Extraction Technologies for Medicinal and Aromatic Plants

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Preface

Medicinal plants are the richest bioresource of drugs for traditional systems of medicine, modern medicines, nutraceuticals, food supplements, folk medicines, pharmaceutical intermediates and chemical entities for synthetic drugs. Aromatic plants are a source of fragrances, flavors, cosmeceuticals, health beverages and chemical terpenes. Medicinal and aromatic plants (MAPs) are traded as such in bulk from many developing countries for further value addition in developed countries. The first step in the value addition of MAP bioresources is the production of herbal drug preparations (i.e. extracts), using a variety of methods from simple traditional technologies to advanced extraction techniques.

Extraction (as the term is pharmaceutically used) is the separation of medicinally active portions of plant (and animal) tissues using selective solvents through standard procedures. Such extraction techniques separate the soluble plant metabolites and leave behind the insoluble cellular marc. The products so obtained from plants are relatively complex mixtures of metabolites, in liquid or semisolid state or (after removing the solvent) in dry powder form, and are intended for oral or external use. These include classes of preparations known as decoctions, infusions, fluid extracts, tinctures, pilular (semisolid) extracts or powdered extracts. Such preparations have been popularly called galenicals, named after Galen, the second century Greek physician. The purpose of standardized extraction procedures for crude drugs (medicinal plant parts) is to attain the therapeutically desired portions and to eliminate unwanted material by treatment with a selective solvent known as menstruum. The extract thus obtained, after standardization, may be used as medicinal agent as such in the form of tinctures or fluid extracts or further processed to be incorporated in any dosage form such as tablets and capsules. These products all contain complex mixture of many medicinal plant metabolites, such as alkaloids, glycosides, terpenoids, flavonoids and lignans. In order to be used as a modern drug, an extract may be further processed through various techniques of fractionation to isolate individual chemical entities such as vincristine, vinblastine, hyoscyamine, hyoscine, pilocarpine, forskolin and codeine.

The industrial processing of MAPs starts with the extraction of the active components using various technologies. The general techniques of medicinal plant extraction include maceration, infusion, percolation, digestion, decoction, hot continuous extraction (Soxhlet), aqueous-alcoholic extraction by fermentation, counter-current extraction, microwave-assisted extraction, ultrasound extraction (sonication), supercritical fluid extraction, and phytonic extraction (with hydrofluorocarbon solvents). For aromatic plants, hydrodistillation techniques (water distillation, steam distillation, water and steam distillation), hydrolytic maceration followed by distillation, expression and enfleurage (cold fat extraction) may be employed. Some of

the latest extraction methods for aromatic plants include headspace trapping, solid phase micro-extraction, protoplast extraction, microdistillation, thermomicrodistillation and molecular distillation.

With the increasing demand for herbal medicinal products, nutraceuticals, and natural products for health care all over the world, medicinal plant extract manufacturers and essential oil producers have started using the most appropriate extraction technologies in order to produce extracts and essential oils of defined quality with the least variations from batch to batch. Such approach has to be adopted by MAP-rich developing countries in order to meet the increasing requirement of good quality extracts and essential oils for better revenue generation within the country, as well as for capturing this market in developed countries.

The basic parameters influencing the quality of an extract are the plant parts used as starting material, the solvent used for extraction, the manufacturing process (extraction technology) used with the type of equipment employed, and the crude-drug:extract ratio. The use of appropriate extraction technology, plant material, manufacturing equipment, extraction method and solvent and the adherence to good manufacturing practices certainly help to produce a good quality extract. From laboratory scale to pilot scale, all the conditions and parameters can be modelled using process simulation for successful industrial-scale production.

With the advances in extraction technologies and better knowledge for maintaining quality parameters, it has become absolutely necessary to disseminate such information to emerging and developing countries with a rich MAP biodiversity for the best industrial utilization of MAP resources.

The experts at the South-East Asian (SEA) Regional Workshop entitled ″Extraction Technologies for Medicinal and Aromatic Plants,″ held in 2006 in Lucknow, India, agreed to prepare a publication on extraction principles, technologies and analytical techniques for quality control of raw materials and processed products in the form of extracts and essential oils for medicinal and aromatic plants. This book focuses on different techniques of hydrodistillation, steam distillation, cohobation and fractional distillation of volatile oils from aromatic plants and on water-solvent extraction and supercritical fluid extraction for medicinal plant extracts. It also discusses general, specific and advanced technologies for preparing extracts of medicinal plants and the extraction of volatile oils and fragrances from aromatic plants. This book is intended to equip emerging and developing countries with techniques of extraction that can help them to produce economical and globally competitive quality extracts.

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1 An Overview of Extraction Techniques for Medicinal and Aromatic Plants

S. S. Handa

Abstract

A wide range of technologies is available for the extraction of active components and essential oils from medicinal and aromatic plants. The choice depends on the economic feasibility and suitability of the process to the particular situation. The various processes of production of medicinal plant extracts and essential oils are reviewed in this paper.

1.1 Introduction

Asia is the largest continent and has 60% of the world's population. It has abundant medicinal and aromatic plant species, well documented traditional knowledge, a long-standing practice of traditional medicine, and the potential for social and economic development of medicinal and aromatic plants (MAPs). Asia is one of the largest biodiversity regions in the world, containing some of the richest countries in plant resources. The continent has diverse plant flora but species richness is concentrated mainly in tropical and subtropical regions. Six of the world's 18 biodiversity hot spots, namely eastern Himalaya, North Borneo, Peninsular Malaysia, Sri Lanka, Philippines and the Western Ghats of South India, lie in Asia. The countries of the region have large flora: China has 30,000 species of higher plants; Indonesia, 20,000; India, 17,000; Myanmar, 14,000; Malaysia, 12,000; and Thailand, 12,000. The total numbers of plant species and the endemics in the region are given below:

Region	Species	Endemics
South East Asia	42-50,000	40,000
China and East Asia	45,000	18,650
Indian Subcontinent	25,000	12,000
South West Asia	23,000	7,100

Sustainable industrial exploitation of such a valuable bioresource, through use of appropriate technologies, can substantially contribute to the socio-economic growth of Asian countries. The International Centre for Science and High Technology (ICS-UNIDO) has thus organized this regional workshop on "extraction technologies for medicinal and aromatic plants" for South East Asian countries.

1.2 Medicinal Plant Extracts

Extraction, as the term is used pharmaceutically, involves the separation of medicinally active portions of plant or animal tissues from the inactive or inert components by using selective solvents in standard extraction procedures. The products so obtained from plants are relatively impure liquids, semisolids or powders intended only for oral or external use. These include classes of preparations known as decoctions, infusions, fluid extracts, tinctures, pilular (semisolid) extracts and powdered extracts. Such preparations popularly have been called galenicals, named after Galen, the second century Greek physician.

The purposes of standardized extraction procedures for crude drugs are to attain the therapeutically desired portion and to eliminate the inert material by treatment with a selective solvent known as *menstruum*. The extract thus obtained may be ready for use as a medicinal agent in the form of tinctures and fluid extracts, it may be further processed to be incorporated in any dosage form such as tablets or capsules, or it may be fractionated to isolate individual chemical entities such as ajmalicine, hyoscine and vincristine, which are modem drugs. Thus, standardization of extraction procedures contributes significantly to the final quality of the herbal drug.

1.2.1 General Methods of Extraction of Medicinal Plants

1.2.1.1 Maceration

In this process, the whole or coarsely powdered crude drug is placed in a stoppered container with the solvent and allowed to stand at room temperature for a period of at least 3 days with frequent agitation until the soluble matter has dissolved. The mixture then is strained, the marc (the damp solid material) is pressed, and the combined liquids are clarified by filtration or decantation after standing.

1.2.1.2 Infusion

Fresh infusions are prepared by macerating the crude drug for a short period of time with cold or boiling water. These are dilute solutions of the readily soluble constituents of crude drugs.

1.2.1.3 Digestion

This is a form of maceration in which gentle heat is used during the process of extraction. It is used when moderately elevated temperature is not objectionable. The solvent efficiency of the menstruum is thereby increased.

1.2.1.4 Decoction

In this process, the crude drug is boiled in a specified volume of water for a defined time; it is then cooled and strained or filtered. This procedure is suitable for extracting water-soluble, heat-stable constituents. This process is typically used in preparation of Ayurvedic extracts called “quath” or “kawath”. The starting ratio of crude drug to water is fixed, e.g. 1:4 or 1:16; the volume is then brought down to one-fourth its original volume by boiling during the extraction procedure. Then, the concentrated extract is filtered and used as such or processed further.

1.2.1.5 Percolation

This is the procedure used most frequently to extract active ingredients in the preparation of tinctures and fluid extracts. A percolator (a narrow, cone-shaped vessel open at both ends) is generally used (Figure 1). The solid ingredients are moistened with an appropriate amount of the specified menstruum and allowed to stand for approximately 4 h in a well-closed container, after which the mass is packed and the top of the percolator is closed. Additional menstruum is added to form a shallow layer above the mass, and the mixture is allowed to macerate in the closed percolator for 24 h. The outlet of the percolator then is opened and the liquid contained therein is allowed to drip slowly. Additional menstruum is added as required, until the percolate measures about three-quarters of the required volume of the finished product. The marc is then pressed and the expressed liquid is added to the percolate. Sufficient menstruum is added to produce the required volume, and the mixed liquid is clarified by filtration or by standing followed by decanting.

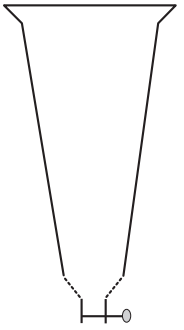

Figure 1: Percolator

1.2.1.6 Hot Continuous Extraction (Soxhlet)

In this method, the finely ground crude drug is placed in a porous bag or “thimble” made of strong filter paper, which is placed in chamber E of the Soxhlet apparatus (Figure 2). The extracting solvent in flask A is heated,

and its vapors condense in condenser D. The condensed extractant drips into the thimble containing the crude drug, and extracts it by contact. When the level of liquid in chamber E rises to the top of siphon tube C, the liquid contents of chamber E siphon into flask A. This process is continuous and is carried out until a drop of solvent from the siphon tube does not leave residue when evaporated. The advantage of this method, compared to previously described methods, is that large amounts of drug can be extracted with a much smaller quantity of solvent. This effects tremendous economy in terms of time, energy and consequently financial inputs. At small scale, it is employed as a batch process only, but it becomes much more economical and viable when converted into a continuous extraction procedure on medium or large scale.

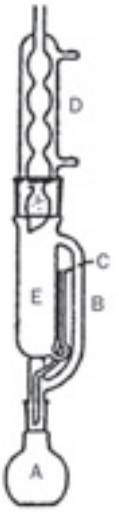

Figure 2: Soxhlet apparatus

1.2.1.7 Aqueous Alcoholic Extraction by Fermentation

Some medicinal preparations of Ayurveda (like *asava* and *arista*) adopt the technique of fermentation for extracting the active principles. The extraction procedure involves soaking the crude drug, in the form of either a powder or a decoction (*kasaya*), for a specified period of time, during which it undergoes fermentation and generates alcohol in situ; this facilitates the extraction of the active constituents contained in the plant material. The alcohol thus generated also serves as a preservative. If the fermentation is to be carried out in an earthen vessel, it should not be new: water should first be boiled in the vessel. In large-scale manufacture, wooden vats, porcelain jars or metal vessels are used in place of earthen vessels. Some examples of such preparations are *karpurasava, kanakasava, dasmularista*. In Ayurveda, this method is not yet standardized but, with the extraordinarily high degree of

advancement in fermentation technology, it should not be difficult to standardize this technique of extraction for the production of herbal drug extracts.

1.2.1.8 Counter-current Extraction

In counter-current extraction (CCE), wet raw material is pulverized using toothed disc disintegrators to produce a fine slurry. In this process, the material to be extracted is moved in one direction (generally in the form of a fine slurry) within a cylindrical extractor where it comes in contact with extraction solvent. The further the starting material moves, the more concentrated the extract becomes. Complete extraction is thus possible when the quantities of solvent and material and their flow rates are optimized. The process is highly efficient, requiring little time and posing no risk from high temperature. Finally, sufficiently concentrated extract comes out at one end of the extractor while the marc (practically free of visible solvent) falls out from the other end.

This extraction process has significant advantages:

i) A unit quantity of the plant material can be extracted with much smaller volume of solvent as compared to other methods like maceration, decoction, percolation.
ii) CCE is commonly done at room temperature, which spares the thermolabile constituents from exposure to heat which is employed in most other techniques.
iii) As the pulverization of the drug is done under wet conditions, the heat generated during comminution is neutralized by water. This again spares the thermolabile constituents from exposure to heat.
iv) The extraction procedure has been rated to be more efficient and effective than continuous hot extraction.

1.2.1.9 Ultrasound Extraction (Sonication)

The procedure involves the use of ultrasound with frequencies ranging from 20 kHz to 2000 kHz; this increases the permeability of cell walls and produces cavitation. Although the process is useful in some cases, like extraction of rauwolfia root, its large-scale application is limited due to the higher costs. One disadvantage of the procedure is the occasional but known deleterious effect of ultrasound energy (more than 20 kHz) on the active constituents of medicinal plants through formation of free radicals and consequently undesirable changes in the drug molecules.

1.2.1.10 Supercritical Fluid Extraction

Supercritical fluid extraction (SFE) is an alternative sample preparation method with general goals of reduced use of organic solvents and increased sample throughput. The factors to consider include temperature, pressure, sample volume, analyte collection, modifier (cosolvent) addition,

flow and pressure control, and restrictors. Generally, cylindrical extraction vessels are used for SFE and their performance is good beyond any doubt. The collection of the extracted analyte following SFE is another important step: significant analyte loss can occur during this step, leading the analyst to believe that the actual efficiency was poor.

There are many advantages to the use of CO_2 as the extracting fluid. In addition to its favorable physical properties, carbon dioxide is inexpensive, safe and abundant. But while carbon dioxide is the preferred fluid for SFE, it possesses several polarity limitations. Solvent polarity is important when extracting polar solutes and when strong analyte-matrix interactions are present. Organic solvents are frequently added to the carbon dioxide extracting fluid to alleviate the polarity limitations. Of late, instead of carbon dioxide, argon is being used because it is inexpensive and more inert. The component recovery rates generally increase with increasing pressure or temperature: the highest recovery rates in case of argon are obtained at 500 atm and 150° C.

The extraction procedure possesses distinct advantages:

i) The extraction of constituents at low temperature, which strictly avoids damage from heat and some organic solvents.
ii) No solvent residues.
iii) Environmentally friendly extraction procedure.

The largest area of growth in the development of SFE has been the rapid expansion of its applications. SFE finds extensive application in the extraction of pesticides, environmental samples, foods and fragrances, essential oils, polymers and natural products. The major deterrent in the commercial application of the extraction process is its prohibitive capital investment.

1.2.1.11 Phytonics Process

A new solvent based on hydrofluorocarbon-134a and a new technology to optimize its remarkable properties in the extraction of plant materials offer significant environmental advantages and health and safety benefits over traditional processes for the production of high quality natural fragrant oils, flavors and biological extracts.

Advanced Phytonics Limited (Manchester, UK) has developed this patented technology termed “phytonics process”. The products mostly extracted by this process are fragrant components of essential oils and biological or phytopharmacological extracts which can be used directly without further physical or chemical treatment.

The properties of the new generation of fluorocarbon solvents have been applied to the extraction of plant materials. The core of the solvent is 1,1,2,2-tetrafluoroethane, better known as hydrofluorocarbon-134a (HFC-134a). This product was developed as a replacement for chlorofluorocarbons. The boiling point of this solvent is -25° C. It is not flammable or toxic. Unlike chlorofluorocarbons, it does not deplete the ozone layer. It has a vapor pressure of 5.6 bar at ambient temperature. By most standards this is a poor solvent. For example, it does not mix with mineral oils or triglycerides and it does not dissolve plant wastes.

The process is advantageous in that the solvents can be customized: by using modified solvents with HFC-134a, the process can be made highly selective in extracting a specific class of phytoconstituents. Similarly, other modified solvents can be used to extract a broader spectrum of components. The biological products made by this process have extremely low residual solvent. The residuals are invariably less than 20 parts per billion and are frequently below levels of detection. These solvents are neither acidic nor alkaline and, therefore, have only minimal potential reaction effects on the botanical materials. The processing plant is totally sealed so that the solvents are continually recycled and fully recovered at the end of each production cycle. The only utility needed to operate these systems is electricity and, even then, they do no consume much energy. There is no scope for the escape of the solvents. Even if some solvents do escape, they contain no chlorine and therefore pose no threat to the ozone layer. The waste biomass from these plants is dry and “ecofriendly” to handle.

1.2.1.11.1 Advantages of the Process

- Unlike other processes that employ high temperatures, the phytonics process is cool and gentle and its products are never damaged by exposure to temperatures in excess of ambient.
- No vacuum stripping is needed which, in other processes, leads to the loss of precious volatiles.
- The process is carried out entirely at neutral pH and, in the absence of oxygen, the products never suffer acid hydrolysis damage or oxidation.
- The technique is highly selective, offering a choice of operating conditions and hence a choice of end products.
- It is less threatening to the environment.
- It requires a minimum amount of electrical energy.
- It releases no harmful emissions into the atmosphere and the resultant waste products (spent biomass) are innocuous and pose no effluent disposal problems.
- The solvents used in the technique are not flammable, toxic or ozone depleting.
- The solvents are completely recycled within the system.

