less risk of contamination, and ease of automation. The preconcentration is also possible by means of the online coprecipitation dissolution; (3) sequential injection; the solvent extraction of metal complexed with ammonium pyrrolidinedithiocarbamate is performed with isobuthyl methylketone. For back extraction dilute nitric acid containing Hg(2) is used. With reference to GF-AAS, a small aliquot of the analytical solution is injected into a graphite tube (pyrolytically or nonpyrolytically coated depending on the element), usually having a graphite platform, and is heated electrically in three stages to dry the solution, ash or pyrolize the residue, and atomize the analyte. Usually, a chemical matrix modifier (diammonium hydrogen phosphate, palladium or magnesium based) is added with the analytical solution to avoid the formation of arsenical volatile compounds, to accelerate the sample digestion, to produce spongier ash, and to facilitate their dissolution. As reported in Table 22.9, the GF-AAS has more sensitivity than F-AAS for Cd and Pb even if it is relatively more expensive and more extended in terms of time of analysis. In this context, Ulrich et al. quantified Cd and Pb with F-AAS after redissolution of ashes from pig offal. They obtained sensitivities of 0.005 and 0.05 mg/kg for Cd and Pb, respectively, and reproducibility below 10% for both metals [62]. Similar detection limits for Cd (0.003 mg/kg) and Pb (0.05 mg/kg) were also found by Doganoc with F-AAS [65]. Sapunar-Postružnik et al. obtained a detection limit of 0.01 mg/kg for Cd by F-AAS [85], whereas Beldomenico et al. found a higher limit equal to 0.05 mg/kg [63].

The use of GF-AAS in the analysis of edible animal by-products generally provides lower limits (up to 10-fold) than F-AAS. In the quantification of Cd and Pb in liver and kidney of horse, sheep, lamb, and deer, GF-AAS gave detection limits, calculated on blanks, of 0.0005 mg/kg for Cd and 0.002 mg/kg for Pb. In addition, the accuracy calculated on the CRM bovine liver (NIST 1577b) was 104% for Cd and 98% for Pb [68]. López Alonso et al. found detection limits of 0.002 mg/kg for Cd and 0.02 mg/kg for Pb in liver and 0.002 mg/kg for Cd and 0.0136 mg/kg for Pb in kidney. Accuracy was calculated on the CRM pig kidney (CRM 186, IRMM) and resulted to be 99.4% for Cd and 92.2% for Pb. The precision was ca. 5% for Cd and ca. 8% for Pb [72]. Rudy obtained detection limits equal to 0.002 mg/kg for Cd and 0.012 mg/kg for Pb in cattle liver, and recoveries on the CRM bovine liver (CRM 185R, IRMM) were 96.7% for Cd and 94.8% for Pb [88]. Lazarus et al. using a mixture of $Mg(NO_3)_2$ and $Pd(NO_3)_2$ as matrix modifier for Cd and Pb analyses in deer organs by GF-AAS recovered approximately 100% of the certified values in bovine liver CRM (NIST 1577b) [67].

The ICP-MS technique is more costly than AAS instruments, but it is able to quantify Cd and Pb simultaneously at masses free of interferences. In addition, this instrumentation supplies low detection limits, reliability, wide dynamic range, and it allows to perform several samples per hour with respect to the AAS approach. Waegeneers et al. found limits of 0.002 mg/kg for Cd and 0.006 mg/kg for Pb with ICP-MS [80]. Using the high-resolution spectrometer (i.e., the SF-ICP-MS), very low detection limits (i.e., 0.003 and 0.002 mg/kg for Cd and Pb, respectively) and a good reproducibility calculated on replicates of bovine liver (2% and 6.5% for Cd and Pb) were obtained [87]. López Alonso et al. reported an accuracy level on pig kidney (CRM 186, IRMM) equal to 91.1% for Cd and 107% for Pb by ICP-MS [83].

Total Hg in edible animal by-products is most frequently analyzed by CV-AAS after acidic digestion (see Table 22.7) [58–60,73,82,86,88,90,96]. In this context, Hg vapor is generated from the analytical solution by reducing the Hg(2) to Hg(0), which is what is ultimately detected by the UV system. The main advantage of this technique is the separation of the analyte from the potentially interfering sample matrix. With reference to the reductant, sodium borohydride in alkaline medium reduces both inorganic Hg and organic Hg, whereas stannous chloride in both acid and alkaline

solution only reduces inorganic Hg. This step undergoes the interference of volatile nitrogen oxides as a reduction product of nitric acid during sample decomposition. The nitrogen oxides can completely oxidize stannous chloride before the completion of the Hg reduction, causing a substantial absorbance decrease. This process can be eliminated by resting the solution 1 day after the digestion purging with Ar or reducing the oxides with sulfamidic acid or urea [97,98]. López Alonso et al. with CV-AAS obtained a detection limit for Hg of 0.001 mg/kg in cattle liver and of 0.001 mg/kg in kidney. The precision of the method was ca. 5% and the recovery from pig kidney (CRM 186, IRMM) was 91.5% [73]. Rudy found a 10-fold lower detection limit in cattle liver (0.0001 mg/kg) and recovery (NIST 8414, bovine muscle) of 90% [88].

A further enhancement of sensitivity by two orders of magnitude and better selectivity may be obtained by using the fluorescence property of the atoms. In particular, the Hg is detected by the CV combined with the atomic fluorescence spectroscopy (CV-AFS) instead of AAS. Ullrich et al. were able to quantify Hg in offal by CV-AFS with a detection limit of 0.005 mg/kg [78].

Another approach to detect Hg in edible animal by-products is represented by the AMA-254. This instrument is suitable for the direct analysis of solid samples without the need for any sample pretreatment. In principle, the solid sample is placed into a combustion boat where it is first dried and then thermally decomposed at high temperature. The Hg vapors are trapped by the Au-amalgamator separating them from the combustion gases. An additional heating of the amalgamator permits the release of the Hg further revealed by the detector. The capability of AMA-254 to analyze Hg in offal of animals has been demonstrated. Caggiano et al. quantified Hg from dried solid samples of ovine liver and kidney obtaining an instrumental detection limit of 0.01 ng [84]. Wet digestion of organs of deer and analysis with AMA-254 provided an accuracy of 98.2% on horse kidney CRM (IAEA H8) [67]. Ulrich et al. analyzing offal of swine with AMA-254 obtained a sensitivity of 0.001 mg/kg and a reproducibility of 1.5% [62].

A better sensitivity and greater selectivity for Hg analysis in food of animal origin can be obtained with ICP-MS, but it suffers a certain memory effect. This means that between samples a washing procedure for tenths of seconds is recommended; this, of course, increases the time of analysis. In respect of ICP-MS, CV-AAS and CV-AFS have the advantage of being comparatively low cost and of simple operations. Another approach is the coupling of CV technique with ICP-MS for Hg detection in offal; Ysart et al. (2000) obtained detection limit ranging between 0.0004 and 0.003 mg/kg [15].

As alternatives to the quantification techniques cited above, there are neutron activation analysis (NAA), ICP-OES, and anodic stripping voltammetry (ASV). Advantages and disadvantages of these techniques can be found in Table 22.9. The nuclear properties of NAA give interferences different from that of the atomic-based techniques. NAA has limited use because of the limited number of nuclear reactors providing this service and the need to dispose of radioactive waste. The sample is exposed to neutrons that cause formation of radioactive isotopes of the element and the gamma rays emitted during the decay of these isotopes (or isotopes of decay product of the element) are measured by gamma-ray spectrometry. It is useful in performing both qualitative and quantitative multielement analysis, and for many elements it offers very high sensitivities. It is chosen as the reference method when new procedures are being developed or when other methods yield results that do not agree. In addition, it is a nondestructive technique; in fact, solid samples are analyzed. The principle of ICP-OES is the atomization of the analyte ion in the Ar plasma gas with the further metal emission at the proper wavelength then collected by the detector. With this technique, it is possible to couple the HG or the CV approaches to detect As. For example, the paper by Chibunda et al. used CV-ICP-OES for Hg detection in liver of Tanzanian cattle and recovery on the CRM based on bovine muscle (Promochem) ranged from 96% to 100% and the limit of detection was 0.02 mg/kg [71]. In general, ICP-OES has detection limits between 1 and 10 µg/kg range for most of the metals, it is a time-saving technique, and it has a wide working range. On the other hand, the cost is higher than the GF-AAS and it undergoes some chemical interferences. Finally, a less used technique to detect metals in edible animal by-products is ASV. This is based on the different

potential of oxidation of metals and their deposition on the electrode. ASV is able to analyze liquid samples only and it requires too much time to analyze each single sample, but it has a good sensitivity and specificity.