1.2.1.11.2 Applications

The phytonics process can be used for extraction in biotechnology (e.g for the production of antibiotics), in the herbal drug industry, in the food, essential oil and flavor industries, and in the production of other pharmacologically active products. In particular, it is used in the production of top-quality pharmaceutical-grade extracts, pharmacologically active intermediates, antibiotic extracts and phytopharmaceuticals. However, the fact that it is used in all these areas in no way prevents its use in other areas. The technique is being used in the extraction of high-quality essential oils, oleoresins, natural food colors, flavors and aromatic oils from all manner of plant materials. The technique is also used in refining crude products obtained from other extraction processes. It provides extraction without waxes or other contaminants. It helps remove many biocides from contaminated biomass.

1.2.1.12 Parameters for Selecting an Appropriate Extraction Method

i) Authentication of plant material should be done before performing extraction. Any foreign matter should be completely eliminated.
ii) Use the right plant part and, for quality control purposes, record the age of plant and the time, season and place of collection.
iii) Conditions used for drying the plant material largely depend on the nature of its chemical constituents. Hot or cold blowing air flow for drying is generally preferred. If a crude drug with high moisture content is to be used for extraction, suitable weight corrections should be incorporated.
iv) Grinding methods should be specified and techniques that generate heat should be avoided as much as possible.
v) Powdered plant material should be passed through suitable sieves to get the required particles of uniform size.
vi) Nature of constituents:
    a) If the therapeutic value lies in non-polar constituents, a non-polar solvent may be used. For example, lupeol is the active constituent of *Crataeva nurvala* and, for its extraction, hexane is generally used. Likewise, for plants like *Bacopa monnieri* and *Centella asiatica*, the active constituents are glycosides and hence a polar solvent like aqueous methanol may be used.
    b) If the constituents are thermolabile, extraction methods like cold maceration, percolation and CCE are preferred. For thermostable constituents, Soxhlet extraction (if non-aqueous solvents are used) and decoction (if water is the menstruum) are useful.
    c) Suitable precautions should be taken when dealing with constituents that degrade while being kept in organic solvents, e.g. flavonoids and phenyl propanoids.

d) In case of hot extraction, higher than required temperature should be avoided. Some glycosides are likely to break upon continuous exposure to higher temperature.
e) Standardization of time of extraction is important, as:
   - Insufficient time means incomplete extraction.
   - If the extraction time is longer, unwanted constituents may also be extracted. For example, if tea is boiled for too long, tannins are extracted which impart astringency to the final preparation.
f) The number of extractions required for complete extraction is as important as the duration of each extraction.

vii) The quality of water or menstruum used should be specified and controlled.
viii) Concentration and drying procedures should ensure the safety and stability of the active constituents. Drying under reduced pressure (e.g. using a Rotavapor) is widely used. Lyophilization, although expensive, is increasingly employed.
ix) The design and material of fabrication of the extractor are also to be taken into consideration.
x) Analytical parameters of the final extract, such as TLC and HPLC fingerprints, should be documented to monitor the quality of different batches of the extracts.

1.2.2 Steps Involved in the Extraction of Medicinal Plants

In order to extract medicinal ingredients from plant material, the following sequential steps are involved:

1. Size reduction
2. Extraction
3. Filtration
4. Concentration
5. Drying

1.2.2.1 Size Reduction

The dried plant material is disintegrated by feeding it into a hammer mill or a disc pulverizer which has built-in sieves. The particle size is controlled by varying the speed of the rotor clearance between the hammers and the lining of the grinder and also by varying the opening of the discharge of the mill. Usually, the plant material is reduced to a size between 30 and 40 mesh, but this can be changed if the need arises. The objective for powdering the plant material is to rupture its organ, tissue and cell structures so that its medicinal ingredients are exposed to the extraction solvent. Furthermore, size reduction maximizes the surface area, which in turn enhances the mass transfer of active principle from plant material to the solvent. The 30-40 mesh size is optimal, while smaller particles may become slimy during extraction and create difficulty during filtration.

1.2.2.2 Extraction

Extraction of the plant material is carried out in three ways:

i) Cold aqueous percolation
ii) Hot aqueous extraction (decoction)
iii) Solvent extraction (cold or hot)

1.2.2.2.1 Cold Aqueous Percolation

The powdered material is macerated with water and then poured into a tall column. Cold water is added until the powdered material is completely immersed. It is allowed to stand for 24 h so that water-soluble ingredients attain equilibrium in the water. The enriched aqueous extract is concentrated in multiple-effect evaporators to a particular concentration. Some diluents and excipients are added to this concentrated extract, which is then ready for medicinal use.

1.2.2.2.2 Hot Aqueous Extraction (Decoction)

This is done in an open-type extractor. The extractor is a cylindrical vessel made from type 316 stainless steel and has a diameter (D) greater than the height (H), i.e. the H/D ratio is approximately 0.5. The bottom of the vessel is welded to the dished end and is provided with an inside false bottom with a filter cloth. The outside vessel has a steam jacket and a discharge valve at the bottom.

One part powdered plant material and sixteen parts demineralized water are fed into the extractor. Heating is done by injecting steam into the jacket. The material is allowed to boil until the volume of water is reduced to one-fourth its original volume. By this time the medicinal ingredients present in the plant material have been extracted out.

1.2.2.2.3 Filtration

The extract so obtained is separated out from the marc (exhausted plant material) by allowing it to trickle into a holding tank through the built-in false bottom of the extractor, which is covered with a filter cloth. The marc is retained at the false bottom, and the extract is received in the holding tank. From the holding tank, the extract is pumped into a sparkler filter to remove fine or colloidal particles from the extract.

1.2.2.2.4 Spray Drying

The filtered extract is subjected to spray drying with a high pressure pump at a controlled feed rate and temperature, to get dry powder. The desired particle size of the product is obtained by controlling the inside

temperature of the chamber and by varying the pressure of the pump. The dry powder is mixed with suitable diluents or excipients and blended in a double cone mixer to obtain a homogeneous powder that can be straightaway used, for example, for filling in capsules or making tablets.

1.2.2.3 Solvent Extraction

The principle of solid-liquid extraction is that when a solid material comes in contact with a solvent, the soluble components in the solid material move to the solvent. Thus, solvent extraction of plant material results in the mass transfer of soluble active principle (medicinal ingredient) to the solvent, and this takes place in a concentration gradient. The rate of mass transfer decreases as the concentration of active principle in the solvent increases, until equilibrium is reached, i.e. the concentrations of active principle in the solid material and the solvent are the same. Thereafter, there will no longer be a mass transfer of the active principle from plant material to the solvent.

Since mass transfer of the active principle also depends on its solubility in the solvent, heating the solvent can enhances the mass transfer. Moreover, if the solvent in equilibrium with the plant material is replaced with fresh solvent, the concentration gradient is changed. This gives rise to different types of extractions: cold percolation, hot percolation and concentration.

1.2.2.3.1 Cold Percolation

The extraction of plant material is carried out in a percolator which is a tall cylindrical vessel with a conical bottom and a built-in false bottom with a filter cloth. The percolator is connected to a condenser and a receiver for stripping solvent from the marc.

The powdered material is fed into the percolator along with a suitable solvent (ethyl alcohol or another non-polar solvent). The material is left in contact with the solvent until equilibrium of the active principle is achieved. The solvent extract, known as *miscella*, is taken out from the bottom discharge valve of the percolator. Fresh solvent is added into the percolator and the miscella is drained out after acquiring equilibrium. Overall, the plant material is washed four to five times until it gets exhausted. All washes from the percolator are pooled and concentrated.

The solvent in the marc is stripped out by passing steam from the bottom of the percolator. The solvent and steam vapors rise and are condensed in a tubular condenser. The condensate, which is a mixture of alcohol and water, is collected in a receiver and then subjected to fractional distillation to get 95% pure ethyl alcohol which is again used as a fresh solvent.

This type of percolation is not efficient as it takes a long time to reach equilibrium due to the slow mass transfer rate. The mass transfer

rate can be enhanced if some sort of movement is created between the particles and the solvent.

This can be achieved either by providing inside agitation with a mechanical stirrer or by repeated circulation of the extract back to the percolator. The first method is cumbersome and power intensive whereas the latter has been successful. A circulation pump that continuously circulates the miscella back to the top of the percolator gives a better mass transfer rate and reduces the equilibrium time considerably. Still, this type of percolation is energy-consuming as large amounts of miscella from multiple washes must be concentrated to remove the solvent.

To overcome this problem, a battery of percolators can be connected in series. If three washes are required for completion of the extraction, four percolators are connected in series with their respective miscella storage tanks.

At a particular time, one percolator is out of circuit, for charging and discharging the material and also for stripping solvent from the marc, whereas the other three percolators are in operation. Material is fed into all the percolators and the solvent is fed into the first percolator. When the equilibrium in the first percolator is reached, the extract from the first percolator is sent to the second percolator. The first percolator is again filled with fresh solvent. The extract of second percolator is transferred to the third, the extract of first is transferred to second, and fresh solvent is added to the first. The extract of the third percolator is transfered to the fourth percolator. After attaining equilibrium, the extract from the fourth percolator is drained off. The extract of the third percolator goes to fourth, the extract of second goes to third, and the extract of first goes to second percolator. The material of the first percolator, which has received three washes, is completely exhausted. This percolator is taken out of the system for stripping the solvent and discharging the extracted marc. This is again filled with fresh plant material and the sequence is repeated with other percolators. In this way, solvent of each percolator comes in contact three times with solid material and gets fully enriched with active principle. The enriched extract is sent for solvent recovery and concentration. Thus, instead of concentrating three volumes of solvent, only one volume has to be concentrated; this saves energy and the process is efficient.

1.2.2.3.2 Hot Percolation

Increasing the temperature of the solvent increases the solubility of the active principle, which increases the concentration gradient and therefore enhances the mass transfer of active principle from solid material to the solvent, provided the active principle is not heat sensitive. This is achieved by incorporating a heat exchanger between the circulation pump and the feed inlet of the percolator. The extract is continuously pumped into

a tubular heat exchanger which is heated by steam. The temperature of the extract in the percolator is controlled by a steam solenoid valve through a temperature indicator controller. This sort of arrangement can be incorporated in single percolators or in a battery of percolators as needed.

The percolators that are tall cylindrical towers must be housed in sheds of relatively great height. Tall towers are difficult to operate, especially when charging material and discharging the marc from the top and bottom manholes, which are time-consuming and labor-intensive procedures. Tall towers have been replaced by extractors of smaller height for which the H/D ratio is not more than 1.5.

These extractors have perforated baskets in which the material to be extracted is charged. These perforated baskets, when loaded outside, can be inserted into the extractor with a chain pulley block and, after the extraction, they can be lifted out from the extractor for discharging the marc. Some extractors have an electrical hoist for the charging the material and discharging the marc, which makes the operation less labor-intensive, quick and efficient.

The other type of instrument for extraction of medicinal ingredients from plant material is the Soxhlet apparatus, which consists of an extractor, a distillation still, a tubular condenser for the distillation still, a tubular condenser for the recovery of solvent from the marc, a receiver for collecting the condensate from the condenser, and a solvent storage tank. The plant material is fed into the extractor, and solvent is added until it reaches the siphon point of the extractor. Then, the extract is siphoned out into the distillation still, which is heated with steam. The solvent vapors go to the distillation condenser, get condensed and return to the extractor. The level of the solvent in the extractor again rises to the siphon point and the extract is siphoned out into the distillation still. In this way, fresh solvent comes in contact with the plant material a number of times, until the plant material is completely extracted. The final extract in the distillation still, which is rich in active principle, is concentrated and the solvent is recovered.

1.2.2.3.3 Concentration

The enriched extract from percolators or extractors, known as miscella, is fed into a wiped film evaporator where it is concentrated under vacuum to produce a thick concentrated extract. The concentrated extract is further fed into a vacuum chamber dryer to produce a solid mass free from solvent. The solvent recovered from the wiped film evaporator and vacuum chamber dryer is recycled back to the percolator or extractor for the next batch of plant material. The solid mass thus obtained is pulverized and used directly for the desired pharmaceutical formulations or further processed for isolation of its phytoconstituents.

1.3 Aromatic Plant Extracts

The types of volatile isolates that are obtained commercially from aromatic plants are essential oils, concretes, absolutes, pomades and resinoids. Essential oils are isolated from plant material by distillation whereas other volatile isolates are obtained by solvent extraction.

1.3.1 Concrete

This is an extract of fresh flowers, herbs, leaves and the flowering tops of plants obtained by the use of a hydrocarbon solvent such as butane, pentane, hexane and petroleum ether. Concrete is rich in hydrocarbon-soluble material and devoid of water-soluble components. It is generally a waxy, semisolid, dark-colored material free from the original solvent.

In practice, concretes are produced in static extractors. These extractors are fitted with numerous perforated trays so that the flowers do not get compressed by their own weight. Each perforated tray has a spacer so the number and distance between them are predetermined.

The set of perforated trays can be within a removable cylindrical basket. In the centre of the lower tray, there is a rod on which the spacers and the perforated trays are fitted while at the top there is a ring or a hook so that the entire contents of the extractor can be readily removed by a chain pulley block.

While stacking the flowers on these trays, care should be taken to minimize bruising and damage of the flowers, because such damage can result in the release of enzymes in the flower juice which deteriorates the quality of concrete. The basket stacked with flowers is inserted into the extractor and the solvent of choice is introduced from the bottom into the extractor until the material on the perforated disc assembly is completely immersed. Four to five such washes are given until the material is exhausted.

The enriched solvent from the extractor is pumped into an evaporator for solvent recovery and the solvent content is reduced to about one-tenth the original volume. The recovered solvent is pumped to the solvent tanks to be used again. The concentrated material from the evaporator is pumped into a vacuum evaporator where the solvent is removed more carefully under high vacuum and the recovered solvent is returned to the solvent tanks for repeated use. The resultant concrete has an odor similar to but stronger than the material from which it was extracted.

In concrete manufacturing, it is a normal practice to circulate fresh solvent through a battery of extractors. At each cycle, the solvent becomes more enriched with the flower volatiles until extraction is complete.

The number of extractors has to be synchronized with the number of solvent washes.

1.3.2 Absolutes

Concretes are not widely used in perfumery in their native form but are generally converted into an alcohol-soluble volatile concentrate known as an absolute, i.e. they have to be extracted with alcohol.

To make an absolute, the concrete is mixed with absolute alcohol and agitated thoroughly in a vessel with an agitator. During agitation, the temperature is kept at 40°-60° C and the concrete is immersed in the solution. The solution is cooled down to -5° to -10° C to precipitate out the wax, since waxes are normally insoluble in alcohol below -1° C. The precipitated wax is removed by passing the solution through a rotary filter. The filtrate from the rotary filter is pumped into a primary evaporator, where it is concentrated to about 10% alcohol content. Finally, the concentrated extract is pumped into an agitating-type evaporator, where the alcohol is carefully removed under high vacuum.

1.3.3 Resinoids

Resinoid is an extract of naturally resinous material, made with a hydrocarbon solvent. Resinoids are usually obtained from dry materials. The extraction process is same as that of concrete production, except that perforated discs are not used for stacking the material; instead powder from dry plant material is fed into the extractor.

1.3.4 Pomades

Pomades are obtained by a process known as enfleurage, which is a cold fat extraction method. The fat is spread out on glass plates contained in wooden frames, leaving a clear margin near the edges. The absorptive surface of the fat is increased by surface grooves made with a wooden spatula.

Fresh flowers are spread out on the surface of the fat and the frames are stacked in piles. After the perfume oils have been absorbed from the flowers, the spent flowers are removed by hand. Fresh flowers are again spread on the fat surface. This is repeated until the fat surface is completely enriched with perfume oils. The pomade so obtained is ready for cold alcoholic extraction.

1.3.5 Essential Oils

Essential oils are used in a wide variety of consumer goods such as detergents, soaps, toilet products, cosmetics, pharmaceuticals, perfumes,

confectionery food products, soft drinks, distilled alcoholic beverages (hard drinks) and insecticides. The world production and consumption of essential oils and perfumes are increasing very fast. Production technology is an essential element to improve the overall yield and quality of essential oil. The traditional technologies pertaining to essential oil processing are of great significance and are still being used in many parts of the globe. Water distillation, water and steam distillation, steam distillation, cohobation, maceration and enfleurage are the most traditional and commonly used methods. Maceration is adaptable when oil yield from distillation is poor. Distillation methods are good for powdered almonds, rose petals and rose blossoms, whereas solvent extraction is suitable for expensive, delicate and thermally unstable materials like jasmine, tuberose, and hyacinth. Water distillation is the most favored method of production of citronella oil from plant material.

1.3.5.1 Sources of Natural Essential Oils

Plant organs containing natural essential oils are illustrated in Figure 3. Essential oils are generally derived from one or more plant parts, such as flowers (e.g. rose, jasmine, carnation, clove, mimosa, rosemary, lavander), leaves (e.g. mint, *Ocimum* spp., lemongrass, jamrosa), leaves and stems (e.g. geranium, patchouli, petitgrain, verbena, cinnamon), bark (e.g. cinnamon, cassia, canella), wood (e.g. cedar, sandal, pine), roots (e.g. angelica, sassafras, vetiver, saussurea, valerian), seeds (e.g fennel, coriander, caraway, dill, nutmeg), fruits (bergamot, orange, lemon, juniper), rhizomes (e.g. ginger, calamus, curcuma, orris) and gums or oleoresin exudations (e.g. balsam of Peru, balsam of Tolu, storax, myrrh, benzoin).

Specialized plant structures that produce and store essential oils are shown in Figure 4. Depending upon the plant family, essential oils may occur in specialized secretary structures such as glandular hairs (Labiatae, Verbenaceace, Geraniaceae), modified parenchymal cells (Piperaceae), resin canals (conifers), oil tubes called vittae (Umbelliferae), lysigenous cavities (Rutaceae), schizogenous passages (Myrtaceae, Graminae, Compositae) or gum canals (Cistacae, Burseraceae). It is well known that when a geranium leaf is lightly touched, an odor is emitted because the long stalked oil glands are fragile. Similarly, the application of slight pressure on a peppermint leaf will rupture the oil gland and release oil. In contrast, pine needles and eucalyptus leaves do not release their oils until the epidermis of the leaf is broken. Hence, the types of structures in which oil is contained differ depending on the plant type and are plant-family specific. Unfortunately, not enough is known even today about these oil secretary structures to carefully categorize them. From the practical standpoint, they can be categorized into superficial and subcutaneous oils. Based on the currently available information, it may be inferred that oils of the Labiatae, Verbenaceae and Geraniaceae families are the only superficial oils known; consequently, the others are considered subcutaneous oils.

During handling, some flowers continue to produce aroma while other quickly loose their odor. Flowers collected at different times may also give different perfumery values. Regarding the rose, half-open flowers with plump anthers give higher oil yield than fully opened flowers with shrivelled anthers. Humidity, wind, rain and surface temperature also affect the oil yield considerably. Harvesting schedule affects both quantity and quality of the oil.

1.3.5.2 Essential Oil Constituents

Major constituents of essential oils are shown in Figure 5, from which it is clear that most essential oils consist of hydrocarbons, esters, terpenes, lactones, phenols, aldehydes, acids, alcohols, ketones, and esters. Among these, the oxygenated compounds (alcohols, esters, aldehydes, ketones, lactones, phenols) are the principal odor source. They are more stable against oxidizing and resinifying influences than other constituents. On the other hand, unsaturated constituents like monoterpenes and sesquiterpenes have the tendency to oxidize or resinify in the presence of air and light. The knowledge of individual constituents and their physical characteristics, such as boiling point, thermal stability and vapor-pressure-temperature relationship, is of paramount importance in technology development of oxygenated compounds.

1.3.5.3 Methods of Producing Essential Oils

Methods for producing essential oils from plant materials are summarized in Figure 6. Regarding hydrodistillation, the essential oils industry has developed terminology to distinguish three types: water distillation; water and steam distillation; and direct steam distillation.

Originally introduced by Von Rechenberg, these terms have become established in the essential oil industry. All three methods are subject to the same theoretical considerations which deal with distillation of two-phase systems. The differences lie mainly in the methods of handling the material.

Some volatile oils cannot be distilled without decomposition and thus are usually obtained by expression (lemon oil, orange oil) or by other mechanical means. In certain countries, the general method for obtaining citrus oil involves puncturing the oil glands by rolling the fruit over a trough lined with sharp projections that are long enough to penetrate the epidermis and pierce the oil glands located within outer portion of the peel (*ecuelle* method). A pressing action on the fruit removes the oil from the glands, and a fine spray of water washes the oil from the mashed peel while the juice is extracted through a central tube that cores the fruit. The resulting oil-water emulsion is separated by centrifugation. A variation of this process is to remove the peel from the fruit before the oil is extracted.

Often, the volatile oil content of fresh plant parts (flower petals) is so small that oil removal is not commercially feasible by the aforementioned

methods. In such instances, an odorless, bland, fixed oil or fat is spread in a thin layer on glass plates. The flower petals are placed on the fat for a few hours; then repeatedly, the oil petals are removed, and a new layer of petals is introduced. After the fat has absorbed as much fragrance as possible, the oil may be removed by extraction with alcohol. This process, known as enfleurage, was formerly used extensively in the production of perfumes and pomades.

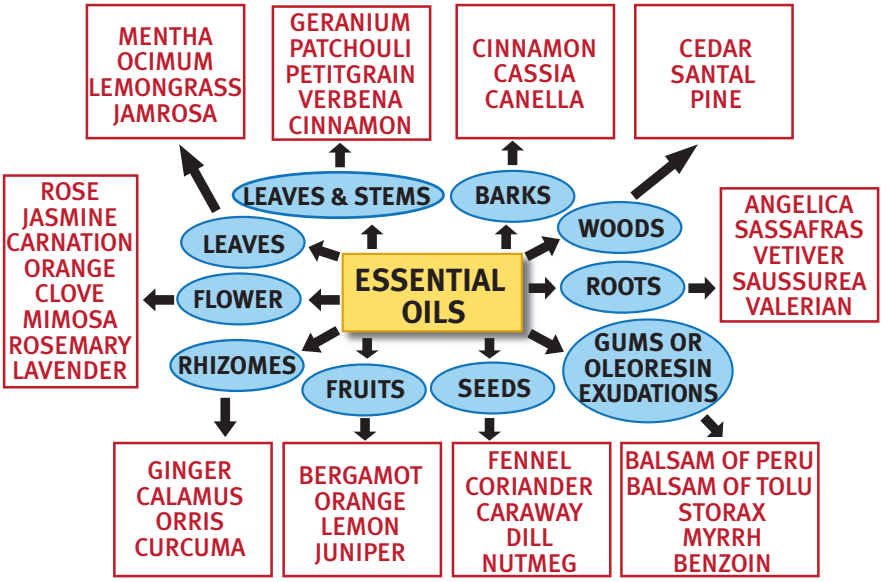

Figure 3: Plant organs containing essential oils

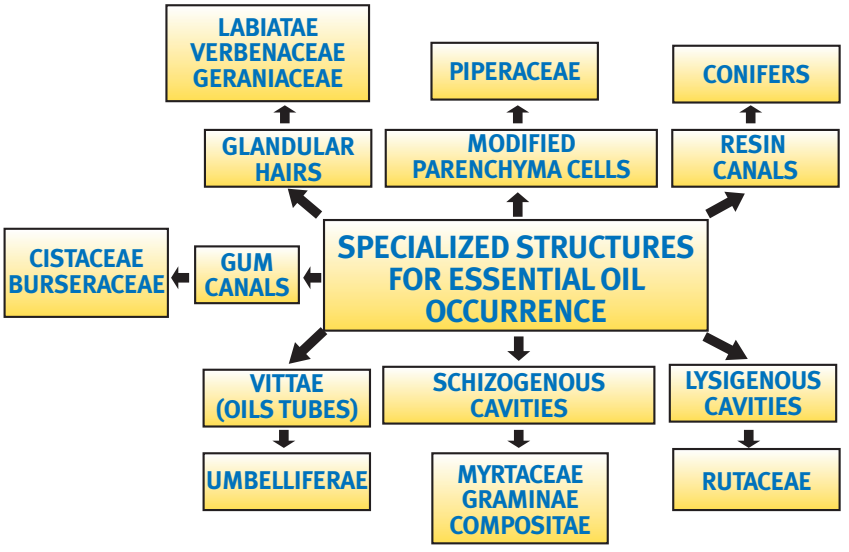

Figure 4: Family-specific plant tissues responsible for producing or storing essential oil

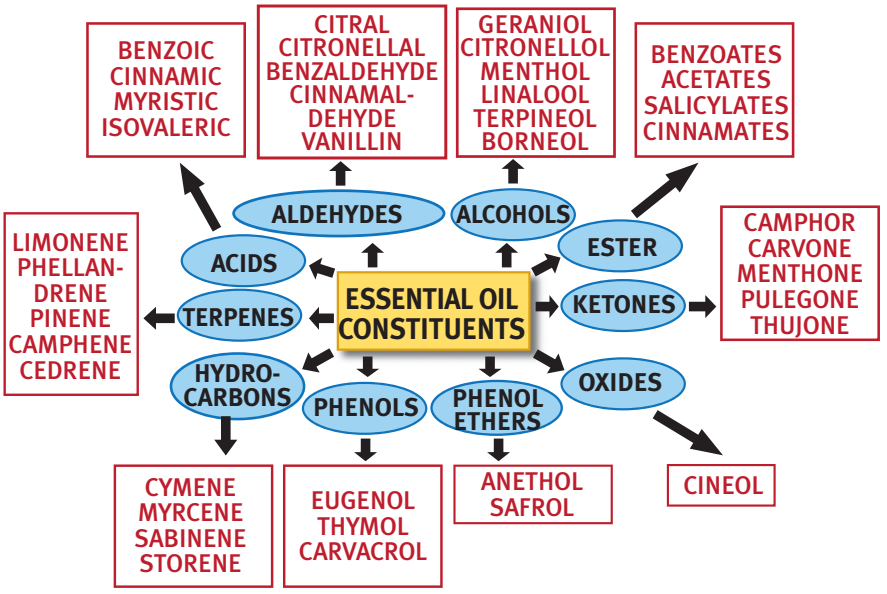

Figure 5: Heterogeneous chemical groups present in essential oil

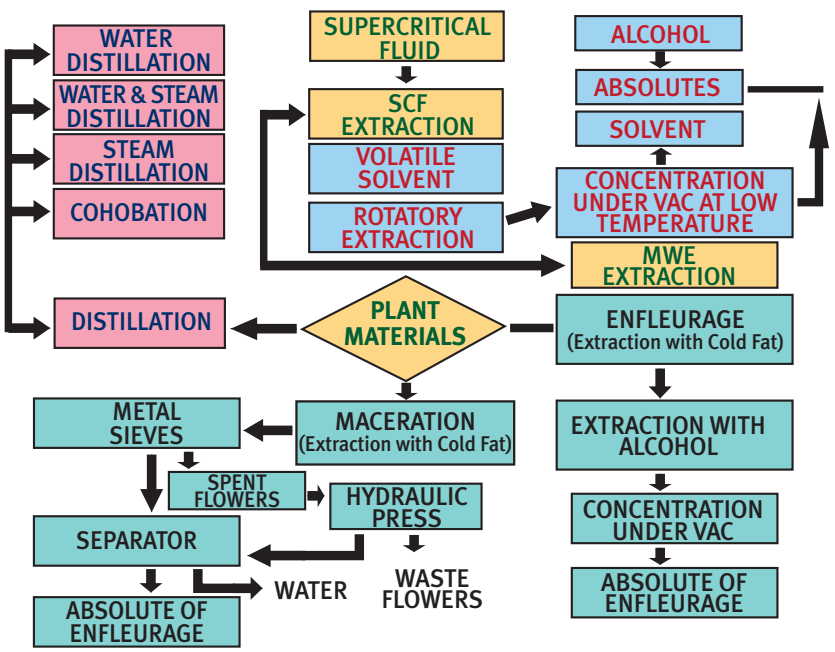

Figure 6: Methods of producing essential oils from plant materials

In the perfume industry, most modern essential oil production is accomplished by extraction, using volatile solvents such as petroleum ether and hexane. The chief advantages of extraction over distillation is that uniform

temperature (usually 50° C) can be maintained during the process, As a result, extracted oils have a more natural odor that is unmatched by distilled oils, which may have undergone chemical alteration by the high temperature. This feature is of considerable importance to the perfume industry; however, the established distillation method is of lower cost than the extraction process.

Destructive distillation means distilling volatile oil in the absence of air. When wood or resin of members of the Pinaceae or Cupressaceae is heated without air, decomposition takes place and a number of volatile compounds are driven off. The residual mass is charcoal. The condensed volatile matter usually separates into 2 layers: an aqueous layer containing wood naptha (methyl alcohol) and pyroligneous acid (crude acetic), and a tarry liquid in the form of pine tar, juniper tar, or other tars, depending on the wood used. This dry distillation is usually conducted in retorts and, if the wood is chipped or coarsely ground and the heat is applied rapidly, the yield often represents about 10% of the wood weight used.

1.3.5.3.1 Hydrodistillation

In order to isolate essential oils by hydrodistillation, the aromatic plant material is packed in a still and a sufficient quantity of water is added and brought to a boil; alternatively, live steam is injected into the plant charge. Due to the influence of hot water and steam, the essential oil is freed from the oil glands in the plant tissue. The vapor mixture of water and oil is condensed by indirect cooling with water. From the condenser, distillate flows into a separator, where oil separates automatically from the distillate water.

1.3.5.3.1.1 Mechanism of Distillation

Hydrodistillation of plant material involves the following main physicochemical processes:

i) Hydrodiffusion
ii) Hydrolysis
iii) Decomposition by heat

1.3.5.3.1.1.1 Hydrodiffusion

Diffusion of essential oils and hot water through plant membranes is known as hydrodiffusion. In steam distillation, the steam does not actually penetrate the dry cell membranes. Therefore, dry plant material can be exhausted with dry steam only when all the volatile oil has been freed from the oil-bearing cells by first thorough comminution of the plant material. But, when the plant material is soaked with water, exchange of vapors within the tissue is based on their permeability while in swollen condition. Membranes of plant cells are almost impermeable to volatile oils. Therefore, in

the actual process, at the temperature of boiling water, a part of volatile oil dissolves in the water present within the glands, and this oil-water solution permeates, by osmosis, the swollen membranes and finally reaches the outer surface, where the oil is vaporized by passing steam.

Another aspect of hydrodiffusion is that the speed of oil vaporization is not influenced by the volatility of the oil components, but by their degree of solubility in water. Therefore, the high-boiling but more water-soluble constituents of oil in plant tissue distill before the low-boiling but less water-soluble constituents. Since hydrodiffusion rates are slow, distillation of uncomminuted material takes longer time than comminuted material.

1.3.5.3.1.1.2 Hydrolysis

Hydrolysis in the present context is defined as a chemical reaction between water and certain constituents of essential oils. Esters are constituents of essential oils and, in the presence of water, especially at high temperatures, they tend to react with water to form acids and alcohols. However, the reactions are not complete in either direction and the relationship between the molal concentrations of various constituents at equilibrium is written as:

$$K = \frac{(\text{alcohol}) \times (\text{acid})}{(\text{ester}) \times (\text{water})}$$

where K is the equilibrium constant.

Therefore, if the amount of water is large, the amounts of alcohol and acid will also be large, resulting in a decreased yield of essential oil. Furthermore, since this is a time-dependent reaction, the extent to which hydrolysis proceeds depends on the time of contact between oil and water. This is one of the disadvantages of water distillation.

1.3.5.3.1.1.3 Effect of Heat

Almost all constituents of essential oils are unstable at high temperature. To obtain the best quality oil, distillation must be done at low temperatures. The temperature in steam distillation is determined entirely by the operating pressure, whereas in water distillation and in water and steam distillation the operating pressure is usually atmospheric.

All the previously described three effects, i.e. hydrodiffusion, hydrolysis and thermal decomposition, occur simultaneously and affect one another. The rate of diffusion usually increases with temperatures as does the solubility of essential oils in water. The same is true for the rate and extent of hydrolysis. However, it is possible to obtain better yield and quality of oils by: (1) maintaining the temperature as low as possible, (2) using as

little water as possible, in the case of steam distillation, and (3) thoroughly comminuting the plant material and packing it uniformly before distillation.

1.3.5.3.2 Three Types of Hydrodistillation

Three are three types of hydrodistillation for isolating essential oils from plant materials:

1. Water distillation
2. Water and steam distillation
3. Direct steam distillation

1.3.5.3.2.1 Water Distillation

In this method, the material is completely immersed in water, which is boiled by applying heat by direct fire, steam jacket, closed steam jacket, closed steam coil or open steam coil. The main characteristic of this process is that there is direct contact between boiling water and plant material.

When the still is heated by direct fire, adequate precautions are necessary to prevent the charge from overheating. When a steam jacket or closed steam coil is used, there is less danger of overheating; with open steam coils this danger is avoided. But with open steam, care must be taken to prevent accumulation of condensed water within the still. Therefore, the still should be well insulated. The plant material in the still must be agitated as the water boils, otherwise agglomerations of dense material will settle on the bottom and become thermally degraded. Certain plant materials like cinnamon bark, which are rich in mucilage, must be powdered so that the charge can readily disperse in the water; as the temperature of the water increases, the mucilage will be leached from the ground cinnamon. This greatly increases the viscosity of the water-charge mixture, thereby allowing it to char. Consequently, before any field distillation is done, a small-scale water distillation in glassware should be performed to observe whether any changes take place during the distillation process. From this laboratory trial, the yield of oil from a known weight of the plant material can be determined. The laboratory apparatus recommended for trial distillations is the Clevenger system (Figure 7).

During water distillation, all parts of the plant charge must be kept in motion by boiling water; this is possible when the distillation material is charged loosely and remains loose in the boiling water. For this reason only, water distillation possesses one distinct advantage, i.e. that it permits processing of finely powdered material or plant parts that, by contact with live steam, would otherwise form lumps through which the steam cannot penetrate. Other practical advantages of water distillation are that the stills

are inexpensive, easy to construct and suitable for field operation. These are still widely used with portable equipment in many countries.