22.5 QUALITY ASSURANCE IN HEAVY METALS ANALYSIS

Quality assurance is one of a number of concerted measures that analytical chemists can take to ensure that the data produced in the laboratory are fit for their intended purposes. Internal quality control comprises the routine practical procedures that enable the chemist to accept a result or group of results as fit for the purpose or reject the results and repeat the analysis. In practice, the fitness for purpose of analytical methods is commonly evaluated by means of method validation studies. Such studies produce data on overall performance and on individual influence factors, which can be applied to the estimation of uncertainty associated with the results of the method in normal use. Validation studies for quantitative analytical methods consist in the assessment of a set of performance parameters and their conformity to well-defined criteria of acceptability [99].

22.5.1 Validation Parameters

Validation studies for analytical methods that quantify heavy metals typically determine some or all of the following parameters: sensitivity, specificity, limits of detection and quantification, linearity, trueness and precision, and robustness. The sensitivity is the change in the response of an instrumental measurement divided by the corresponding change in the amount of the metal [100]. A method is called sensitive if a small change in concentration causes a large change in the measured signal [101]. It is calculated using samples containing a low concentration of the metal of interest. For example, in ICP-MS analysis of heavy metals, it is expressed as instrumental counts per second (cps) relative to a defined concentration in the selected matrix. The specificity ensures that the method responds to the specific metal of interest only, and not to other interferents [100]. This characteristic is predominantly a function of the measuring technique used, but can vary according to the class of compound or matrix. For example, in ICP-MS measurements of heavy metals, specificity involves the process of selection of the analytical mass and the instrumental resolution and confirmation that, at those conditions, interferences are not significant. To this end, samples are spiked with various suspected interferences in the presence of the metal of interest. After the analysis, if the presence of the interfering ion leads to a false identification of the metal and if the quantification is influenced notably, adequate correction factors are mathematically calculated. The order of correction is arranged so that only the interference-free values are quantified. For the limit of detection (LoD) a lot of definitions exist. The Association of Official Analytical Chemists (AOAC) refers to LoD as "the lowest content that can be measured with reasonable statistical certainty" [102], while the ISO prefers the general term "minimum detectable net concentration" [103] and International Union of Pure and Applied Chemistry (IUPAC) uses "minimum detectable (true) value" [104]. Another definition for the LoD is "the lowest concentration of the analyte in a sample that can be detected, but not necessarily quantified under the stated conditions of the test" [105]. The Eurachem/CG reported that for validation purposes it is sufficient to provide an indication of the level at which detection becomes problematic and the "mean sample value + 3 standard deviation (SD)" approach is commonly used [99]. Following this approach, the LoD is determined by repeating the analysis of 10 independent test samples measured once each and is expressed as the analyte concentration, the response of which is equivalent to the mean sample response + 3 SD. The limit of quantification (LoQ) is the lowest concentration of analyte that can be determined with an acceptable level of precision and accuracy and it is expressed as the analyte concentration corresponding to the sample test value + 10 SD [99]. It is calculated on 10 independent test samples measured once each and is dependent on the type of test sample. The ability of the method to obtain

proportional responses to different analyte concentrations is defined as the linearity representing the concentration range over which the method may be applied, and it can vary passing from matrix to matrix [99]. At the lower end of the concentration range the limiting factors are the values of the LoD and/or LoQ. At the upper end of the concentration range limitations will be imposed by various effects, depending on the instrument response system. In general, the linearity checks require points at least 10 different concentration values. The accuracy is defined as the closeness of agreement between a quantity value obtained by measurement and the true value of the measurand [106]. It is determined by determining trueness and precision [107]. The trueness is the closeness of agreement between the average value obtained from a large series of test results and an accepted reference value [107,108]. Two basic techniques for practical assessment of the trueness are available: checking against certified values given by the CRMs or checking against typical materials spiked with known concentration of pure certified reference standards (the recovery tests) [100]. A CRM means a material that has a specified metal content assigned to it [109]. The ideal CRM is a natural matrix reference material, closely similar to the samples of interest. To check trueness using a CRM, the mean and the SD of independent test samples are determined and compared with the certified value. The trueness is then calculated by dividing the estimated mean concentration by the certified value and multiply by 100—to express the result as a percentage. When a suitable CRM is not available, the recovery can be determined and it represents the percentage of the true concentration of the metal recovered during the analytical procedure [100]. The recovery (%) is calculated by fortifying independent aliquots of real matrix with relevant concentrations of the metals and expressed as (Concentration found – Concentration originally present) × 100/(Concentration theoretical). The precision is the closeness of agreement between independent test results obtained under stipulated conditions [105,108]. It is expressed as repeatability and within-laboratory reproducibility and computed as the relative SD (RSD) of the test result. Less precision is determined by a larger RSD. The repeatability (r) requires that mutually independent test results are obtained with the same method on a test material in the same laboratory with the same equipment by the same operator within a short interval of time [108]. The within-laboratory reproducibility (R) requires that test results are carried out within the same laboratory, over a longer period of time, by different analysts, using different reagent lots, in different environmental conditions and even using different instrumentation [108]. Both the repeatability and within-laboratory reproducibility are dependent on the metal concentration and so should be determined at a number of concentrations. The robustness is the capacity of a method to remain unaffected by deliberate variations in the analytical protocol [100]. It is necessary to identify the variables in the method that have the most significant effect and ensure that, when using the method, they are closely controlled. In the case of heavy metal analysis using MW digestion and ICP-MS quantification, parameters such as concentration of reagents, MW irradiation power and time, radiofrequency power, flow gas rates, spray chamber temperature can be varied. Practically, 10 independent test samples are processed under standard operative conditions and under dissimilar instrumental working conditions and concentration values obtained are statistically compared for differences.

22.5.2 Acceptability Criteria

The methods validated should specify if they support a regulatory requirement or are in compliance with a defined specification. In some cases, there are guidelines that specify the validation acceptability criteria. In other cases, it is left to the analyst's discretion to decide what performance is required from the method. As regards the sensitivity and the specificity, these parameters must be demonstrated only. The analyst must decide if the change in the instrument response is adequate for the purpose and how much the presence of the interferent enhance or inhibit the quantification of the metals. If the sensitivity is not adequate and the quantification is inhibited by the interferences, further method development will be required. With regard to the LoD, the Commission Regulation 333/2007 about the methods of analysis for the official control of Cd and Pb in food specifies that

the LoD must be no more than one-tenth of the ML values, reported in the Commission Regulation 1881/2006. If the ML for Pb is <0.1 mg/kg, the LoD should be no more than one-fifth of that value [7,11]. The LoQ value recommended by the Commission Regulation 333/2007 should be no more than one-fifth of the ML values, given in the Commission Regulation 1881/2006, and no more than two-fifth if the ML for Pb is <0.1 mg/kg [7,11]. To establish the linearity, a visual inspection of the line and the calculation of the relevant regression coefficient may be sufficient. In the ICP-MS analysis of heavy metals, a correlation coefficient of >0.999 is considered as evidence of acceptable fit of the data to the regression line. Concerning the trueness, the deviation of the estimated mean content from the certified value shall not lie outside the limit ±10%, and the recovery data are only acceptable when they are within ±10% of the target value, as recommended by the Commission Decision 2002/657/EC. The RSD_R for the within-laboratory reproducibility must be equal to or less than 20% when the metal concentration ranges from 10 to 100 ng/g and as better as possible for concentrations <10 ng/g [109]. The results obtained for the robustness can be compared through different approaches. The Youden approach has been suggested by the Commission Decision 2002/657/EC [109]. With this method, it is possible to identify the variables that have the most significant effect and ensure that, when using the method, they are closely controlled. Where there is a need to improve the method further, improvements can probably be made by concentrating on those parts of the method known to be critical.