The main disadvantage of water distillation is that complete extraction is not possible. Besides, certain esters are partly hydrolyzed and sensitive substances like aldehydes tend to polymerize. Water distillation requires a greater number of stills, more space and more fuel. It demands considerable experience and familiarity with the method. The high-boiling and somewhat water-soluble oil constituents cannot be completely vaporized or they require large quantities of steam. Thus, the process becomes uneconomical. For these reasons, water distillation is used only in cases in which the plant material by its very nature cannot be processed by water and steam distillation or by direct steam distillation.

Figure 7: Clevenger-type laboratory-scale hydrodistillation apparatus

1.3.5.3.2.1.1 Traditional Method of Producing Attar Using Hydrodistillation

Floral attars are defined as the distillates obtained by hydrodistillation of flowers (such as saffron, marigold, rose, jasmine, pandanus) in sandal wood oil or other base materials like paraffin.

Attar manufacturing takes place in remote places because the flowers must be processed quickly after collection. The apparatus and equipment used to manufacture attar are light, flexible, easy to repair, and have a fair degree of efficiency. Keeping in view these facts, the traditional "deg and bhapka" process has been used for centuries and is used even now with the following traditional equipment (Figure 8).

- Deg (still)
- Bhapka (receiver)
- Chonga (bamboo condenser)

- Traditional bhatti (furnace)
- Gachchi (cooling water tank)
- Kuppi (leather bottle)

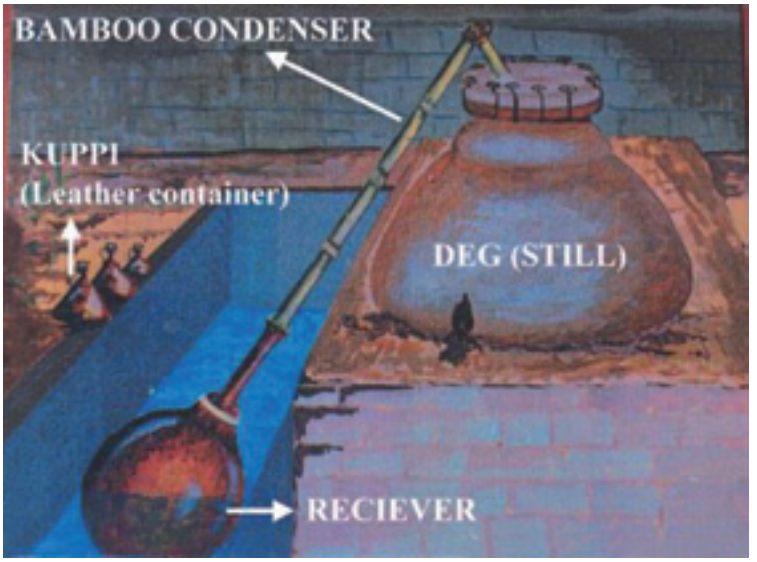

Figure 8: Traditional process of water distillation for making attar

1.3.5.3.2.1.2 Disadvantages of Water Distillation

- Oil components like esters are sensitive to hydrolysis while others like acyclic monoterpene hydrocarbons and aldehydes are susceptible to polymerization (since the pH of water is often reduced during distillation, hydrolytic reactions are facilitated).
- Oxygenated components such as phenols have a tendency to dissolve in the still water, so their complete removal by distillation is not possible.
- As water distillation tends to be a small operation (operated by one or two persons), it takes a long time to accumulate much oil, so good quality oil is often mixed with bad quality oil.
- The distillation process is treated as an art by local distillers, who rarely try to optimize both oil yield or quality.
- Water distillation is a slower process than either water and steam distillation or direct steam distillation.

1.3.5.3.2.2 Water and Steam Distillation

In water and steam distillation, the steam can be generated either in a satellite boiler or within the still, although separated from the

plant material. Like water distillation, water and steam distillation is widely used in rural areas. Moreover, it does not require a great deal more capital expenditure than water distillation. Also, the equipment used is generally similar to that used in water distillation, but the plant material is supported above the boiling water on a perforated grid. In fact, it is common that persons performing water distillation eventually progress to water and steam distillation.

It follows that once rural distillers have produced a few batches of oil by water distillation, they realize that the quality of oil is not very good because of its still notes (subdued aroma). As a result, some modifications are made. Using the same still, a perforated grid or plate is fashioned so that the plant material is raised above the water. This reduces the capacity of the still but affords a better quality of oil. If the amount of water is not sufficient to allow the completion of distillation, a cohobation tube is attached and condensate water is added back to the still manually, thereby ensuring that the water, which is being used as the steam source, will never run out. It is also believed that this will, to some extent, control the loss of dissolved oxygenated constituents in the condensate water because the re-used condensate water will allow it to become saturated with dissolved constituents, after which more oil will dissolve in it.

1.3.5.3.2.2.1 Cohobation

Cohobation is a procedure that can only be used during water distillation or water and steam distillation. It uses the practice of returning the distillate water to the still after the oil has been separated from it so that it can be re-boiled. The principal behind it is to minimize the losses of oxygenated components, particularly phenols which dissolve to some extent in the distillate water. For most oils, this level of oil loss through solution in water is less than 0.2%, whereas for phenol-rich oils the amount of oil dissolved in the distillate water is 0.2%-0.7%. As this material is being constantly re-vaporized, condensed and re-vaporized again, any dissolved oxygenated constituents will promote hydrolysis and degradation of themselves or other oil constituents. Similarly, if an oxygenated component is constantly brought in contact with a direct heat source or side of a still, which is considerably hotter than 100° C, then the chances of degradation are enhanced. As a result, the practice of cohobation is not recommended unless the temperature to which oxygenated constituents in the distillate are exposed is no higher than 100° C.

In steam and water distillation, the plant material cannot be in direct contact with the fire source beneath the still; however, the walls of the still are good conductors of heat so that still notes can also be obtained from the thermal degradation reactions of plant material that is touching the sides of the still. As the steam in the steam and water distillation process is wet, a major drawback of this type of distillation is that it will make the plant

material quite wet. This slows down distillation as the steam has to vaporize the water to allow it to condense further up the still. One way to prevent the lower plant material resting on the grid from becoming waterlogged is to use a baffle to prevent the water from boiling too vigorously and coming in direct contact with the plant material.

1.3.5.3.2.2.2 Advantages of Water and Steam Distillation over Water Distillation

- Higher oil yield.
- Components of the volatile oil are less susceptible to hydrolysis and polymerization (the control of wetness on the bottom of the still affects hydrolysis, whereas the thermal conductivity of the still walls affects polymerization).
- If refluxing is controlled, then the loss of polar compounds is minimized.
- Oil quality produced by steam and water distillation is more reproducible.
- Steam and water distillation is faster than water distillation, so it is more energy efficient.

Many oils are currently produced by steam and water distillation, for example lemongrass is produced in Bhutan with a rural steam and water distillation system.

1.3.5.3.2.2.3 Disadvantages of Water and Steam Distillation

- Due to the low pressure of rising steam, oils of high-boiling range require a greater quantity of steam for vaporization - hence longer hours of distillation.
- The plant material becomes wet, which slows down distillation as the steam has to vaporize the water to allow it to condense further up the still.
- To avoid that the lower plant material resting on the grid becomes waterlogged, a baffle is used to prevent the water from boiling too vigorously and coming in direct contact with the plant material.

1.3.5.3.2.3 Direct Steam Distillation

As the name suggests, direct steam distillation is the process of distilling plant material with steam generated outside the still in a satellite steam generator generally referred to as a boiler. As in water and steam distillation, the plant material is supported on a perforated grid above the steam inlet. A real advantage of satellite steam generation is that the amount of steam can be readily controlled. Because steam is generated in a satellite boiler, the plant material is heated no higher than 100° C and, consequently, it should not undergo thermal degradation. Steam distillation

is the most widely accepted process for the production of essential oils on large scale. Throughout the flavor and fragrance supply business, it is a standard practice.

An obvious drawback to steam distillation is the much higher capital expenditure needed to build such a facility. In some situations, such as the large-scale production of low-cost oils (e.g. rosemary, Chinese cedarwood, lemongrass, litsea cubeba, spike lavender, eucalyptus, citronella, cornmint), the world market prices of the oils are barely high enough to justify their production by steam distillation without amortizing the capital expenditure required to build the facility over a period of 10 years or more.

1.3.5.3.2.3.1 Advantages of Direct Steam Distillation

- Amount of steam can be readily controlled.
- No thermal decomposition of oil constituents.
- Most widely accepted process for large-scale oil production, superior to the other two processes.

1.3.5.3.2.3.2 Disadvantage of Direct Steam Distillation

- Much higher capital expenditure needed to establish this activity than for the other two processes.

1.3.5.3.3 Essential Oil Extraction by Hydrolytic Maceration Distillation

Certain plant materials require maceration in warm water before they release their essential oils, as their volatile components are glycosidically bound. For example, leaves of wintergreen (*Gaultheria procumbens*) contain the precursor gaultherin and the enzyme primeverosidase; when the leaves are macerated in warm water, the enzyme acts on the gaultherin and liberates free methyl salicylate and primeverose. Other similar examples include brown mustard (sinigrin), bitter almonds (amygdalin) and garlic (alliin).

1.3.5.3.4 Essential Oil Extraction by Expression

Expression or cold pressing, as it is also known, is only used in the production of citrus oils. The term expression refers to any physical process in which the essential oil glands in the peel are crushed or broken to release the oil. One method that was practiced many years ago, particularly in Sicily (*spugna* method), commenced with halving the citrus fruit followed by pulp removal with the aid of sharpened spoon-knife (known as a *rastrello*). The oil was removed from the peel either by pressing the peel against a hard object of baked clay (*concolina*) which was placed under a large natural sponge or by bending the peel into the sponge. The oil

emulsion absorbed by the sponge was removed by squeezing it into the *concolina* or some other container. It is reported that oil produced this way contains more of the fruit odor character than oil produced by any other method.

A second method known as equaling (or the *scodella* method), uses a shallow bowl of copper (or sometimes brass) with a hollow central tube; the equaling tool is similar in shape to a shallow funnel. The bowl is equipped with brass points with blunt ends across which the whole citrus fruit is rolled by hand with some pressure until all of the oil glands have burst. The oil and aqueous cell contents are allowed to dribble down the hollow tube into a container from which the oil is separated by decantation. Obviously, hand pressing is impractical because it is an extremely slow process, e.g. on average only 2-4 lbs oil per day can be produced by a single person using one of these hand methods. As a result, over the years a number of machines have been designed to either crush the peel of a citrus fruit or crush the whole fruit and then separate the oil from the juice.

1.3.5.3.4.1 Pelatrice Process

In the *pelatrice* process, citrus fruits are fed from a hopper into the abrasive shell of the machine. The fruits are rotated against the abrasive shell by a slow-moving Archimedian screw whose surface rasps the fruit surfaces causing some of the essential oil cavities on the peel to burst and release their oil-water emulsion. This screw further transports the fruit into a hopper in which rollers covered with abrasive spikes burst the remaining oil cavities. The oil and water emulsion is washed away from the fruit by a fine spray of water. The emulsion next passes through a separator where any solids are removed, after which it passes through two centrifugal separators working in series to yield the pure oil. Most bergamot oil and some lemon oil are produced this way in Italy.

1.3.5.3.4.2 Sfumatrice Process

The *sfumatrice* equipment consists of a metallic chain that is drawn by two horizontal ribbed rollers. The peels are conveyed through these rollers during which time they are pressed and bent to release their oil. As in *pelatrice*, the oil is washed away from the *sfumatrice* rollers by fine sprays of water. Again, the oil is initially passed through a separator prior to being sent to two centrifuges in series, so that purified oil can be produced. At one time, *sfumatrice* was the most popular process for citrus oil isolation in Italy; however, today the *pelatrice* method appears more popular.

1.3.5.3.5 Essential Oil Extraction with Cold Fat (Enfleurage)

Despite the introduction of the modern process of extraction with volatile solvents, the old fashioned method of enfleurage, as passed on

from father to son and perfected in the course of generations, still plays an important role. Enfleurage on a large scale is today carried out only in the Grasse region of France, with the possible exception of isolated instances in India where the process has remained primitive.

The principles of enfleurage are simple. Certain flowers (e.g. tuberose and jasmine) continue the physiological activities of developing and giving off perfume even after picking. Every jasmine and tuberose flower resembles, so to speak, a tiny factory continually emitting minute quantities of perfume. Fat possesses a high power of absorption and, when brought in contact with fragrant flowers, readily absorbs the perfume emitted. This principle, methodically applied on a large scale, constitutes enfleurage. During the entire period of harvest, which lasts for eight to ten weeks, batches of freshly picked flowers are strewn over the surface of a specially prepared fat base (corps), let there (for 24 h in the case of jasmine and longer in the case of tuberose), and then replaced by fresh flowers. At the end of the harvest, the fat, which is not renewed during the process, is saturated with flower oil. Thereafter, the oil is extracted from the fat with alcohol and then isolated.

The success of enfleurage depends to a great extent upon the quality of the fat base employed. Utmost care must be exercised when preparing the corps. It must be practically odorless and of proper consistency. If the corps is too hard, the blossoms will not have sufficient contact with the fat, curtailing its power of absorption and resulting in a subnormal yield of flower oil. On the other, if it is too soft, it will tend to engulf the flowers and the exhausted ones will adhere; when removed, the flowers will retain adhering fat, resulting in considerable shrinkage and loss of corps. The consistency of the corps must, therefore, be such that it offers a semihard surface from which the exhausted flowers can easily be removed. The process of enfleurage is carried out in cool cellars, and every manufacturer must prepare the corps according to the prevailing temperature in the cellars during the months of the flower harvest.

Many years of experience have proved that a mixture of one part of highly purified tallow and two parts of lard is eminently suitable for enfleurage. This mixture assures a suitable consistency of the corps in conjunction with high power of absorption. The fat corps thus prepared is white, smooth, absolutely of uniform consistency, free of water and practically odorless. Some manufacturers also add small quantities of orange flower or rose water when preparing the corps. This seems to be done for the sake of convention. Such additions somewhat shade the odor of the finished product by imparting a slight orange blossom or rose note.

1.3.5.3.5.1 Enfleurage and Defleurage

Every enfleurage building is equipped with thousands of so-called chassis, which serve as vehicles for holding the fat corps during the process. A chassis consists of a rectangular wooden frame. The frame holds a glass plate upon both sides of which the fat corps is applied with a spatula at the beginning of the enfleurage process. When piled one above the other, the chassis form airtight compartments, with a layer of fat on the upper and lower side of each glass plate.

Every morning during the harvest the freshly picked flowers arrive, and after being cleaned of impurities, such as leaves and stalks, are strewn by hand on top of the fat layer of each glass plate. Blossoms wet from dew or rain must never be employed, as any trace of moisture will turn the corps rancid. The chassis are then piled up and left in the cellars for 24 h or longer, depending upon the type of flowers. The latter rest in direct contact with one fat layer (the lower one), which acts as a direct solvent whereas the other fat layer (beneath the glass plate of the chassis above) absorbs only the volatile perfume given off by the flowers.

After 24 h, the flowers have emitted most of their oil and start to wither, developing an objectionable odor. They must then be removed from the corps, which process, despite all efforts to introduce labor-saving devices, is still done by hand. Careful removal of the flower (defleurage) is almost more important than charging the corps on the chassis with fresh flowers (enfleurage) and, therefore, the persons doing this work must be experienced and skilled. Most of the exhausted flowers will fall from the fat layer on the chassis glass plate when the chassis is struck lightly against the working table, but since it is necessary to remove every single flower and every particle of the flower, tweezers are used for this delicate operation. Immediately following defleurage, that is, every 24 h, the chassis are recharged with fresh flowers. For this purpose the chassis are turned over and the fat layer, which in the previous operation formed the top (ceiling) of the small chamber, is now directly charged with flowers. In the case of jasmine, the entire enfleurage process lasts about 70 days: daily the exhausted flowers are removed and the chassis are recharged with fresh ones. At the beginning of, and several times during, the harvest, the fat on the chassis is scratched over with metal combs and tiny furrows are drawn in order change and increase the surface of absorption.

At the end of the harvest, the fat is relatively saturated with flower oil and possesses the typical fragrance. The perfumed fat must then be removed from the glass plates between the chassis. For this purpose, it is scraped off with a spatula and then carefully melted and bulked in closed containers. The final product is called pomade (pomade de jasmine, pomade de tuberous, pomade de violet, etc.). The most highly saturated pomade is pomade no. 36, because the corps on the chassis have been

treated with fresh flowers 36 times during the whole process of enfleurage. At the beginning of the harvest, every chassis is charged with about 360 g fat corps on each side of the glass plate, in other words, with 720 g per chassis. Every kilogram of fat corps should be in contact with about 2.5 kg (preferably with 3.0 kg) of jasmine flowers for the entire period of enfleurage, which lasts from 8 to 10 weeks. The quantities differ somewhat for different flowers. At the end of enfleurage, the fat corps has lost about 10% of its weight because of the various manipulations.

1.3.5.3.5.2 Hot Maceration Process

In this process, the long enfleurage time is reduced by the immersion of petals in molten fat heated at 45°-60° C for 1 to 2 h, depending upon the plant species. After each immersion, the fat is filtered and separated from the petals. After 10 to 20 immersions, the fat is separated from waste flowers and water. Absolute of maceration is then produced from fat containing oil through the process of extraction and concentration under reduced pressure. It is mainly used for highly delicate flowers whose physiological activities are lost rapidly after their harvest, such as lily of valley.

1.3.6 Modern (Non-traditional) Methods of Extraction of Essential Oils

Traditional methods of extraction of essential oils have been discussed and these are the methods most widely used on commercial scale. However, with technological advancement, new techniques have been developed which may not necessarily be widely used for commercial production of essential oils but are considered valuable in certain situations, such as the production of costly essential oils in a natural state without any alteration of their thermosensitive components or the extraction of essential oils for micro-analysis. These techniques are as follows:

- Headspace trapping techniques
  - Static headspace technique
  - Vacuum headspace technique
  - Dynamic headspace technique
- Solid phase micro-extraction (SPME)
- Supercritical fluid extraction (SFE)
- Phytosol (phytol) extraction
- Protoplast technique
- Simultaneous distillation extraction (SDE)
- Microwave distillation
- Controlled instantaneous decomposition (CID)
- Thermomicrodistillation
- Microdistillation
- Molecular spinning band distillation
- Membrane extraction

Some of these techniques are discussed in other chapters. Here, a few important, relevant references are provided.

1.4 Conclusions

Some of the major constraints in sustainable industrial exploitation of medicinal and aromatic plants (MAPs) are due to the fact that the countries of South East Asia have poor agricultural practices for MAPs, unscientific and indiscriminate gathering practices from the wild, poor post-harvest and post-gathering practices leading to poor quality raw material, lack of research for the development of high-yielding varieties of MAPs, poor propagation methods, inefficient processing techniques, poor quality control procedures, lack of research on process and product development, difficulty in marketing, non-availability of trained personnel, lack of facilities and tools to fabricate equipment locally, and finally lack of access to the latest technologies and market information. This calls for co-operation and co-ordination among various institutes and organizations of the region, in order to develop MAPs for sustainable commercial exploitation.

The process of extracting MAPs determines how efficiently we add value to MAP bioresources. In the case of essential oils, the extraction process affects the physical as well as internal composition. External appearance, at times, can result in rejection of the batch even if the analytical results are within acceptable limits. Furthermore, essential oils are evaluated internationally for their olfactory properties by experienced perfumers and these olfactory qualities supersede analytical results. Variations in the chemical constituents of the extracts of medicinal plants may result by using non-standardized procedures of extraction. Efforts should be made to produce batches with quality as consistent as possible (within the narrowest possible range).

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2 Role of Process Simulation in Extraction Technologies for Medicinal and Aromatic Plants

M. Fermeglia

Abstract

This paper illustrates the role of process simulation in the field of extraction technologies for medicinal and aromatic plants. The paper starts with a brief introduction to process simulation fundamentals and the role of process simulation in the industry today. It describes procedures to follow in simulating a process and the benefits of process simulation. In the second part of the paper, phytochemical processes that have been simulated at ICS-UNIDO are listed, followed by two case studies to illustrate the applicability of the methodology proposed: (i) turpentine oil batch distillation and (ii) menthol recovery by crystallization of mentha oil. At the end, recommendations are given advocating the importance of process simulation for developing countries.

2.1 Introduction

Developing countries are rich in medicinal and aromatic plants (MAPs) but, due to difficulty in accessing efficient extraction technologies, value addition to this rich bioresource is difficult. In most cases, and particularly in very poor countries, the technologies used are inappropriate and not economical. The crucial problem is related to the quality of the product: primitive extraction technologies do not guarantee a stable and high-quality product and, in some cases, inappropriate technologies and procedures result in producing contaminated product which has low market value.

In order to assist developing countries to achieve the objective of using rich MAP resource for producing value-added products, dissemination of knowledge of existing extraction technologies and of the latest developments in these technologies is essential.

Commercial process simulation software can be used to predict, on a computer, the real plant and consequently is a useful tool for optimizing the process conditions and enhancing the capacity of managing the phytochemical processes. In particular, process simulation can assist developing and emerging countries in optimizing an advanced process rather than managing a primitive process, which should be substituted by more efficient and standardized procedures. The focus in this case is more related to practical problems such as the quality of the materials and of the water to be used for the extraction. In most cases, developing countries face problems in the type of vessel, quality of water and stability of the product during the processing.

This paper describes the use of process simulation software in the extraction and purification of essential oils at both pilot and industrial scales. Such processes have been developed and are in operation in developing and emerging countries. The goal of this paper is to illustrate a procedure for obtaining better knowledge of the extraction process and, therefore, for optimizing the process in terms of energy use, raw material consumption and environmental impact.

2.2 Process Simulation Goals and Definitions

Simulation is the act of representing some aspects of the real world by numbers or symbols which may be manipulated to facilitate their study. A process simulator is an engineering tool that performs several tasks, including automated calculations, material and energy balances, physical property estimations, design or rating calculations, and process optimization. A process simulator is not a process engineer, and a process engineer is always needed to analyze the problem and the output of a process simulator. A process simulator solves material and energy balances by means of computer code.

In principle, a process simulator for the study of a chemical process goes through the procedure outlined in Figure 1. One starts from the definition of the problem (problem analysis) and then develops the process model, i.e. the system of equations (algebraic or differential). Furthermore, one collects the necessary additional data and solves the model with a suitable method, depending on the system of equations. Finally, the process engineer analyzes the results and perhaps starts over again to develop a more realistic model.

The same picture applies to steady-state simulation, dynamic simulation and optimization problems; only the process model and the method of solution change. Solution of the system of material and energy balance equations is not an easy task because it must be solved considering many components, complex thermophysical models for phase equilibrium calculations, a large number of subsystems (equipment), rather complex equipment (e.g. distillation columns), recycle streams and control loops.

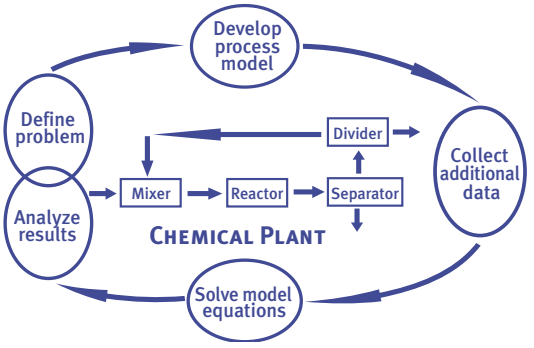


Figure 1: Fundamental steps in running process simulation software

A typical process simulation scheme, with the most important elements and their connections, is shown in Figure 2. Clearly, a process simulator includes cost estimation as well as economic evaluation. The importance of the database is shown in the figure as a necessary source of information for different objects in the structure.

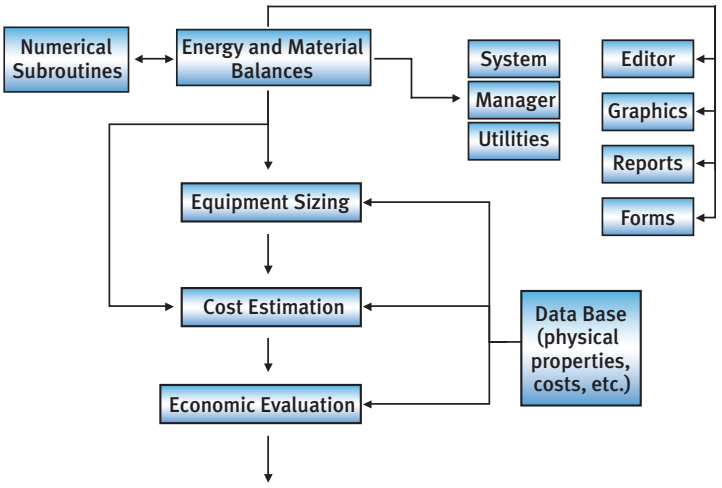


Figure 2: General scheme of a steady-state process simulator

The following approaches are available in process simulation:

- Steady state simulation, which considers a snapshot in time of the process.
- Dynamic simulation, which considers the evolution in the time domain of the equations describing the process.
- Integrated steady-state–dynamic simulation, which combines the previous two approaches.

These three approaches may be used in different ways when dealing with process simulation. One possibility is to perform *process analysis*, in which an existing process is studied and alternative conditions as well as dynamic behavior are investigated in the appraisal of effectiveness of the design. The second is *process synthesis*, in which different process configurations are compared in order to identify the best choice of units and the connections between them. The third possibility is *process design and simulation*, which aims at establishing the optimal operating conditions of a given process.

In all these possibilities, impact on industry is pervasive rather than restricted to a single moment in the development of the process. Process simulation has strongly affected the way engineering knowledge is used in processes. The traditional way of using process simulation was mainly focused on designing flowsheets and on defining critical equipment parameters, such as distillation column stages and column diameter. Today,

engineers are oriented to a more comprehensive use of process simulation in the entire "life" of the plant, as in designing control strategies, optimizing process parameters, studying process time evolution for understanding startup and shutdown procedures, performing risk analysis, training operators, and defining procedures to reduce non-steady-state operations.

The main benefits gained from such a comprehensive use of process simulation are the partial or total replacement of pilot plants (reduction of the number of runs and planning), the reduction of time to market for the development of new processes, and the fast screening of process alternatives to select the best solution in terms of economics, environmental aspects, energy consumption and flexibility. Due to the high complexity of chemical processes, to get these benefits one must critically simplify the process and apply process simulation techniques in the entire life cycle of a process.

Steady-state simulators are considered the core products of process simulation and are used for designing processes, evaluating process changes and analyzing what-if scenarios. Steady-state simulation is normally performed before all other kinds of simulation: dynamic simulation, process synthesis with pinch technology, detailed equipment design, off-line and on-line equation-based optimization, and application technologies for vertical markets (e.g. polymers).

The problems involved in a process simulation run are the definition of an accurate thermodynamic model (equations of state or excess Gibbs energy model), the necessity of defining dummy operations (not always easy to identify), and the tear streams identification to achieve rapid convergence.

The logical procedure for performing a simulation is as follows. First, one defines all the components to be used in the simulation, including conventional and non-conventional components. Next, the most important step in the definition of the simulation is the selection of physicochemical properties to be used in the calculations. Having done this, one proceeds to flowsheet connectivity and to the definition of the feed conditions. The next step is the unit operation internal definitions. At this stage, it is possible to run a base case and check that the system is converging.

Process specification definition, control parameters, and equipment hold-up definition are added later to refine the simulation and to obtain results similar to the reality. Various different results are obtained from a simulation run. The most important are the validation of phase equilibria models for the real system to be used in similar conditions, the verification of the process operating conditions, information on intermediate streams and enthalpy balance, verification of plant specifications, and influence of the operative parameters on the process specifications.

All this information is useful for de-bottlenecking the entire process, or part of it, for identifying process control strategies, and for tuning the instrumentation. This is important since it allows one to verify the behavior of security systems when process conditions are varied.

As far as dynamic simulation is concerned, applications can be found in continuous processes, concurrent process and control design, evaluation of alternative control strategies, troubleshooting process operability, and verification of process safety.

The most important benefits of dynamic modeling are: the capital avoidance and lower operating costs through better engineering decisions; the throughput, product quality, safety and environmental improvements through improved process understanding; and the increased productivity through enhanced integration of engineering work processes.

2.3 Biotechnological and Phytochemical Processes Studied at ICS-UNIDO

This section summarizes ongoing work involving the simulation of important biotechnological and phytochemical processes.

2.3.1 Brief of Biotechnological Processes

2.3.1.1 Alcohol Production from Biomass

The goal of the process is the steady-state simulation of the production of ethanol from biomass fermentation. The process is divided into two parts: (i) biomass fermentation that produces a mixture of ethanol, water and other components, and (ii) separation using a distillation column that concentrates the ethanol. The modelled reactors are continuous stirred tank reactors in series or parallel.

2.3.1.2 Soybean Oil Refining and Treatment of the Waste

The goal of this work is to simulate the soybean oil refining process. This is a complex biotechnological process that involves many reactions and the treatment of solids. The process is divided into three sections: (i) degumming and neutralization, (ii) bleaching, and (iii) deodorization. The main objective of the simulation is to reduce the consumption of steam by applying the pinch technology concept. Steam is consumed in the heat exchangers to heat the oil, in the bleacher equipment, and in the deodorizer. The difficulty of this simulation lies in the large number of undefined components that must be characterized in order to obtain a reliable simulation. In addition, an alternative way of reducing the free acids, by using extraction

with ethanol, is being examined. Key features are the achievements of the required product quality by minimizing the capital and operating costs.

2.3.1.3 Production of Synthetic Hydrocarbon Fuels from Biomass

Starting from natural gas, coal, or wood, a syngas of suitable composition can be produced by gasification. Then, water-gas shift reaction, Fischer-tropsch synthesis and hydrocracking can be applied to the syngas to obtain a mixture of liquid hydrocarbons that can be used as synthetic fuel. This is a complex process that can be simplified by neglecting the kinetics of the chemical reactions involved. The purpose of this project is to quantify the mass and energy consumption and the emission of carbon dioxide. Key features are: the selection of the thermodynamic model to achieve a realistic simulation; the heat integration among different process sections to minimize the environmental impact during recovery and recycling of the entrainer; and the reduction of energy duties.

2.3.1.4 Production of Bio-ethanol from Corn

By fermentation of sugar cane, corn or wheat, ethanol (bio-ethanol) can be easily produced. This process includes the steps of liquefaction, cooking, fermentation, distillation, dehydration, evaporation and drying of the solid by-product. By a careful simulation of the operations involved, the needs for water and energy can be minimized, and the use of fossil fuels can be avoided. Key features are the energy balance starting from the content in the feedstock biomass, and the water saving.

A second problem can be addressed by accurately simulating the distillation and dehydration aspects, which have the highest energy demand of this process. Key feature is the use of pressure as an operating variable.

2.3.2 Brief of Phytochemical Processes

2.3.2.1 Citral Recovery by Distillation of Lemon Peel Oil

The goal of this process is the production of citral. Lemon peel oil is fractioned by a traditional method of separation to get an oxygenated substance (citral). A simulation model of the distillation helps identify the optimal operating conditions. The objective of this simulation is to separate the oxygenated compounds from terpenes.

2.3.2.2 Menthol Recovery by Crystallization of Mentha Oil

Mentha oil contains menthol, a commercially important product. Menthol is separated from the other components on the basis of differences in melting temperatures. Crystallization from solution is an industrially important unit operation due to its ability to provide high purity separations.

The crystal growth and nucleation kinetic parameters must be determined experimentally before systematically designing a crystallizer and computing optimal operations and control procedures.

2.3.2.3 Carvone Recovery from Spearmint Oil

Spearmint oil contains the major component carvone that must be separated from other components. The separation of carvone is done by continuous distillation and the process is optimized after identification of the relevant parameters worked out by sensitivity analysis. The objective of this simulation is to obtain carvone at 95% purity or more.

2.3.2.4 Peppermint Oil Extraction by Steam Distillation

For the steam distillation of peppermint, the peppermint leaves are placed at the bottom of a distillation flask and steam is percolated through. The peppermint oil evaporates, and the emerging mixture of vaporized water and oil moves through a coil, usually cooled with running water, where the steam condenses. The mixture of condensed water and essential oil is collected and separated by decantation or, in rare cases, by centrifugation.

2.3.2.5 Multiple-effect Evaporation of Milk Serum

Evaporation is a widely used operation for the recovery of valuable products from dilute aqueous mixtures, such as milk serum. In this case, a four-effect process helps minimize the energy consumption and makes this process economically attractive. Key features are the effects of both pressure and heat transfer coefficients on the overall performance of the apparatus.

2.3.3 Case Study: Turpentine Oil Batch Distillation

Turpentine oil obtained from species of *Pinus* (family Pinaceae) is mainly used in paint and soap manufacturing industries, and in the pharmaceutical industry its use is limited to balms and oil bases. Semi-fluid mixtures of resins remain dissolved in the volatile oil, thus it is produced by fractional distillation. The objectives of the process simulation are to: (i) develop the process simulation base case, (ii) understand how to obtain complete fractionation of the oil, (iii) optimize the composition of pinene, carene and longifolene in the product streams, and (iii) optimize the time and energy consumption of the process.

Figure 3a shows the fractional composition profiles of pinene, carene and longifolene versus time obtained in the top of the distillation column. Figure 3b shows the instantaneous energy consumption of a constant reflux operation.

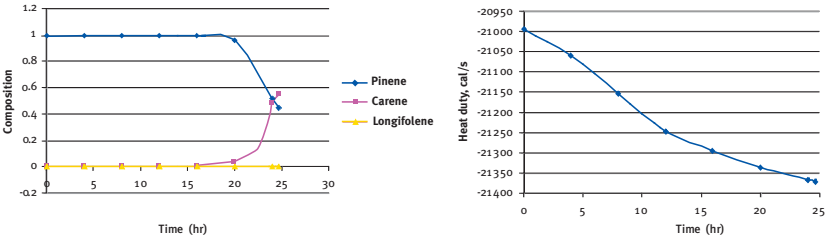


Figure 3: a) Fractional composition versus time, and b) Energy consumption versus time for the base case

Figure 4 shows the same process in which the reflux ratio is varied in order to obtain a constant composition at the top of the column. It is interesting to note that the distillation time as well as energy consumption are greatly reduced.