22.5.3 Measurement Uncertainty

Uncertainty can be obtained either by calculating all the sources of uncertainty individually or by grouping different sources of uncertainty when possible [110]. However, identifying and quantifying all the sources of uncertainty individually is not simple, so, in this view, approaches based on calculating uncertainty using information from the in-house validation process have been proposed [99,111–113]. These documents planned to include the estimates of reproducibility SD and of the bias coming from CRM/recovery study as the main components of the overall uncertainty. Then, uncertainty includes any other sources of uncertainty that are not covered by the data from the validation study. Following this approach, the relative combined uncertainty (u_{comb}) is calculated by the following formula:

$$u_{comb} = \sqrt{u_R^2 + u_{tru/rec}^2 + u_{other}^2}$$

where u_R is the contribution from long-term random variability estimated from the within-laboratory reproducibility experiments. It is the SD_R obtained during the reproducibility test. $u_{tru/rec}$ is the systematic variability typically associated to the analysis of CRMs or spiked samples. The u_{tru} is calculated with the following formula:

$$\left(\frac{u_{CRM}}{Conc_{CRM}}\right)^2 + \left(\frac{SD_{estimated}}{Conc_{estimated}\sqrt{n}}\right)^2$$

The first term is the uncertainty of the CRM given by the formula $SD_{STD}/\sqrt{3}$ (the SD_{CRM} was available in the manufacture certificate and $\sqrt{6}$ represented the triangular distribution). In the absence of the CRM, the uncertainty on the standard solution purity (u_{STD}) used for spiking samples that follows a rectangular distribution (i.e., $SD_{STD}/\sqrt{3}$) is used to calculate the u_{rec}. The second term was the uncertainty of the estimated value of the CRM expressed as the RSD on replicated measurements. The n was the number of replicates on each CRM or on fortified samples. u_{other} includes uncertainty sources coming from any other components associated to effects not completely accounted for in the validation study

(operator effects, calibration uncertainty, reagent batches, ancillary equipment, etc.). These effects are included in the reproducibility SD, thus, this term can be reasonably considered unnecessary.

Finally, the relative expanded uncertainty (U) is calculated by multiplying the u_{comb} by a coverage factor of 2, which accounts for an appropriate level of confidence of 95%.

22.6 CONCENTRATION OF HEAVY METALS IN EDIBLE ANIMAL BY-PRODUCTS

22.6.1 Arsenic

Concentration of As in edible animal by-products is reported in Table 22.10 [14,58,59,72,74,83,90]. Arsenic content in liver and kidney of bovine, sheep, and horse never exceeded the 0.1 mg/kg. In general, tissues from pig and poultry showed higher As (0.013–0.36 mg/kg) than in those from cattle; this is possibly because As concentration in forages is low compared with commercial compound feeds. In Canada, pig tissues contained high As concentration because of the use of organoarsenical as growth promoters [90]. In fact, in past years, additives as organoarsenicals (such as arsanilic acid, 3-nitro-4-hydroxyphenylarsonic acid and 4-nitrophenylarsonic acid) were used as growth promoters in pigs and chickens or for medicinal purposes, such as the control of scour in pigs. However, nowadays, these arsenic compounds are not allowed and As in pig feeds depend mainly on the content of products derived from other sources; for example, in Sweden, it is reported that pig feed contained a certain amount of fish meal, which was a source of As [59]. The accumulation of As in animals exposed to the influence of emissions from industrial plants are reported in Table 22.11 [77,80,92]. It should be noted that, in Belgium and Spain, the environmental pollution effect was weak and bovine liver and kidney had As concentration within the normal ranges [80,92]. On the contrary, in Pakistan the As concentration in liver and kidney of beef, mutton, and poultry was strikingly high and indicative of As pollution in the environment, which might be due to copper smelting, coal combustion, burning of firewoods, and cow dung [77].

22.6.2 Cadmium

Concentration of Cd in edible animal by-products are reported in Table 22.12 showing a higher rate of accumulation in the kidney than in the liver of animals [38,40,58,60–63,65–68,72,74–76, 81,83,84,86–88,95]. In bovine, ovine, and poultry kidney, the amount of Cd ranged between

TABLE 22.10
Content (mg/kg) of As in Edible Animal By-Products

Country [Reference]	Liver	mg/kg	Kidney	mg/kg
Canada [90]	Cattle	0.03	Cattle	0.03
	Horse	0.03	Horse	0.03
	Pig	0.26	Pig	0.17
	Poultry	0.36	Poultry	0.15
	Sheep/lamb	0.04	Sheep/lamb	0.03
	Veal	0.03	Veal	0.05
Croatia [14]	Bovine	0.006	Bovine	0.001
	Porcine	0.001	Porcine	0.001
Jamaica [74]	Cattle	0.05	Cattle	0.11
Spain [72]	Cow	0.010	Cow	0.015
Spain [83]	Pig	0.013	Pig	0.011
Sweden [59]	Bovine	<0.015	Bovine	<0.015
	Pig	0.023	Pig	0.019
The Netherlands [58]	Sheep	0.005	Sheep	0.011

TABLE 22.11
Accumulation (mg/kg) of As in Animals from Polluted Area

Country [Reference]	Organ	Control Area	Polluted Area	Source of Contamination
Belgium [80]	Bovine kidney	0.043	0.093	Zinc smelters; metals refining production plants
	Bovine liver	0.017	0.037	
Pakistan [77]	Beef kidney	N/A	46.9	Copper smelting; coal combustion; burning of firewood and cow dung
	Beef liver	N/A	52.4	
	Mutton kidney	N/A	40.8	
	Mutton liver	N/A	42.7	
	Poultry liver	N/A	46.7	
Spain [92]	Calf kidney	0.010	0.013	Industrialized area
	Calf liver	0.011	0.012	

0.03 and 0.79 mg/kg. In bovine, ovine, and poultry liver, the amount of Cd ranged between 0.01 and 0.191 mg/kg. The levels of elements in organs depend on the age of animals, kind of fodder, grazing environment, etc. Horses may reach higher levels, ca. 2.5 mg/kg in liver and ca. 20 mg/kg in kidney, than other slaughter animals [63,81,88]. This can be the consequence of their diet and lifespan (horses are often slaughtered at a later age than other animals). Moreover, horses have kidneys with longer proximal tubules and can more effectively reabsorb and store many toxic metals [114]. Deer had high levels of Cd in the kidney (ca. 2.7 mg/kg) [67,68]; deer have feeding habits that deviate considerably from the other animals because they also eat perennial plants like lichens that are known to accumulate toxic metals. Also, big game animals like bison accumulated in kidney and liver more Cd than domestic animals, and two-fold higher Cd levels in the grasses eaten by bison in comparisons with those eaten by cattle were found [76]. Organs such as lung, spleen, and heart of calf and lamb contained Cd at a very low level (<0.003 mg/kg) [87]. Gizzards and intestine of chicken accumulated 0.019 and 0.069 mg/kg of Cd, respectively [66].