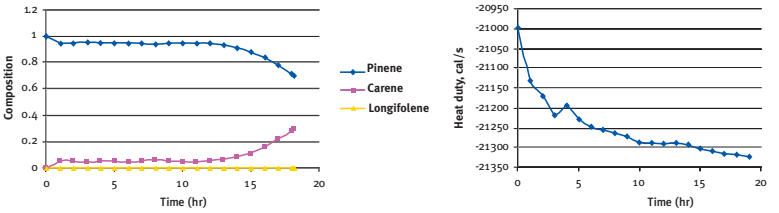


Figure 4: a) Fractional composition versus time, and b) Energy consumption versus time for the constant composition case

Figure 5 shows that a total separation of the oil constituents is achievable.

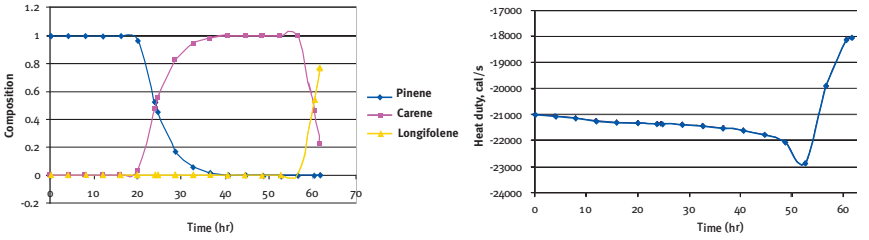


Figure 5: a) Fractional composition versus time, and b) Energy consumption versus time for the total fractionation case

These simulations show that, in the base case with a reflux ratio of 15 and a high consumption of energy (Figure 4), the distillate accumulator collects a high percentage of pinene (93%). If a PID controller is introduced to maintain the concentration constant, the composition of pinene is around 90% and it takes only 12 h and a reflux ratio of 5 to achieve the desired value, thus saving time and energy. In the case of complete fractionation, we can collect 93% of pinene, 88% of carene and 5.7% of dump products at the end of the process.

2.3.4 Case Study: Menthol Recovery by Crystallization of Mentha Oil

Crystallization from solution is an industrially important operation due to its ability to provide high-purity separations. The menthol crystallization process using menthe oil is rather simple, and consists of a cascade crystallization as shown in Figure 6.

The objective of the simulation is to optimize the menthol crystallization process. The oil, composed of 75% menthol and also containing menthyl acetate, limonene and menthone (Table 1), is fed into the first crystallizer where the temperature is 35° F. The menthol crystals produced here are separated by decantation. The decanted liquid is passed to the second crystallizer and the crystals obtained in this second stage are also separated from the liquid by decantation. Thus, the assumptions made are: (i) limonene is present in all the fractions, and (ii) the separation of solid material from the liquid portion is complete. Furthermore, the thermophysical properties of menthone and menthyl acetate are included in the software's database. The feed stream conditions are: temperature, 80° F; pressure, 1 atm; and flow rate, 50 lb · mol/h.

Table 1: Major constituents of mentha oil

Constituent	Concentration
Menthol	75%
Menthyl acetate	11%
Limonene	8%
Menthone	6%

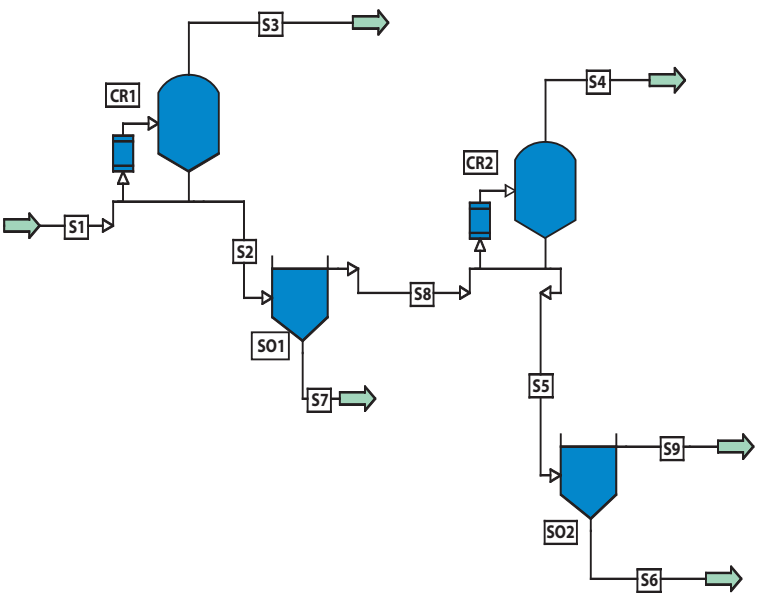
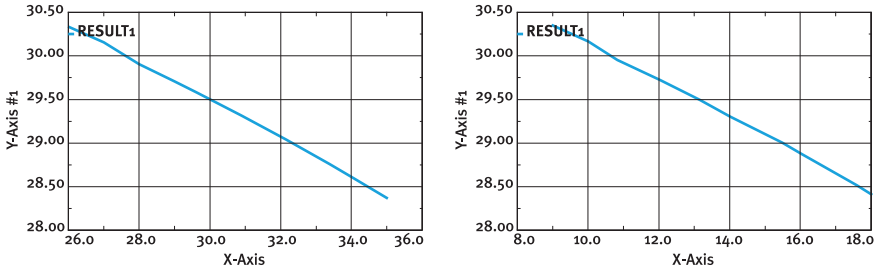


Figure 6: Flowsheet for the menthol crystallization process



a *b*

Figure 7: Variation of menthol flow rate (Y axis, lb mol/hr) as product of both crystallizers (S6 + S7) versus T (X axis, °F). a) Crystallizer 1. b) Crystallizer 2

Figures 7a and b show on the Y axes the total flow rate (lb mol/hr) of menthol crystal produced in both crystallizers (the combination of S6+S7 of Figure 6). It is evident from the sensitivity analysis that the temperature of the crystallizers has an effect on the total amount of pure product obtained, and consequently on the product yield. In fact, the base case (Table 2) reports a figure of 28.469 lb mol/hr and this amount can be raised linearly if the temperature of the crystallizers is lowered. The sensitivity analysis helps the engineer to select the right temperatures of the crystallizers for a given production.

The material and energy balance of the menthol extraction plant for the base case is listed in Table 2. This is an example of the simulator's output and these values may change if the process conditions are changed. In the first crystallizer, the menthol produced is equal to 5.633 lb mol/hr (purity one) and in the second crystallizer the amount is 22.836 lb mol/hr (purity one). If we compare the total amount of menthol produced by the two crystallizers (22.836 + 5.633 = 28.469 lb mol/hr) with the total amount of menthol fed to the process (37.5 lb mol/hr), we obtain a recovery (amount of pure menthol produced/total amount of menthol fed to the process) of 75.91%, which is rather satisfactory. Table 2 also reports the temperature, pressure, total flow and composition of each single stream considered in the process.

Table 2: Material and energy balance for the menthol crystallization process. The stream names refer to Figure 6

Stream name		**S1**	**S2**	**S5**	**S6**	**S7**	**S8**	**S9**
Phase		**Liquid**	**S/L**	**S/L**	**Solid**	**Solid**	**Liquid**	**Liquid**
Temperature	F	80	35	18	18	35	35	18
Pressure	PSIA	14.696	14.696	14.696	14.696	14.696	14.696	14.696
Molecular weight		159.170	159.170	161.608	156.270	156.270	161.608	163.004
Total	lb mol/hr	50.000	50.000	27.164	5.633	22.836	27.164	21.531
Component mole fractions								
D-limene		0.0800	0.0800	0.1472			0.1473	0.1858
Menthol		0.7500	0.7500	0.5399	1.000	1.0000	0. 5398	0.4195
Menthyl acetate		0.1100	0.1100	0.2025			0.2025	0.2554
Menthone		0.0600	0.0600	0.1104			0.1104	0.1393

2.4 Conclusions

There are two important benefits of the application of process simulation to phytochemical processes of industrial interest.

The first is to improve process knowledge. This is achieved by verifying "in silico" the operating conditions and the estimates of data for intermediate streams, which are difficult to measure. Process knowledge also includes: (i) enthalpy balance information, (ii) verification of plant specifications, (iii) influence of operative parameters on process specifications, (iv) validation of phase equilibrium models for the real system to be used in similar conditions, and finally (v) process de-bottlenecking for each section.

The second important benefit is process optimization, in terms of: (i) consumption of energy and raw materials, (ii) identification of process control strategies, and (iii) clarification of security system behavior when process conditions are varied.

Running process simulation software requires: (i) availability of thermodynamic properties for all components involved, (ii) definition of an accurate thermodynamic model (equations of state or excess Gibbs energy model) for binary and multi-component mixtures, (iii) availability of all necessary interaction parameters, (iv) availability of all necessary unit operation modules, and (v) identification of tear streams to achieve rapid convergence in case of recycles. Furthermore, sometimes it is necessary to define and develop user models and user thermodynamic models.

It is necessary to stress some important principles. First, the program is an aid in making calculations and decisions: the process engineer must ensure that it is "fit for purpose" and is responsible for the results generated and for any use which is made of the results. Second, it is the professional, ethical and legal responsibility of the process engineer to take care and to exercise good judgment. Process simulation is, in essence, a program.

Nonetheless, process simulation is important since it: (i) has high accuracy, (ii) allows one to focus on the interpretation of the results rather than on the methods for obtaining the results, (iii) allows a global vision of the process by assembling theories and models, (iv) is essential in the design of new and existing processes, (v) is essential in the analysis of existing plants in terms of environmental impact, and (vi) is a simple tool for treating real cases.

Process simulation is a well established tool in the chemical industry and has been used for a decade in the petrochemical industry. Process simulation is now applicable in many different fields besides the

petrochemical and fine chemical industries, and is particularly interesting for biotechnological and phytochemical processes.

In summary, process simulation may play an important role in the optimization of phytochemical processes and, therefore, application of process simulation can assist in the development of advanced processes. This paper showed that it is possible to achieve energy reduction and maximization of product yield.

Process simulation perhaps is not a good tool for countries that are using primitive techniques, since they should aim at reaching a stable quality of the product rather than an optimization of energy consumption and environmental issues. Moreover, they should focus on practical problems such as the quality of the materials and the water to be used for extraction.

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3 Maceration, Percolation and Infusion Techniques for the Extraction of Medicinal and Aromatic Plants

J. Singh

Abstract

Techniques of maceration, percolation and infusion have been traditionally used for making galenicals and tinctures from medicinal and aromatic plants (MAPs). This article describes the underlying principals and mechanisms of these extraction techniques, and discusses the various modifications made for the small- and large-scale extraction of MAPs, the factors affecting the extraction process, and the quality of the extracts produced.

3.1 Introduction

Before the nineteenth century, there was no real progress in methods of extraction of plant materials for industrial use. Nonetheless, the various classes of preparations involving simple expression and extraction techniques were in vogue for a long time for the preparation of medicines used in traditional medicine and in complimentary and alternative medicine, practiced throughout the world. The techniques available were limited to expression, aqueous extraction and evaporation; later on, the use of extraction processes was extended by using alcohol as a solvent. Such techniques were highly successful in the phytochemical field and, consequently, single pure molecules were isolated for industrial and medicinal uses. After the nineteenth century, rapid progress was made in extraction processes which led to the isolation and characterization of many groups of plant metabolites of therapeutic importance, including both single chemical constituents as well as standardized extracts of crude drugs. In manufacturing various classes of medicinal plant preparations, such as decoctions, infusions, fluid extracts, tinctures, semisolid extracts (pilular) and powdered extracts, popularly known as galenicals, both simple traditional methods and advanced technologies are used, conforming to the official procedures and specifications as laid down in various pharmacopoeias and codices of the world.

Maceration, percolation and infusion are the general techniques used for the extraction of medicinal plants and are mostly applied for galenical preparations. The sole purpose of such basic extraction procedures is to obtain the therapeutically desirable portion and eliminate the inert material by treatment with a selective solvent known as menstruum. These techniques also play a decisive role in the qualitative and quantitative evaluation of the extracts. The standardized extracts thus obtained are further processed for inclusion in other solid and semisolid herbal dosage

forms. These extracts are also used as sources of therapeutically active chemical constituents for various dosage forms of modern medicines.

Historically, galenical preparations were much more extensively used than they are at the present time. Nonetheless, due to resurgence in interest of herbal drugs throughout the world, these extraction procedures are still relevant and are mentioned in official and unofficial monographs about drug preparations. The preparations involving these procedures are primarily intended for extemporaneous dispensing and must be freshly prepared, due to the fact that they rapidly produce a deposit because of coagulation of inert colloidal material and readily support microbial growth due to absence of preservatives.

This article describes the principal methods of extraction by maceration, percolation and infusion as well as the modifications in these procedures for small-scale, official and large-scale extraction. In addition, the paper discusses the choice of extraction method, quality assurance, and factors affecting the extraction procedures.

3.2 General Principles and Mechanisms Involved in Crude Drug Extraction by Maceration, Percolation and Infusion

The general principles and mechanisms involved in maceration, percolation and infusion for the extraction of the crude drugs are same as to those for the extraction of soluble constituents from solid materials using solvent, which is generally referred to as leaching. The processes of leaching may involve simple physical solution or dissolution. The extraction procedures are affected by various factors, namely the rate of transport of solvent into the mass, the rate of solubilization of the soluble constituents by the solvent, the rate of transport of solution out of the insoluble material. The extraction of crude drugs is mostly favored by increasing the surface area of the material to be extracted and decreasing the radial distances traversed between the solids (crude drug particle). Mass transfer theory states that the maximum surface area is obtained by size reductions which entail reduction of material into individual cells. However, this is not possible or desirable in many cases of vegetable material. It has been demonstrated that even 200 mesh particles contain hundred of unbroken cells with intact cell wall. Therefore, it is pertinent to carry out extraction with unbroken cells to obtain an extract with a high degree of purity and to allow enough time for the diffusion of solvent through the cell wall for dissolution of the desired solute (groups of constituents) and for diffusion of the solution (extract) to the surface of the cell wall.

3.3 Factors Affecting the Choice of Extraction Process

The choice of the process to be used for the extraction of a drug depends on a number of factors.

3.3.1 Nature of the Crude Drug

The choice to use maceration or percolation primarily depends upon the nature and characteristics of the crude drugs to be extracted. Therefore, knowledge of the type of organs and tissues of the plant matter is essential for achieving the best result.

3.3.2 Stability of the Crude Drug

Continuous hot extraction procedures should be avoided when constituents of the drug are thermolabile.

3.3.3 Cost of the Crude Drug

When the crude drug is expensive (e.g. ginger), it is desirable to obtain complete extraction. Therefore, from the economic point of view, percolation should be used. For inexpensive drugs, maceration, despite its lower efficiency, is acceptable in view of its lower cost.

3.3.4 Solvent

Selection of the solvent depends on the solubility of the desired components of the material. If the constituents demand a solvent other than a pure boiling solvent or an azeotrope, continuous extraction should be used.

3.3.5 Concentration of the Product

Dilute products such as tinctures can be made by maceration or percolation. For semi-concentrated preparations, the more efficient percolation process is used. Concentrated preparations, such as liquid or dry extracts, are made by percolation.

3.3.6 Recovery of Solvent

Solvent is preferably recovered under reduced pressure to save thermolabile constituents.

3.4 Quality Assurance: the Extraction Process and Solvent

The type of extraction procedure also plays a decisive role in determining the qualitative and quantitative composition of the extract. Some important points regarding the quality of the extracts need to be considered:

i) The more exhaustive the extraction, the better is the yield of the constituents from the herbal drugs.
ii) If maceration is facilitated by stirring and by use of comminuted material, the additional stirring and shearing forces may lead to better extraction.
iii) Other factors determining the quality of the extracts are extraction time, temperature and solvent volume.
iv) Some drugs (e.g. Hypericum spp.) are extracted very slowly so that exhaustive extraction can only be achieved by percolation or multistage motion extraction. In many cases, the transfer of quality-relevant constituents from the herbal drugs to the extract (i.e. extraction rate) can be considerably improved by raising the temperature. Hypericin, pseudohypericin and biapigenin are extracted better at higher temperature and with longer extraction times.
v) The quality of the extracts and the spectrum of constituents obtained by maceration or digestion (i.e. maceration at higher temperature) are also influenced by the ratio of herbal drug to solvent. The quantity of extracted matter increases with the volume of extraction solvent. For example, maceration of Salvia officinalis flowers achieves almost exhaustive extraction and thus the full spectrum of constituents obtained with percolation can be achieved with a drug:solvent ratio of 1:20.
vi) The composition of an herbal extract depends on the type, concentration and elution strength of the solvent. The spectrum of constituents may vary considerably depending on the hydrophilic or lipophilic nature of the solvent.

3.5 Maceration Processes (Steady-state Extraction)

3.5.1 General Procedure

The general process of maceration on a small scale consists of placing the suitably crushed plant material, or a moderately coarse powder made from it, in a closed vessel and adding the selected solvent called menstruum. The system is allowed to stand for seven days, with occasional shaking. The liquid is then strained off and the solid residue, called marc,

is pressed to recover as much occluded solution as possible. The strained and expressed liquid thus obtained is mixed and clarified by filtration. Plant material in fine powder form is never used, as it makes subsequent clarification of the extract difficult. In the case of vegetable drugs, sufficient time is allowed for the menstruum to diffuse through the cell wall to solubilize the constituents within the cells and for the resulting solution to diffuse out. As the system is static, except for occasional shaking, the process of extraction works by molecular diffusion, which is very slow. Occasional shaking assists diffusion and also ensures dispersal of the concentrated solution accumulating around the surface of the particles, thereby bringing fresh menstruum to the particle surface for further extraction. A closed vessel is used to prevent evaporation of the menstruum during the extraction period and thus avoids batch to batch variation.