Values for contaminated liver and kidneys of slaughtered animals are reported in Table 22.13 [64,69,70,77,80,85,92,94,119]. The application on pastures and hay fields of phosphate fertilizers and sewage sludge from industrial sources are important causes of Cd contamination [115]. Some phosphate fertilizers can contain up to 300 mg Cd/kg [116]. Increases in soil Cd content result in an increase in the uptake of Cd by plants; some plants readily extract Cd from the soil rendering this metal available for consumption, as demonstrated by McLaughlin et al. (1997), when comparing the Cd concentration in clover grown in soils fertilized with high and low Cd rock phosphate fertilizers [117]. When cattle are allowed to graze pastures treated with anaerobically digested sewage sludge for up to 8 years, Cd accumulated consistently in the tissues of cattle [118]. Similarly, when corn silage or corn that was grown on sewage sludge fertilized fields were fed to sheep or pigs, significant increases in kidney Cd concentrations were measured [119]. Another study performed in Sweden found a positive correlation between the Cd in feed and pig kidneys [61]. In an additional study in the Netherlands, bulls fed with diets containing high concentrations of Cd had increased levels of the metal in kidney and liver [120]. Another case reported that the premix for pigs containing 898 mg/kg of Cd caused a heavy contamination of pig liver and kidney [85]. It has been found that Cd levels in complete dairy feed rations were very close to the U.S. maximum acceptable concentrations, suggesting that Cd will exceed the limit if Cd concentration in feeds increases in the future [121]. In Morocco, very high levels in animal organs were caused by untreated wastewater, which are discarded on fields; the wastes came from craft industries using chemical products as fungicides and bactericides in the treatment of wool and leather [70]. Wet and dry deposition of Cd from the atmosphere may also contribute to large amounts of Cd in soils from areas surrounding nonferrous mines and smelters [122]. Concentration of this element in kidneys were 2.5-fold higher in cattle from the areas contaminated by emissions from the nonferrous metal industry than in regions with

TABLE 22.12
Content (mg/kg) of Cd in Edible Animal By-Products

Country [Reference]	Liver	mg/kg	Kidney	mg/kg	Other Organs, mg/kg
Argentina [63]	N/A	N/A	Horse	21.5	N/A
Croatia [67]	Deer	0.17	Deer	2.65	N/A
Croatia [38]	Bovine	0.066	Bovine	0.26	N/A
	Porcine	0.068	Porcine	0.41	
Czech Republic [62]	Pig	0.020	Pig	0.086	N/A
Denmark [40]	Calf	0.042	Calf	0.19	N/A
	Chicken	0.024	Ox	0.78	
	Duck	0.13	Pig	0.26	
	Ox	0.10			
	Pig	0.042			
	Turkey	0.035			
Finland [60]	Cattle	0.061	Cattle	0.35	N/A
	Pig	0.028	Pig	0.17	
Greece [95]	Cow	0.024	Lamb	0.022 (medulla)	N/A
	Lamb	0.016		0.009 (cortex)	
Iceland [86]	Lamb	0.045	Lamb	0.058	N/A
Italy [81]	Horse	2.46	Horse	20	N/A
Italy [84]	Ovine	0.33	Ovine	6.71	N/A
Italy [87]	Calf	0.058	Calf	0.096	Calf lung, <0.003
	Pig	0.11			Calf spleen, <0.003
					Lamb heart, <0.003
					Lamb lung, <0.003
Jamaica [74]	Cattle	0.010	Cattle	0.033	N/A
Kuwait [75]	Chicken	0.089	Goat	0.44	N/A
	Goat	0.047	Sheep	0.30	
	Sheep	0.044			
Philippines [66]	Chicken	0.029	N/A	N/A	Chicken gizzard, 0.019
					Chicken intestine, 0.069
Poland [88]	Cattle	0.19	N/A	N/A	N/A
Poland [76]	Bison	0.45	Bison	2.79	N/A
	Cattle	0.20	Cattle	1.30	
Slovenia [65]	Cattle	0.09	Cattle	0.37	N/A
	Pig	0.09	Pig	0.39	
Spain [72]	Cow	0.083	Cow	0.38	N/A
Spain [83]	Pig	0.073	Pig	0.30	N/A
Sweden [61]	N/A	N/A	Pig	0.11	N/A
Sweden [68]	Deer	0.6	Deer	2.7	N/A
	Horse	2.5	Horse	18	
	Lamb	0.031	Lamb	0.12	
			Sheep	1.0	
The Netherlands [58]	Sheep	0.089	Sheep	0.29	N/A

normal background metal contamination levels, in Belgium [80]. Similarly, tissues from bovine, sheep, and horse in the vicinity of nonferrous metal smelter in China had Cd significantly (up to 25-times) higher than in controls [69,94]. In Egypt, organs such as heart and spleen accumulated high levels of Cd because animals were exposed to the influence of air pollution from industrial processes for long periods [64].

TABLE 22.13
Accumulation (mg/kg) of Cd in Animals from Polluted Area

Country [Reference]	Organ	Control Area	Polluted Area	Source of Contamination
Belgium [80]	Bovine kidney	1.14	2.86	Zinc smelters; metals refining production plants
	Bovine liver	0.19	0.44	
China [94]	Bovine kidney	2.12	38.3	Lead-zinc smelter; highway
	Bovine liver	0.21	2.47	
China [69]	Horse heart	0.79	2.13	Nonferrous metal smelters
	Horse kidney	1.02	27.2	
	Horse liver	1.13	8.30	
	Sheep heart	0.30	0.38	
	Sheep kidney	1.83	25.4	
	Sheep liver	0.49	7.92	
	Sheep lung	0.61	3.02	
	Sheep spleen	0.45	0.59	
Croatia [85]	Pig kidney	0.19–0.63	8.54	Cadmium-polluted feeds
	Pig liver	0.03–0.10	0.97	
Egypt [64]	Bovine heart	0.01	0.28	Metallurgic and manufacturing processes (cars, fertilizers, ceramics, refrigerators); incineration of refuse and ferrous scrap
	Bovine kidney	0.22	0.56	
	Bovine liver	0.11	0.31	
	Bovine spleen	0.09	0.16	
	Buffalo heart	0.091	0.19	
	Buffalo kidney	0.17	0.58	
	Buffalo liver	0.08	0.34	
	Buffalo spleen	0.076	0.089	
	Elk heart	0.08	0.36	
	Elk kidney	0.84	1.01	
	Elk liver	0.68	0.96	
	Elk spleen	0.09	0.093	
	Goat heart	0.061	0.09	
	Goat kidney	0.98	0.91	
	Goat liver	0.091	0.26	
	Goat spleen	0.041	0.05	
	Sheep heart	0.08	0.08	
	Sheep kidney	0.88	0.82	
	Sheep liver	0.082	0.26	
	Sheep spleen	0.061	0.062	
Morocco [70]	Cattle bone	0.2	0.47	Animal grazing on wastewater spreading fields
	Cattle kidney	2.2	10.3	
	Cattle liver	1.7	5.1	
Pakistan [77]	Beef kidney	N/A	0.91	N/A
	Beef liver	N/A	0.42	
	Mutton kidney	N/A	0.45	
	Mutton liver	N/A	0.41	
	Poultry liver	N/A	0.49	
Spain [92]	Calf kidney	0.096	0.16	Industrialized area
	Calf liver	0.023	0.03	
United States [119]	Pig kidney	0.64	1.50	Fed corn grown on municipal sewage sludge soil
	Pig liver	0.13	0.21	

22.6.3 Lead

The content of Pb in edible animal by-products is reported in Table 22.14 [38,40,58,60,62, 65–68,72,74,75,83,84,87,88]. In general, the ranges of Pb concentration in internal organs are comparable (0.004–1.5 mg/kg in liver; 0.008–2.0 mg/kg in kidney) among the countries considered. Maximum values were found in ovine from Italy (1.5 mg/kg in liver and 2.0 mg/kg in kidney), in sheep from the Netherlands (0.96 mg/kg in liver and 0.42 mg/kg in kidney), in cattle from Jamaica (0.523 mg/kg in kidney), in goat from Kuwait (0.43 mg/kg in kidney), and in lamb from Italy (0.28 mg/kg in liver). Organs such as lung, spleen, and heart of animals collected in Italy contained Pb from 0.004 to 0.072 mg/kg [87]. Gizzard and intestine of chickens farmed in Philippines contained Pb at concentration of 0.129 and 0.164 mg/kg, respectively [66]. Comparisons between samples collected in different countries are problematic because differences may depend on the age of the animals, the feedings as well as variations in the exposure to environmental Pb.

TABLE 22.14
Content (mg/kg) of Pb in Edible Animal By-Products

Country [Reference]	Liver	mg/kg	Kidney	mg/kg	Other Organs, mg/kg
Croatia [67]	Deer	0.095	Deer	0.093	N/A
Croatia [38]	Bovine	0.20	Bovine	0.13	N/A
	Porcine	0.13	Porcine	0.18	
Czech Republic [62]	Pig	0.12	Pig	0.027	N/A
Denmark [40]	Calf	0.017	Calf	0.053	N/A
	Chicken	<0.016	Ox	0.089	
	Duck	<0.016	Pig	<0.016	
	Ox	0.043			
	Pig	<0.016			
	Turkey	<0.016			
Finland [60]	Cattle	0.057	Cattle	0.11	N/A
	Pig	0.038	Pig	0.040	
Italy [84]	Ovine	1.5	Ovine	2.0	N/A
Italy [87]	Calf	0.032	Calf	0.023	Calf lung, 0.004
	Lamb	0.28	Lamb	0.17	Calf spleen, 0.004
	Pig	0.009			Lamb heart, 0.005
					Lamb lung, 0.072
Jamaica [74]	Cattle	0.16	Cattle	0.52	N/A
Kuwait [75]	Chicken	0.10	Goat	0.43	N/A
	Goat	0.13	Sheep	0.14	
	Sheep	0.13	N/A	N/A	
Philippines [66]	Chicken	0.082	N/A	N/A	Chicken gizzard, 0.13
					Chicken intestine, 0.16
Poland [88]	Cattle	0.18	N/A	N/A	N/A
Slovenia [65]	Cattle	0.10	Cattle	0.14	N/A
	Pig	0.06	Pig	0.06	
Spain [72]	Cow	0.048	Cow	0.058	N/A
Spain [83]	Pig	0.004	Pig	0.008	N/A
Sweden [68]	Deer	0.13	Deer	0.13	N/A
	Horse	0.13	Horse	0.047	
	Lamb	0.031	Lamb	0.05	
			Sheep	0.046	
The Netherlands [58]	Sheep	0.96	Sheep	0.42	N/A

TABLE 22.15
Accumulation (mg/kg) of Pb in Animals from Polluted Areas

Country [Reference]	Organ	Control Area	Polluted Area	Source of Contamination
Belgium [80]	Bovine kidney	0.19	0.37	Zinc smelters; metals refining production plants
	Bovine liver	0.082	0.21	
China [94]	Bovine kidney	0.07	0.8	Lead-zinc smelter; highway
	Bovine liver	0.05	1.0	
China [69]	Horse heart	2.12	3.27	Non-ferrous metal smelters
	Horse kidney	3.21	68.8	
	Horse liver	1.05	23.7	
	Sheep heart	1.54	4.30	
	Sheep kidney	0.96	39.5	
	Sheep liver	0.72	15.3	
	Sheep lung	1.45	4.35	
	Sheep spleen	1.01	3.94	
Egypt [64]	Bovine heart	0.03	0.21	Metallurgic and manufacturing process (cars, fertilizers, ceramics, refrigerators); incineration of refuse and ferrous scrap
	Bovine kidney	0.22	0.71	
	Bovine liver	0.12	0.56	
	Bovine spleen	0.021	0.042	
	Buffalo heart	0.02	0.14	
	Buffalo kidney	0.21	0.72	
	Buffalo liver	0.11	0.40	
	Buffalo spleen	0.011	0.032	
	Elk heart	0.043	0.081	
	Elk kidney	0.18	0.62	
	Elk liver	0.082	0.46	
	Elk spleen	0.012	0.042	
	Goat heart	0.01	0.061	
	Goat kidney	0.11	0.68	
	Goat liver	0.08	0.46	
	Goat spleen	0.01	0.042	
	Sheep heart	0.02	0.091	
	Sheep kidney	0.18	0.54	
	Sheep liver	0.08	0.42	
	Sheep spleen	0.012	0.083	
Pakistan [77]	Beef kidney	N/A	2.02	Car exhaust gases (antiknocking agents in gasoline), waste water of industries
	Beef liver	N/A	2.18	
	Mutton kidney	N/A	3.85	
	Mutton liver	N/A	4.25	
	Poultry liver	N/A	3.15	
Spain [92]	Calf kidney	0.016	0.038	Industrialized area
	Calf liver	0.021	0.038	
Spain [93]	Horse kidney	N/A	1.7–6.75	Battery recycling plant
	Horse liver	N/A	2.5–15	
United Kingdom [79]	Cattle kidney	0.79	2.49	Lead-polluted feeds
	Cattle liver	0.89	2.72	

The concentration of Pb in samples collected from contaminated areas is reported in Table 22.15 [64,69,77,79,80,92–94]. Nowadays, if on one hand the regulations reduced the direct exposure of animals to Pb reducing the Pb poisoning, on the other, the environmental contamination with Pb is not easy to solve; in fact, poisoning of animals from contaminated forages, soils, and water is still frequent. Areas near Pb industrial establishments (Pb mining, Pb smelting, and

battery treatment) may be enriched by aerial deposition of Pb particles, and wastewater discharged from industries are used to irrigate agricultural soils in the vicinity. Moreover, some feed mixtures containing very high level of Pb are used in cattle feed. As a consequence, animals eating contaminated feeds and vegetations can accumulate enough Pb to produce clinical signs of Pb poisoning. In China, there were deaths of a large number of grazing sheep and horses on farmland in the vicinity of metallurgic industries that had a few decades of intensive development [69]. Approximately 200 cases of Pb poisoning occurred naturally in British cattle [123]. Feed imported into the United Kingdom have been contaminated at very high Pb level (up to 12,400 mg/kg) by a lead-zinc composite during transportation, and animals can contain 2.5 mg Pb/kg in the kidney and 2.72 mg Pb/kg in the liver [79]. In China, feed had markedly higher Pb concentration from 3.6 to 132 mg/kg in highly polluted areas (lead-zinc smelters and highway), and in the same areas Pb liver increased from 0.05 to 1.0 mg/kg and kidney from 0.07 to 0.8 mg/kg [94]. In Egypt, the industrial area, employing intense manufacturing processes such as cars, fertilizers, ceramics, refrigerators, incineration of refuse and ferrous scrap as well as metallurgic processes, had a strong influence in the Pb level of organs of bovine, buffalo, elk, sheep, and goat [64]. In the same way, in Belgium and Spain, the average Pb in liver and kidney of cattle resulted to be from 2 to 2.5 times higher in areas contaminated by smelters and refining production plants in respect to a clean environment [80,92]. Equines appear to be one of the most sensitive among all animal species. Pb ingestion rate for horses, estimated using Pb levels of grasses grown in the nearest soils to a recycling plant was considerably higher than the fatal dosage for horses reported in literature (i.e., >10 and >15 mg/kg in the liver and kidneys, respectively) [93,124]. Also in China, Pb levels in horses that ingested grasses growing in sites near smelters surpassed the fatal dosage. The liver of horses was found to accumulate up to 23.7 mg/kg and the kidney up to 68.8 mg/kg and a clear decrease with the increasing distance from the smelters was observed confirming the aerial dispersion of Pb particles as the main source of contamination of soils and forages [69]. Emissions of Pb from leaded petrol have caused the progressive increase in soil Pb concentrations, at least until the removal of Pb from petrol. In recent years, there has been remarkable decline, >10-fold, in Pb levels of animal tissues throughout the world [125,126]. However, in developing countries Pb continues to be used in fuel, and animals grazing near roads where vehicles use leaded petrol had increased Pb concentrations. In beef, mutton, and poultry organs of animals from Pakistan, the major source of Pb pollution was found to be automobile exhaust fumes and Pb values ranged from 2.0 to 4.2 mg/kg [77].

22.6.4 Mercury

Mercury residues in organs of animals have been little studied in comparison with Cd and Pb, because the EU has not established acceptable maximum concentration for Hg in meat and offal so far. It is important to report that in the mammalian system various form of Hg are interchangeable, e.g., inorganic Hg can be methylated and vice versa. In this context, it is of concern that MeHg tend to be accumulated in the animal present at the top of the food chain. Therefore, poisoning by Hg can occur after consumption of meat or grain contaminated or by excessive exposure to inorganic or organic Hg compounds. In industrialized areas, contamination occurs predominantly from the combustion of fossil fuels. Moreover, although the use of alkyl mercury fungicides (particularly as seed dressings) is being discouraged, there may still be possibilities, in the absence of appropriate regulatory measures, that traces of such fungicides will get into cereals by accident. Phosphate fertilizers and sewage sludge also contain Hg [127].