At the end of the maceration process, when equilibrium has been reached, the solution is filtered through a cloth; the marc may be strained through a special press. The concentrations of active constituents in the strained and expressed liquids, sometimes called *miscella*, are the same and hence they can be combined. The expressed liquid may be cloudy with colloidal and small particles, and sufficient time (perhaps several weeks) is necessary for coagulation and settling. The settled matter is filtered through a filter press or any other suitable equipment.

3.5.2 Maceration Process for Organized and Unorganized Crude Drugs

Organized drugs have a defined cellular structure whereas unorganized drugs are non-cellular. Bark and roots are examples of organized crude drugs, while gum and resin are unorganized crude drugs. The processes of maceration for organized and unorganized drugs are slightly different, as shown in Table 1.

Table 1: Four differentiating steps of the maceration process, for organized and unorganized crude drugs

Organized drugs	Unorganized drugs
(i) Drug + entire volume of menstruum	(i) Drug + four-fifths of menstruum (in most cases)
(ii) Shake occasionally for 7 days	(ii) Shake occasionally on days 2 to 7, as specified
(iii) Strain liquid, press the marc	(iii) Decant the liquid. Marc is not pressed
(iv) Mix the liquids, clarify by subsidence for filtration. Filtrate is not adjusted for volume	(iv) Filter the liquid and add remaining menstruum through the filter

During maceration of organized drugs, the marc is pressed because a considerable proportion of liquid adheres to it and cannot otherwise be separated. Moreover, the volume is not adjusted because a variable amount of liquid containing soluble matter is left in the marc. If the volume is adjusted, a weak product will result. Omitting adjustment, the volume of liquid expressed influences the product yield and the percentage of soluble matter, regardless of the efficiency with which the marc is pressed in a hand press, screw press or hydraulic press; the strength of the product is not affected. Preparations made by this processes include vinegar of squill (British Pharmaceutical Codex, BPC), oxymel of squill (BPC), tincture of orange (Indian Pharmacopoeia, IP), tincture of capsicum (BPC), compound tincture of gentian, tincture of lemon, and tincture of squill (BPC).

In maceration of unorganized drugs, the marc is not pressed because the desirable material is mostly dissolved and the remaining marc is gummy and slimy. Thus, it is neither practicable nor necessary to press it. Moreover, the volume is adjusted because the clear upper layer is easily separated by filtration from the lower layer. The solution contains practically all the soluble matter of the drug; the small amount adherent to the gummy matter is recovered when the marc is washed by menstruum in the filter. Therefore, adjustment of volume leads to uniformity. Preparations made by this process include compound tincture of benzoin, tincture of myrrh (BPC), and tincture of tolu (BPC).

3.5.3 Modifications to the General Processes of Maceration

Repeated maceration may be more efficient than a single maceration process, as described earlier, because an appreciable amount of active principle may be left behind in the first pressing of the marc. Double maceration is used when the active constituents are particularly valuable and also when the concentrated infusions contain volatile oil. Where the marc cannot be pressed, a process of triple maceration is sometimes employed. The total volume of solvent used is, however, large and the second and third macerates are usually mixed and evaporated before being added to the first macerate.

3.5.4 Large-scale Extraction Procedures

For large-scale, industrial extraction, certain modifications are warranted. When the extraction vessel contains a small amount of solvent (500-1000 ml), occasional shaking is no problem. But, for industrial work where a large amount of solvent and huge vessels are involved, shaking the vessels is difficult. Obviously, there are alternative methods of agitation that are just as effective and much simpler to put into practice. In addition, economics become increasingly important and one of the most important objectives is to improve the efficiency of extraction so that less solvent is needed

and evaporation requirements for concentrated products are reduced. Reducing the cost of evaporation has the further advantage of minimizing the heat damage to thermolabile constituents. Some of the modified maceration procedures used for large-scale extraction are described in the next paragraphs.

3.5.4.1 Circulatory Extraction

The efficiency of extraction in a maceration process can be improved by arranging the solvent to be continuously circulated through the drug, as indicated in the Figure 1. Solvent is pumped from the bottom of the vessel (through an outlet) and is distributed by spray nozzles over the surface of the drug. The movement of the solvent reduces boundary layers and the uniform distribution minimizes local concentration in a shorter time.

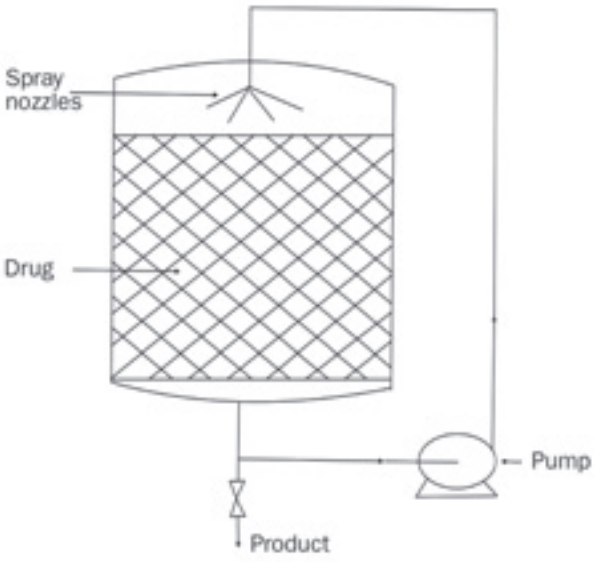


Figure 1: Circulatory extraction

3.5.4.2 Multistage Extraction

In the normal maceration process, extraction is incomplete, since mass transfer ceases when equilibrium is reached. This problem can be overcome using a multistage process. The equipment needed for this method is a vessel for the crude drug, a circulating pump, spray distributors and a number of tanks to receive the extracted solution. The extractor and tanks are connected with piping and valves as shown in Figure 2, so that any of the tanks may be connected to the extractor for transfer of the solution. Each batch of drug is treated several times with solvent and, once a cycle is in process, the receivers contain solution with the strongest in receiver 1 and the weakest in receiver 3.

3.5.4.2.1 Advantages

The crude drug is extracted as many times as there are receivers (in Figure 2 there are three receivers). If more extraction stages are required, it is only necessary to have more receivers. The last treatment of the drug – before it is discharged – is with fresh solvent, giving maximum extraction. The solution is in contact with fresh drug before removal for evaporation, giving the highest possible concentration.

3.5.4.2.2 Procedure

Fill the extractor with crude drug, add solvent and circulate. Run off to receiver 1. Refill the extractor with solvent and circulate. Run off to receiver 2. Refill the extractor with solvent and circulate. Run off to receiver 3. Remove drug from the extractor and recharge. Return solution from receiver 1 to the extractor. Remove for evaporation. Return solution from receiver 2 to the extractor and circulate. Run off to receiver 1. Return solution from receiver 3 to the extractor and circulate. Run off to receiver 2. Add fresh solvent to the extractor and circulate. Run off to receiver 3. Remove drug from the extractor and recharge. Repeat cycle.

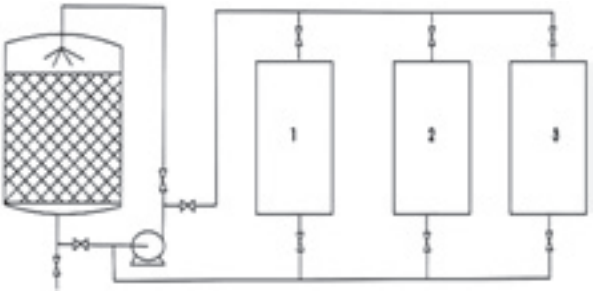


Figure 2: Multistage extraction

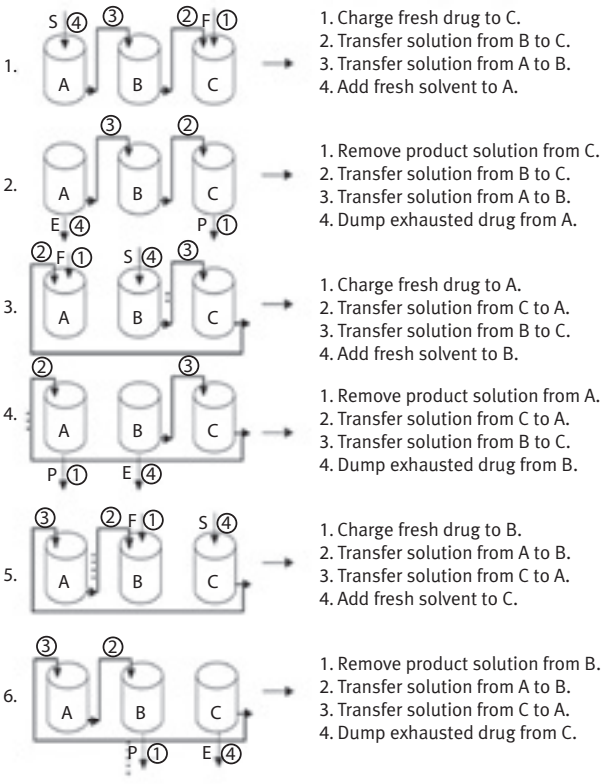
3.5.4.2.3 Extraction Battery

In the normal percolation process, the percolate is a very dilute solution, while the ideal situation is to obtain the maximum concentration possible. Continuous extraction devices of battery type are used when large amounts of a single material are handled. Such devices can be achieved by treating percolation as a multistage process. In an extraction battery process, a series of vessels is used and extraction is semicontinuous.

3.5.4.2.4 Equipment

An extraction battery consists of a number of vessels with interconnecting piping. Vessels are so arranged that solvent can be added to and the product taken from any vessel. These vessels can, therefore, be

made into a series with any of vessels as the first of the series. The use of an extraction battery is illustrated in Figure 3, which shows the simplest arrangement of three vessels.



Arrows indicate transfer of material to or from the vessels. An asterisk(.) is added after each contact stage. Key: F = fresh drug. S = solvent. P = product. E = exhausted drug. A, B, C, extraction vessels. Start with C empty and units A and B containing drug, with A more nearly exhausted.

Figure 3: Extraction battery

3.6 Percolation (Exhaustive Extraction)

3.6.1 General Process of Percolation

In this process, an organized vegetable drug, in a suitably powdered form, is packed in a percolator and the solvent is allowed to percolate through it. Although some materials (e.g. ginger) may be packed directly into the percolator in a dry state, this may cause difficulties with other drugs. With the addition of solvent, the dry material swells and this swelling increases with increasing aqueous nature of the solvent. This swelling reduces or blocks the flow of the solvent, thus seriously affecting the extraction process. Fur-

thermore, if the dry powder is packed, fine particles may be washed down the column and settle at the lower levels, reducing the porosity drastically, blocking the column and making the column nonuniform. The finer particles may even be washed out of the column. These difficulties can be prevented by a preliminary uniform moistening of the raw material with the menstruum for a period of 4 h in a separate closed vessel; this process is called imbibition. During this period, the crude drug is allowed to swell to the maximum extent. Hence, when aqueous solvents are used for extraction, more menstruum is needed during imbibition. Also, the occluded air in the drug powder is replaced by the vapor of the solvent, thereby enabling the material to be more evenly packed and allowing the menstruum to flow more uniformly. Uneven packing permits more solvent to pass through channels offering less resistance to the flow of the solvent, thus resulting in inefficient extraction. After imbibition, the drug is packed evenly into the percolator. The imbibed drug is packed over a loose plug of tow or other suitable material previously moistened with the solvent. Even packing can be achieved by introducing the material layer by layer and pressing it with a suitable implement to give even compression; the pressure exerted on the material depends on the nature of the material and its permeability. After packing is over, a piece of filter paper is placed on the surface followed by a layer of clean sand such that the top layer of the drug is not disturbed when solvent is added for extraction. Sufficient menstruum is now poured over the drug slowly and evenly to saturate it, keeping the tap at the bottom open to allow the occluded gases between particles to pass out. Menstruum should never be poured with the tap closed since the occluded air will escape from the top, disturbing the bed. When the menstruum begins to drip through the tap, the tap is closed; sufficient menstruum is added to maintain a small layer above the drug and allowed to stand for 24 h. The layer of menstruum above the surface of the bed prevents drying of the top layer, which may result in the development of cracks on the top surface of the bed. The 24-h maceration period allows the solvent to diffuse through the drug, solubilize the constituents and leach out the soluble material. In this way, the extraction is more efficient than carrying out percolation without the maceration period.

After the maceration, the outlet is opened and the solvent is percolated at a controlled rate with continuous addition of fresh solvent. The volume of percolate collected depends on the nature of the final product. In general, about 75% of the volume of the finished product is collected, the marc is pressed and the expressed liquid is added to the percolate, giving about 80%-90% of the final volume. After assay, the volume is adjusted with calculated quantities of fresh menstruum. If no assay is available, the volume is adjusted after adding the other constituents, if any. In percolation, the expressed liquid is devoid of active constituents as they are already extracted during the percolation period; pressing the marc is only to recover the valuable solvent. This is in contrast with maceration in which the marc is pressed.

3.6.2 Modifications to the General Process of Percolation

In the general process of percolation, particularly in the manufacture of concentrated preparations like liquid extracts, the following problems may arise:

- If the active substances are thermolabile, evaporation of large volumes of dilute percolate may result in partial loss of the active constituents.
- In the case of alcohol-water mixtures, evaporation results in preferential vaporization of alcohol, leaving behind an almost aqueous concentrate. This may not be able to retain the extracted matter in solution and hence the substances may precipitate.

In such cases, the general process of percolation is modified, as described in the next paragraphs.

3.6.2.1 Reserved Percolation

In this case, extraction is done through the general percolation procedure. At the end, the evaporation is done under reduced pressure in equipment like a climbing evaporator to the consistency of a soft extract (semisolid) such that all the water is removed. This is then dissolved in the reserved portion, which is strongly alcoholic and easily dissolves the evaporated portion with any risk of precipitation.

3.6.2.2 Cover and Run Down Method

This is a process that combines the maceration and percolation techniques. This process cannot be used for materials that contain volatile principles or for those which undergo change during the evaporation stage. This procedure is advantageous because industrial methylated spirit may be used for extraction instead of the costly rectified spirit.

The detailed procedure is as follows. After the imbibition stage, the material is packed in a percolator and macerated for a few hours with a suitable diluted industrial methylated spirit. Then, the liquid is run off and the bed is covered with more menstruum. Maceration is done as before and the second volume of the extract is collected. This process is repeated several times with the later weaker extracts used for extraction of a fresh batch of the drug. More concentrated fractions are evaporated under reduced pressure to eliminate the toxic methanol. After the concentrate is assayed for the active principle or for total solids content, it is diluted with water and ethanol to obtain the correct concentration of alcohol and active principle.

3.6.3 Percolators

Different types of percolators are used for small- and large-scale extraction.

3.6.3.1 Small-scale or Laboratory-scale Extraction

The processes for the manufacture of concentrated preparations, maceration and percolations, are involved in extraction followed by the evaporation of solvents. The two operations are combined in a continuous extraction process.

The general procedures and apparatus used for small- or laboratory-scale extraction are described in the following paragraphs.

3.6.3.1.1 Soxhlet Apparatus

On the laboratory scale, the Soxhlet apparatus is used. It consists of a flask, a Soxhlet extractor and a reflux condenser. The raw material is usually placed in a thimble made of filter paper and inserted into the wide central tube of the extractor. Alternatively, the drug, after imbibition with menstruum, may be packed in the extractor taking care that the bottom outlet for the extract is not blocked. Solvent is placed in the flask and brought to its boiling point. Its vapors pass through the larger right hand tube into the upper part of the extractor and then to the condenser where they condense and drop back onto the drug. During this period, the soluble constituents are extracted. When the level of the extract reaches the top of the syphon tube, the entire volume of extract syphons over into the flask. The process is continued until the drug is completely extracted. The extract in the flask is then processed. This procedure is thus a series of short macerations.

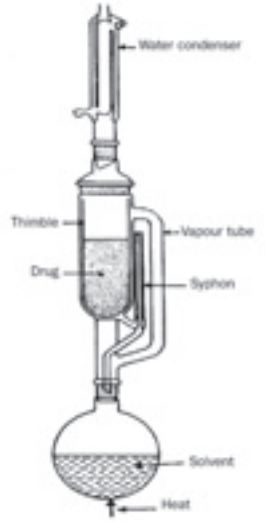


Figure 4: Soxhlet apparatus for hot extraction

3.6.3.1.2 Official Extractor

An official extractor, as described in the official monographs (e.g. IP, British Pharmacopoeia), is illustrated in Figure 5. In this case, the extraction is a continuous percolation procedure. In this apparatus, vapors rise through the extraction chamber passing the drug container; the vapor condenses in the reflux condenser and returns through the drug, taking the soluble constituents to the flask.