Studies reported in Table 22.16, showed that Hg levels are, in general, very low in offal of animals, and a very high percentage of samples of liver and kidney had undetectable Hg concentrations [40,58–60,62,67,73,83,84,86,88,90,128]. Mean values for all animal species were between <0.007 and 0.06 mg/kg for liver and between <0.007 and 0.18 mg/kg for kidney in the different countries selected. In Canada, Hg was not detected in all species, except horses. Around 90% of

TABLE 22.16
Content (mg/kg) of Hg in Edible Animal By-Products

Country [Reference]	Liver	mg/kg	Kidney	mg/kg
Canada [90]	Cattle	0.01	Cattle	0.02
	Horse	0.06	Horse	0.18
	Pig	0.01	Pig	0.02
	Poultry	0.02	Poultry	0.02
	Sheep/lamb	0.02	Sheep/lamb	0.03
	Veal	0.01	Veal	0.02
Croatia [67]	Deer	0.004	Deer	0.009
Czech Republic [62]	Pig	0.004	Pig	0.012
Denmark [40]	Calf	<0.007	Calf	<0.007
	Chicken	<0.007	Ox	0.017
	Duck	<0.007	Pig	0.008
	Ox	<0.007		
	Pig	<0.007		
	Turkey	<0.007		
Finland [60]	Cattle	0.012	Cattle	0.015
	Pig	0.012	Pig	0.014
Iceland [86]	Lamb	0.009	Lamb	0.012
Italy [84]	Ovine	0.005	Ovine	0.009
Poland [128]	Duck	0.003	Chicken	0.003
	Geese	0.002	Rabbit	0.004
	Rabbit	0.002	Sheep	0.019
	Sheep	0.007	Turkey	0.004
	Turkey	0.003		
Poland [88]	Cattle	0.001	N/A	N/A
Spain [73]	Asturian calves	0.001	Asturian calves	0.003
	Galician calves	0.001	Galician calves	0.012
Spain [83]	Pig	0.001	Pig	0.002
Sweden [59]	Bovine	0.006	Bovine	0.010
	Pig	0.015	Pig	0.019
The Netherlands [58]	Sheep	0.004	Sheep	0.009

equine kidneys and 54% of equine liver had Hg > 0.01 mg/kg [90]. It was uncertain the reasons of the exposure of horses, but it might be from the therapeutic use of mercurial liniments. In a pig–cattle comparative study, the Hg concentration was higher in pig samples, which was attributed to the higher Hg content of compound feeds for pigs [59]. The Hg found in liver and kidney of Icelandic lamb was attributed to the volcanic eruptions, which typically characterize Iceland [86]. In Italy, the data showed that liver and kidney of ovine had Hg concentrations similar to that found in fodder [84].

Cases of Hg contamination of animal organs are given in Table 22.17 [71,77,78,82]. Ullrich et al. studied the accumulation of Hg in cow grazing near a chlor-alkali plant [78]. The contamination of grass and lake due to atmospheric discharges of Hg from the plant resulted in Hg concentration of 11 mg/kg in kidney and 6 mg/kg in liver. In Pakistan, industrial wastes, pesticides, and fungicides have been found to be the main contamination sources in liver and kidney of beef, mutton, and poultry, and this last species had a greater accumulation because of Hg-contaminated feeds [77]. In Galicia and Asturias (Spain), the Hg exposure of cattle was associated with atmospheric Hg emissions from coal-fired power plants, which explain the interindividual variability in kidney content of

TABLE 22.17
Accumulation (mg/kg) of Hg in Animals from Polluted Areas

Country [Reference]	Organ	Control Area	Polluted Area	Source of Contamination
Kazakhstan [78]	Cow kidney	<LoD	10.9	Derelict chlor-alkali plant
	Cow liver	<LoD	5.74	
Pakistan [77]	Beef kidney	N/A	50.6	Industrial wastes; pesticides and fungicides
	Beef liver	N/A	31.4	
	Mutton kidney	N/A	63.4	
	Mutton liver	N/A	77.7	
	Poultry liver	N/A	78.9	
Spain [82]	Asturian calves kidney	N/A	0.003	Coal burning power plant
	Galician calves kidney	N/A	0.014	
Tanzania [71]	Cattle liver	0.022	0.049–0.11	Gold mining area

LoD, limit of detection.

animals grazing nearby [82]. Cattle grazing around mining villages had liver with Hg concentration higher than those from cattle grazing in the reference area [71].

22.7 CONCLUSIONS

Being present in all Earth's elements, As, Cd, Hg, and Pb are naturally ubiquitous, but, because of their wide use in industrial productions, agricultural practices, pharmaceutical compounds, and animal feeding, they are also recognized as toxicants for animals and humans. Reared animals are the final receptors of these environmental contaminants posing a risk for consumers that eat edible animal by-products as liver, kidney, intestine, spleen, and brain. To minimize the intake of these heavy metals via diet, the EU and some international bodies and health agencies recommend maximum limits for As, Cd, Hg, and Pb in food and feeding stuffs and call countries to plan official controls to check compliance to legislations. On the other hand, laboratories involved in food control are even more asked to develop accurate and precise analytical procedures for the purpose, and to produce data that fulfill specified analytical criteria issued by international bodies such as AOAC, Eurachem, and ISO. The realization of a quality assurance system can surely lead benefits to laboratories in terms of goodness of analytical data and comparison of results among international teams. Last but not least, a special concern should be devoted to the speciation analysis in edible animal by-products, because metals, depending on their chemical form, can have different degrees of toxicity or they cannot be toxic at all. Retrospect through the fact that inorganic As forms are more toxic than organic ones, while organic Hg is more harmful than inorganic Hg. Considering that an ad hoc sample pretreatment procedure and an highly sophisticated quantification technique are mandatory for the speciation analysis, this task represents an exciting challenge for analysts.

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23 Environmental Contaminants: Polychlorinated Biphenyls in Edible Animal By-Products

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CONTENTS

23.1 INTRODUCTION

Edible meat by-products can be defined as non-carcass materials (Anderson 1988, Goldstrand 1988). These products cover a broad range of products that can be used to prepare different meat products. Table 23.1 describes where the most typical meat by-products are used. See also Chapter 1.

The concentrations of organochlorinated compounds in edible meat by-products are not well known. In fact, there are not many data about the content of polychlorinated biphenyls (PCBs) and organochlorinated pesticides in these meat by-products. Meat (muscle) and fat from poultry, pig, calf, lamb, and other animal species have been the most studied. However, there are a lot of studies dealing with wild animals, in special fish, marine mammals, and bivalves like mussels. PCBs and dioxins have been studied in the environmental samples to evaluate the fate and the bio-uptake by living organisms in a whole ecosystem (Eisler and Belisle 1996, Hernandez et al. 1988, 1989). The toxicological effects have been another important activity to classify the compounds on the basis of their toxic effects to correct the concentrations by the so-called toxic equivalent factor (TEF) (Ahlborg et al. 1994, Van den Ber et al. 1998). The presence of PCBs, organochlorinated pesticides, and dioxins has been less studied in farm animals because of the production systems which, in general, keep the animals in a controlled environment. However, it is evident that animal feeding could be a source of organochlorinated compounds and some critical episodes of high contamination by PCBs and dioxins have been reported, which have forced new legislation about the permitted concentrations and the analytical conditions to identify and quantify these compounds.

The analysis of organochlorinated compounds should include organochlorinated pesticides, PCBs, and dioxins. The complexity of the determination of these compounds makes the application of the analytical methods developed for fish, mussel very difficult to the broad range of samples of edible meat by-products. The determination of these compounds in edible meat by-products could be based on the analytical methods developed for fish, mussels, fat, muscle tissues, and environmental samples. However, the broad chemical composition of the edible meat

TABLE 23.1
Description of the Edible Meat By-Products

Raw By-Product	Used in	Other Uses
Brain	Variety of meat	
Liver	Variety of meat	
Heart	Variety of meat	Luncheon meat
Kidneys	Variety of meat	
Stomach		Pet foods
Spleen	Variety of meat	
Sweetbreads	Variety of meat	
Tongue	Variety of meat	Sausages and Luncheon meat
Cheek and head trimmings	Sausage material	
Blood	Sausage	Pet foods
Bones		Pet foods
Intestines	Casing	Pet foods

Sources: Adapted from Souci, S.W. et al., *Food Composition and Nutrition Tables*, 7th edn, Taylor & Francis, Boca Raton, FL, 2008; Mataix Verdú, J. and Mañas Almendros, M., *Tabla de Composición de Alimentos Españoles* (*Table of the Composition of Spanish Foods*), 3rd edn. University of Granada, Granada, Spain, 1998.

by-products makes difficult the direct application of those methods (Table 23.2). The matrix effect is a very important factor to consider in the development of analytical protocols and few reported methods include a complete validation for nontypical samples. Also, the need for expensive equipment to carry out the analysis under the required legislative conditions is an important limitation to gaining information about the fate of PCBs, organochlorinated pesticides, and dioxins in food samples, especially in edible animal by-products. All these elements show the lack of feasible analytical methods for edible meat by-products, relatively cheap, fast and able to be applied for more laboratories to obtain more data about the concentrations of PCBs and other organochlorinated compounds. Important efforts are necessary to simplify the analytical protocols and to provide new tools to facilitate a higher analytical capability. New cleanup procedures, screening methods based on biosensors, immunological procedures, and GCxGC-ECD could help to explore, on the basis of Commission Directive 2002/69/EC, a high number of samples, and to sort them for a more specific and selective analysis.

Edible meat by-products are very important because they need to be valorized to reduce the animal waste. The most important ways are the use in some meat products and in the pet food industry. For example, the fat contents are an element to be considered. The fat contents of a great number of edible by-products are depicted in Table 23.2.

23.2 EXPERIMENTAL DESIGNS

23.2.1 Equipment

The most convenient equipment to determine PCBs and dioxins is GC/MS (HRMS) (gas chromatography/mass spectrometry or high resolution mass spectrometry) (Mullins et al. 1984, EPA Method 1668B [EPA 2008]). GC is the separation technique that allows high efficiency in terms of plate number; however, the typical used apolar phases show a limited selectivity on the basis of Rs and α values for some groups of peaks. New stationary phases or dedicated apolar phases have been introduced to improve the separation: HT8 (SGE), BPX50 (SGE), Zebron ZB5-ms (Phenomenex), Rtx-CL Pesticides (Restek), Rtx-PCB (Restek), DB-35 ms (Agilent), DB-XLB (Agilent), VF-Xms

TABLE 23.2
Mean Fat Content of Some Edible Meat By-Products

Edible Meat By-Products	Fat
Pig tongue	18.30
Beef tongue	15.90
Sheep tongue	14.80
Sheep hearth	10.00
Beef brain	9.63
Sheep brain	9.10
Pig brain	9.00
Calf brain	7.60
Pig lungs	6.67
Calf kidney	6.37
Calf tongue	6.20
Beef hearth	6.00
Pig liver	5.71
Pig kidney	5.20
Beef kidney	5.14
Calf hearth	5.06
Calf liver	4.14
Sheep spleen	4.00
Sheep liver	3.95
Pig spleen	3.64
Pig hearth	3.60
Beef liver	3.10
Sheep kidney	3.03
Calf spleen	3.00
Beef lungs	2.90
Beef spleen	2.90
Sheep lungs	2.30
Calf lungs	2.17

Sources: Adapted from Souci, S.W. et al., *Food Composition and Nutrition Tables*, 7th edn, Taylor & Francis, Boca Raton, FL, 2008; Mataix Verdú, J. and Mañas Almendros, M., *Tabla de Composición de Alimentos Españoles* (*Table of the Composition of Spanish Foods*), 3rd edn. University of Granada, Granada, Spain, 1998.

(Agilent-Varian), MDN-5S (Supelco), SPB-Octyl (Supelco), 007-23 (Quadrex), 007-5 ms (Quadrex). The most important difficulty is the resolution of the capillary column for some critical group of peaks that are different in each kind of stationary phase. This fact implies that the complete separation of PCBs, dioxins, and organochlorinated pesticides is not possible with a single capillary column. The detection systems, ECD and MS, can hide the coelution of other compounds without response. However; these compounds are present in the ion source of the MS and they can produce changes in the ionization of the target compounds. The use of 2D-GC or GC x GC should be considered to reduce the overlapping of those critical PCB congeners (Ratel and Engel 2009). The fractionation of the different groups of compounds (PCBs, dioxins) can help to obtain better results but this increase the time of the analytical protocol.

23.2.2 Target Compounds

Elements to consider for an analysis may be (a) selection of the compounds having the highest toxicity, (b) classification of the edible meat by-products by the content in fat and the protein/collagen total ratio, and (c) edible meat by-products having the highest rate of consumption.

The PCB congeners most often found in foods are PCB77, PCB105, PCB118, PCB126, and PCB169 (WHO-TEF [1998] and Van den Berg et al. [2006]). Other planar PCBs are PCB81, PCB114, PCB123, PCB156 and PCB157, PCB167, PCB189 (Mullins et al. 1984). However, other PCBs should be considered because they have endocrine disruptive action. Organochlorine pesticides, whereas the expected concentrations should be low, can be analyzed in the same extract as PCBs. Organochlorinated pesticides are related compounds that are present in the extract for the analysis of PCBs. The expected concentrations of these pesticides could be low; however, they could be analyzed together with PCBs and dioxins. The maximal limits for dioxins (2002/69/EC [Commission Directive 2002/69/EC 2002]) were in pg WHO-TEQ/g fat for pig 1, for poultry 2, and for lamb and cattle 3. The PCBs concentrations are shown in Table 23.3. Also, for dioxins and dioxin-like PCBs the values were 4.5 (ruminants), 4.0 (poultry), and 1.5 (pigs). Values for liver samples of terrestrial animals and derived products were 12.0 WHO-TEQ/g fat for the dioxins plus dioxin-like PCBs (Commission Directive 2002/69/EC).

The analysis of the high toxic dioxins should be carried out to obtain a complete picture about the contamination by organolchlorine compounds. Multiresidue methods to determine PCBs and dioxins are described but before the application, it is advisable to establish the objectives of the study carefully. Instead, application of the GC/ECD or GC/LRMS (low resolution MS) methods is not considered to be correct for confirmatory analysis, because the lack of structural information to assure the identification and quantization. GC/ECD or GC/LRMS can be used as a first approach to sort samples having concentrations below the permitted maximal levels. There are accredited

TABLE 23.3
PCBs Concentrations Found in Different Foodstuffs in Catalonia, Spain

PCB Congener (ng kg^{-1} Wet Weight)	Fish and Shellfish	Meat and Derivatives	Fats and Oils
28	102.89	48.66	93.17
52	275.43	24.12	123.0
77	18.62	1.58	5.16
101	593.99	13.70	65.17
105	219.86	5.03	10.52
118	877.85	24.38	31.00
126	7.36	0.54	2.62
138	3202.40	88.31	33.50
153	4757.45	111.50	40.50
169	5.79	<0.77	3.23
180	1802.79	56.13	45.33
ng WHO-TEQ/kg wet weight	0.906	0.065	0.299

Source: Adapted from Agència Catalana de Seguretat Alimentària, *Contaminants Químics, Estudi de Dieta Total a Catalunya* (*Chemical Contaminants, Study of the Total Diet in Catalonia*), Eduard Mata, Ed., Gneralitat de Catalunya, Barcelona, Spain, pp. 37–53, 2005.

TEQ PCB = Σ ($TEFi_{1998\ WHO} \cdot Ci_{PCB\ dioxin\text{-}like}$).

methods to be chosen as reference for the analysis of these compounds in edible meat by-products: EPA Method 1668B, EN 1984-3, and JSA JIS K 0312.

23.2.3 Analysis of Edible Meat By-Products and Alternatives

A new approach to be considered is hair analysis (Klein et al. 1992, Schramm 1997, 2008). Several studies have shown the feasibility of this sample to obtain information about contaminants like PCBs. This sample allows the study of contaminants in living animals and some information is available on the ratios between hair concentrations and fat concentrations. Hair analysis can be carried out by treatment with KOH simplifying the sample treatment because PCBs are stable at different pHs (acid and basic). However, other organochlorinated compounds undergo degradation at pH > 7 (e.g., DDT is converted to DDE). Animal feed is the most important source of PCBs. The control of this product is the key factor to avoid the potential contamination. However, geographical conditions should be considered: contaminated areas are another important source of contaminants by the uptake from air (respiration and dermal contact), drinking water, and soils.

In a previous work, a general overview on analytical methods for the analysis of PCBs was reported (Garcia-Regueiro and Castellari 2009). In brief, the analytical conditions for the analysis of PCBs should consider the presence of organochlorinated pesticides to design the cleanup procedure. The use of sulfuric acid can simplify the analysis of PCBs because they are stable at very low pH and other organochlorinated compounds are degraded by acid treatment. However, this approach is not applied in solution; a complex cleanup with a column containing silica and sulfuric acid is preferred, after this column a separation in different fractions containing PCBs and dioxins can be achieved by means of florisil or carbon columns. Despite that, it is very difficult to avoid the cross-contamination of the fractions with compounds of other classes.

The fat content is an important factor to consider in the cleanup protocol. A large content of neutral lipids requires the use of a higher amount of sorbents or the use of smaller quantities of sample extract to avoid the overloading of the cleanup columns. Partition procedures by using acetonitrile/hexane could be applied but this choice is time-consuming and some losses can be produced during the liquid-liquid extractions. Fat could be reduced by careful precipitation at −20°C; the evaluation of the recovery and losses of this operation is necessary. Some works claimed that it is possible to quantify concentrations at 0.01 ng/kg. This means that the amount injected into the capillary columns is lower than 1 pg/μL, in some cases 0.01 pg/μL. In these conditions is very difficult to avoid high standard deviations required by the legislation (%rsd 15 for confirmatory methods and 30% for screening methods). The fat contents of a great number of edible by-products are depicted in Table 23.2. As a comparison the fat content of fish muscles is in the range 1%–15% (Mataix Verdú and Mañas Almendros 1998). So, the analytical methods suitable for fish samples could be applied to edible meat by-products but other aspects like collagen content have to be considered.

The analysis by GC/ECD or GC/LRMS could help to obtain a screening database to increase the information on PCBs concentrations in edible meat by-products. Those methods are more affordable for many laboratories and they allow the management of a higher number of samples. This approach could give a preliminary picture about the contamination of edible meat by-products and to know which edible meat by-products are the less contaminated. After, samples with concentrations higher than the limits allowed should be analyzed by confirmatory methods to establish the trueness of the results.

23.3 PCBs AND OTHER ORGANOCHLORINATED COMPOUNDS IN EDIBLE MEAT BY-PRODUCTS

There are few works dealing with this subject (Alamir et al. 1985, Khalafalla et al. 1993, Kofer and Fuchs 1994, Ulrich and Raszky 2002, Blanco-Penedo et al. 2008). So, not sufficient data are available to allow an overall picture of the presence of PCBs in edible meat by-products. As we know, the study of PCBs, dioxins, and organochlorinated compounds has been performed

in samples taken from the environment: wild fish, marine mammals, marine sediments, water, soils, air, etc. Some studies about concentrations in foods have been carried out; however, in general, the samples are from meat and derivative, and in some cases there are data available from animal species (Hansen et al. 1976, Quick et al. 1989, and Fazekas et al. 2010).

In a study about the contaminants in the total diet of Catalonia, the following concentrations of dioxins were reported (Agència Catalana de Seguretat Alimentària 2005): 0.39 pg WHO-TEQ/g fat (89.9 pg WHO-TEQ/kg wet weight) in pig and derivatives, 1.56 pg WHO-TEQ/g fat (63.8 pg WHO-TEQ/kg wet weight) in poultry, 0.49 pg WHO-TEQ/g fat (57.9 pg WHO-TEQ/kg wet weight) in lamb and 0.52 pg WHO-TEQ/g fat (72.9 pg WHO-TEQ/kg wet weight) in cattle and derivatives. The maximal limits (CE) were in pg WHO-TEQ/g fat of 1 (pig), 2 (poultry), and 3 (lamb and cattle). The PCBs concentrations are shown in Table 23.3.

The content of the PCB congeners 28, 52, 101, 138, 153, and 180 was studied in milk, meat, organs, fat tissue, and eggs in samples from Czechoslovakia (Gajduskova and Ulrich 1992). The results showed low concentrations; the most important aspect of this work was the use of a new cleanup material: Ekosorb (Kavalier Glasswork, Votice) as an alternative to Florisil.

Some organochlorinated compounds like DDTs, isomers, and Dieldrin can be metabolized by the liver to introduce hydroxyl groups in the DDT molecule (Sundtröm et al. 1977). Also, photo-Dieldrin was identified in liver and kidneys of broilers (Gallego-Iniesta and Peritierra-Rimada 1987). These results showed that the analysis of the parent molecules could not show the intake of these compounds by farm animals. The degradation of PCBs by metabolic processes is very difficult due to the high chemical stability of these molecules. As a result, nonmetabolites are expected from PCBs in edible meat by-products.

The episode of PCBs contamination in Belgium in 1999 allowed to study of the patterns of PCBs in poultry and pig. PCBs and PCDD/F patterns were different in contaminated animal feed and chicken and pig fat. PCB congeners 105, 118, 126, and 2,3,4,7,8-PeCDF showed higher concentrations in chicken than in pork; and PCDF congeners with non-2,3,7,8 substitutions were present in chicken fat but not in pork fat (Covaci et al. 2002). These results showed that the uptake and fate is species dependent and probably the fate in organs could be very different. Table 23.2 shows the fat content of the different edible meat by-products. The lipophilic properties of PCBs could be related with an increase in by-products with high content of fat. However, other organs like intestines could retain PCBs despite the high rate of elimination by the feces. A study about the bioavailability of tritiated 2,3,7,8-TCDD was performed to evaluate the bioaccumulation form grain and soil in cows (Jones et al. 1989).

A study about the fate of the PCBs was performed in snapping turtle (Chelydra turtle) during the years 1988 and 1989 (Bryan et al. 1987, Bishop et al. 1991, 1994, Eisler and Belisle 1996).The concentrations of Aroclor 1254 and Arocolor 1260 were higher in brain (82,000 μg/kg of lipids, liver 72,000 μg/kg of lipids, testes 100,000 μg/kg of lipids and fat 1,600,000 μg/kg of lipids. The lower concentrations were found in lungs (13,000 μg/kg of lipids) and the intermediate concentrations were 49,000 μg/kg of lipids (heart), 48,000 μg/kg of lipids (kidney), and 48,000 μg/kg of lipids (pancreas). Though turtle is not a mammal, the results showed a tendency to the accumulation of PCBs in fatter or lipid-rich tissues and in the liver. In particular, it is very important to note the high concentration in the brain. The metabolism in mammals can produce hydroxylation of PCBs with a low content of chloride atoms (Kurtz and George 1977, Sundtröm et al. 1977). These products could be more toxic than parent molecules. Other important result is that herbivores and predators differently metabolized PCBs. In predators were found higher concentrations of highly chlorinated PCBs. The embryos of chicken (*Gallus* sp.) were very sensitive to PCB77 and PCB153 was retained in adipose tissues of pigs. PCB180 is of particular interest to evaluate the fate of PCBs because the high concentrations expected of this congener in many kinds of samples (Hernandez et al. 1989).

23.4 CONCLUSION

Edible meat by-products are non-carcass materials that are used in different ways to produce meat products. The content of organochlorinated compounds in these products is not well known. The fate of PCBs, dioxins, and organochlorinated pesticides has been studied in environmental samples and to a minor extent in farm animals in controlled production systems. The relative unaffordable analytical equipment needed to perform the confirmatory analyses is a bottleneck to obtain more data and data in more types of samples. The cost of an analysis by GC/MS (HRMS) is very expensive to carry out a high number of samples. On the other hand, the cleanup procedure is complex and time-consuming. Only a few laboratories are capable of doing these analyses and their capacity is limited to cover the broad types of food samples to be analyzed. In fact, a specific method will be necessary for each edible meat by-product, due to the chemical differences between them. The application of analytical methods developed for other samples is questionable and a specific validation of the analytical method will be necessary.

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