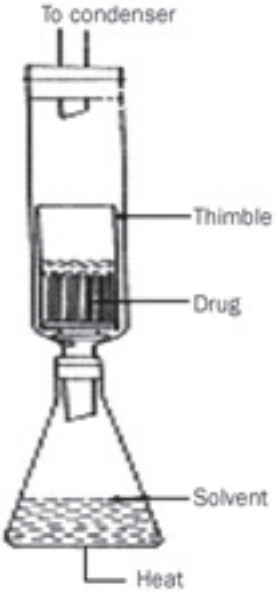


Figure 5: Apparatus for the continuous extraction of drugs

3.6.3.2 Limitations of the Official Extractor

It is not useful when the raw material contains thermolabile active constituents, because the extraction is carried out at an elevated temperature and the extract in the flask is also maintained in the hot condition until the process is complete. It can be used only with pure solvents or with solvent mixtures forming azeotropes. If an ordinary binary mixture is used as the menstruum, the composition of the vapor will be different from the liquid composition.

Similar methods can be used in large-scale production.

A typical industrial set-up for continuous extraction is shown in Figure 6. The principle of operation resembles that of the laboratory equipment.

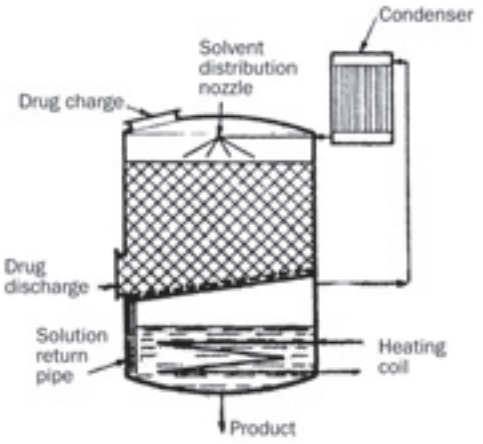


Figure 6: Continuous extraction: large-scale plant

3.6.3.3 Large-scale Extractor

The drug is supported on a perforated metal plate covered with a layer of sacking or straw. The percolator has a removable lid which contains portholes for running the solvent in and for observing the flow of solvent. The outlet from the percolator is fitted with a tap and pipeline. This outlet permits removal of the percolate for subsequent processing or for use as a menstruum in a second percolator in series, resulting in more efficient use of the menstruum by carrying out the extraction in a counter-current manner. On the small scale, copper percolators were originally used but these are now largely replaced with percolators made of glass or stainless steel. A slightly conical percolator is better than a cylindrical one since the sloping sides permit the eventual expansion of the bed and also allow the solvent to permeate the material present near the sides at the bottom, which is a problem with a cylindrical percolator.

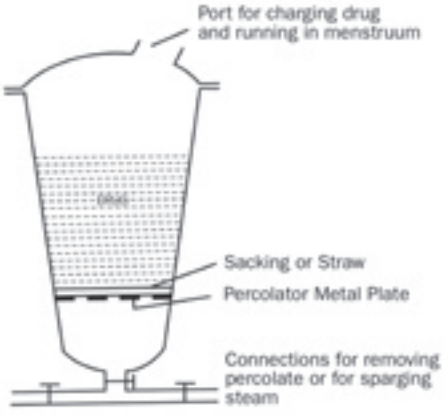


Figure 7: Commercial scale percolator

3.7 Infusion

3.7.1 General Considerations

Infusions are dilute solutions containing the readily soluble constituents of crude drugs. Formerly, fresh infusions, prepared by macerating the drug for a short period in cold or boiling water, were used and diluted to eight volumes but, now, infusions are usually prepared by diluting one volume of a concentrated infusion to ten volumes with water. Concentrated infusions are prepared by a modified percolation or maceration process. After dilution with water, concentrated infusions resemble in potency and aroma the corresponding fresh infusion. Infusions are liable to fungal and bacterial growth, and it is necessary to dispense them within 12 h of their preparation.

3.7.2 General Method for Preparing Fresh Infusions

The coarsely powdered crude drug (50 g) is moistened, in a suitable vessel with a cover, with 50 ml cold water and is allowed to stand for 15 min. Then, 900 ml boiling water is added, and the vessel is covered tightly and allowed to stand for 30 min. The mixture is strained and enough water is passed to make the infusion measure 1000 ml. Some drugs are supplied (accurately weighed) in muslin bags for preparing specific amounts of infusion and as such are used for infusion preparation. If the activity of the infusion is affected by the heat of the boiling water, cold water should be used. As fresh infusions do not keep well, they should be made extemporaneously and in small quantities at the time of use.

3.7.3 Preparation of Concentrated Infusions

The official monographs also recognize certain “concentrated infusions” in which 25% alcohol is added during or after the infusion process and then diluted as per pharmacopoeial (official) requirement. Concentrated infusions are especially prepared in cases in which the active and desirable principles of drug are equally soluble in water and in the menstruum used for both concentrates and infusions.

3.8 Evaporation

One quality-relevant parameter is the evaporation of the eluate from the soft extract. The state of art is cautious vacuum evaporation, in which evaporation temperatures do not exceed 55° C. The temperature in relation to the evaporation time is of special importance for quality of this step, especially if the extract contains volatile or thermolabile constituents.

3.9 Conclusions

The spectrum of constituents obtained by steady-state extraction (simple maceration) differs from that obtained by exhaustive extraction (percolation). With maceration, one can achieve a spectrum of constituents similar to that of percolation. Different extraction procedures may be considered to be equivalent if they respect critical quality parameters and if the analysis of numerous production batches confirms their compliance with standards.

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4 Hydrolytic Maceration, Expression and Cold Fat Extraction

A. K. Singh

Abstract

The incorporation of bioactive ingredients without loss of activity into foods, flavors, pharmaceuticals, pesticides and cosmaceutical products is very important. Extraction of active constituents from raw materials is an important and critical step in maintaining bioactivity. A number of methods are available for extraction, and these are selected in such a way that the activity of the phytoconstituents is retained. This paper discusses the processes of hydrolytic maceration, expression and cold fat extraction.

4.1 Introduction

The extraction of active constituents from plants is one of the most critical steps in the development of natural products for commercial use. The simplest example of extraction may be brewing a cup of coffee, wherein caffeine and tannins are extracted from coffee beans in hot water. All living organisms contain complex mixtures of chemicals, usually held within cellular structural material (protein, lipid, polysaccharides etc.) of which some are desired while others are not. Thus, taking out the desired part from the whole crude drug is referred to as extraction and it is done in solvents where ingredients move from one phase to another.

A number of methods are available for extraction and the choice among them is dictated by the physicochemical properties and stability of the phytoconstituents to be obtained. For the extraction of essential oils, the simplest methods are hydrodistillation and steam distillation while other methods also employed are cold fat extraction, expression, maceration and solvent extraction. Nowadays, more advanced technologies are used, such as supercritical fluid extraction, solid phase micro-extraction and phytonic extraction. The present article deals with extraction by hydrolytic maceration, expression and cold fat extraction.

4.2 Hydrolytic Maceration

The word maceration is derived from the Latin word *maceratus,* which means to soften. In reference to medicinal and aromatic plants, maceration refers to the preparation of a solution by soaking plant material in vegetable oil or water. Maceration methods are based on the immersion of crude drug in bulk solvent, while percolation methods depend on the flow

of solvent through the powdered drug. The rate of extraction depends upon the following:

- The rate of transport of solvent into the mass to be leached.
- The rate of solubilization of soluble constituents into solvent.
- The rate of transport of solute out of the insoluble material and from the surface of the insoluble material into the solution.

In the process of maceration, the powered solid material is placed in a closed vessel (to prevent evaporation) and the chosen solvent (menstruum) is added. It is allowed to stand for a long time (varying from hours to days) with occasional shaking. Sufficient time is allowed for the menstruum to diffuse through the cell wall to solubilize the constituent present in the cells and for the resulting solution to diffuse out. The process takes place only by molecular diffusion. After the desired time, the liquid is strained off; the solid residue (marc) is pressed to recover as much solvent as possible. When the menstruum is water and the period of maceration is long, a small quantity of alcohol may be added to prevent microbial growth.

Cold maceration of crushed grapes is done at room temperature before the onset of fermentation. In this process, the skin and seeds are permitted to soak for one to two days prior to the initiation of fermentation to get more aqueous extraction without the effect of ethanol on grape cells. In certain cases, hydrolysis is done with a suitable agent (enzyme) prior to maceration, e.g. maceration of wine. The quality of wine is judged by its appearance, color, aroma, taste (mouth feel) and flavor. Grape-derived aroma and flavor precursors exist partially as non-volatile, sugar-bound glycosides. Hydrolysis modifies sensory attributes and potentially enhances wine quality. Flavored aglycones potentially affect the wine quality after hydrolysis. Cell wall-degrading enzymes help in the release of grape aroma, when cold maceration prior to fermentation has been carried out.

4.3 Expression Extraction of Essential Oils

Most essential oils are isolated from the respective plant parts by the process of hydrodistillation or steam distillation. A few essential oils such as those present in the citrus fruit peel can be, and in large part are, obtained by pressing, which yields a product of superior quality. The long action of heat with boiling water affects some thermolabile constituents which may decompose due to hydrolysis, polymerization and resinification. Therefore, the essential oil obtained by distillation does not represent the natural oil as it originally occurred in the plant material. In such plant materials, essential oil is extracted by the process of expression or solvent extraction.

Before dealing with the process of expression in citrus oil, it is important to review the structure of a citrus peel. The citrus peel contains numerous oval, balloon-shaped oil sacs and glands or vesicles (0.4-0.6 mm diameter). These are ductless glands which are irregularly distributed in the outer colored (flavedo) portion of the peel of maturing and matured fruit, in the outer mesocarp beneath the epicarp and hypoderm and above the inner mesocarp (albedo), respectively. Albedo is made of cellulose, hemicellulose, lignin, pectin, sugars, glycosides, etc. On maturation, cells of albedo become elongated and branched, with large intracellular spaces which give the ripe peel its spongy texture. The spongy layer plays an important role in the expression of oil, but it easily absorbs the oil ejected from the sacs and causes some mechanical difficulties in oil extraction. The freshness and stage of maturity affect the oil ejection from the peel. The total weight of the peel is about half that of the fruit and the oil content is 0.5%-0.7%. The cells surrounding the oil sac contain salt (colloids) in aqueous solution. In contact with water, the higher osmotic pressure of the cell results in diffusion of water into them, increasing turgor pressure and causing the oil sac to stress from every side. If the spongy tissue is not filled with water, it will absorb the oil as the sacs are broken and hold it with great tenacity. So the pressure exerted yields first aqueous fluid and later oil.

In Sicily, peels are immersed in water for several hours before being subjected to a “sponge” process. A dilute aqueous solution of salt acts as a carrier to prevent loss by spurting. The product of expression is not a simple mixture of oil and water but a thin emulsion which is left to stand; gradually a supernatant layer forms. Filtration through a sponge absorbs the colloidal material and leaves the mixture of oil and water.

4.3.1 Process of Expression

4.3.1.1 Hand Process

In this process, the freshly harvested fruits are cut transversely into two halves. The pulp is removed with a sharp-edged spoon called *rastrello*. The peel is then immersed in water for several hours and finally pressed by hand. The worker holds, with the left hand, either one large or two smaller flat sponges on top of a wooden crossbar and, with the right hand, presses the peel against the upper sponge. Thinner peels can be pressed from inside. The emulsion ejected from the oil sacs is soaked by the sponge, which retains solid matter and absorbs colloidal substances. Squeezing the content of the sponge from time to time, oil is finally decanted and drawn off. This process requires much labor and the yield of this method is 50%-70% of the total oil present in the peel. The quality of oil obtained from hand pressing is near to the quality present in fruit peel. A large number of small units in Sicily and Calabria were formerly responsible for the entire Italian production of lemon and orange oil, but the process is not in use now.

4.3.1.2 Ecuelle Process

This process was common in the south of France. Ecuelle consists of a shallow bowl of copper with a hollow central tube with which it forms a sort of funnel. The bowl is equipped with large brass nails with blunt ends, on which the fruit is rolled by hand with some pressure until the entire surface of fruit yields its oil. The oil and aqueous cell contents drain into the central tube where they are separated by decantation. The yield is only 20% of the total oil present in the peel.

These methods were quite laborious and, with the advance of technology, machines have been invented to do them. Nowadays, rinds are extracted for oil using centrifugal force.

4.3.1.3 Hand Machine

For expression, the peel is placed in a hollow sponge attached with other sponges to a plate actuated by the lever and fixed below with additional sponges to the base. The sponge is fitted with a funnel through which oil and aqueous material pass to the receiving vessel. The part that comes in contact with oil is made of brass or bronze.

4.3.1.4 Sfumatrici and Pelatrici

The machines that treat only the peel after removal of juice and pulp are called *sfumatrici*, while those that process the whole fruit are known as *pelatrici*.

4.3.1.4.1 Special Sfumatrice

This is a specially designed roller type machine in which each peel is bent to expel the maximum quantity of oil. Not much pressure is exerted to expel other contents of the cells. The emulsion is collected and filtered through wool or sponges to yield oil and water. A number of *sfumatrici* have been developed and were in use in many countries.

In expression using *sfumatrici*, two approaches are used. In the first approach (used by Ramino Sfumatrice), only the peel is treated, so halving the fruit, removing the pulp and expressing the peel are the steps involved. In the second approach, the oil is extracted by either puncturing the peel glands or cutting a superficial layer of peel to expose the oil glands; this is followed by washing away the oil with a spray of water.

In whole fruit extraction with a rasping machine, the whole fruit is crushed and oil is subsequently separated from the aqueous phase (juice and cell liquid). As citrus peel oil is a byproduct of the citrus juice industry, both the oil and juice are extracted subsequently, e.g. in a rotatory juice

extractor or Pipkin peel oil press. Here, the fruit is washed automatically, sorted according to size and then cut in two halves. The halved fruit is passed between two cylinders which remove only the juice by gentle pressing without affecting the oil glands on the surface. The residual halved peels are extracted for essential oil. In the Pipkin peel oil press, for example, oil is expressed by two close stainless steel cylinders with capillary grooves running around the circumference. The expressed oil automatically comes out of the grooves and there is no need to spray water..

4.3.1.5 Modern Machines

Nowadays, complete mechanization has been achieved and whole fruit processing machines have been developed. These machines either crush whole fruit and then separate the oil from the aqueous phases by distillation or centrifugation, or they express the oil in such a way that it does not come in contact with the juice during the process. The oil extractor developed by Brown International, California, liberates the essential oil from whole citrus fruit. Oil removal is achieved by lightly puncturing the entire surface of the fruit with over three million sharp stainless steel points configured in the form of rotating rolls. An adjustable speed differential between adjacent rolls controls the amount of pressure applied to the fruit. Whole fruits roll across the brown oil extractor (BOE, Figure 1), which is made of toothed rollers partially set in a flowing bed of water which propels the fruit across the machine and simultaneously punctures the peel to release the oil from the glands. The fruit proceeds to the extractor and the oil-water mixture goes to centrifugation and to the oil recovery chamber.

Figure 1: Brown oil extractor

4.3.1.6 FMC Whole Fruit Extractor

Food Machinery Corporation (FMC) of San José, California, has developed many designs and improved extractors in which both the juice and the volatile oil are extracted without getting mixed with each other. Further details are available at: http://files.asme.org/ASMEORG/Communities/History/Landmarks/5549.pdf.

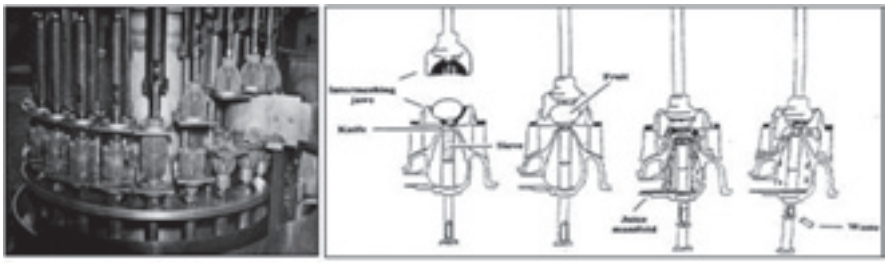

Figure 2: FMC whole fruit extractor

The FMC juice extractor has two inter-meshing jaws which encompass the fruit, crushing it between them. The juice exits through a mesh screen which penetrates the center of the fruit and the juice is separated from the peel, pith and seeds. This crushing action is sufficient to force the oil out of the glands on the surface of the flavado. The FMC machine sprays water onto the surface of the fruit, and the oil-water emulsion is subjected to centrifugation. Traces of water and waxy material are separated by keeping low temperature. About 75% of citrus oil production utilizes this technology. The odor of cold pressed oil is true in nature and similar to that of the oil present in fruit.

4.4 Cold Fat Extraction (Enfleurage)

Certain high-quality odor-producing flowers such as jasmine, tuberose and gardenia yield small quantities of oil and cannot be distilled by hydrodistillation. Furthermore, the oil components are thermolabile and such flowers, even after plucking, continue to emit small quantities of perfume. The oil from these types of flowers is extracted by cold fat extraction, i.e. enfleurage.

Figure 3: Chassis for holding the fat crops

Fat possesses a high power of absorption of volatile oil and, if brought in contact with fragrant flowers, it absorbs the perfume. This principle methodically applied on large scale constitutes enfleurage. The quality of the fat base is important for the quality of the flower oil. It must be odorless and of proper consistency. If the crops (fat) is too hard, the blossoms will not have sufficient contact with it, the absorption will be insufficient, and the quality and yield will be poor. If the crops is loose, it may engulf the flowers so that exhausted ones are difficult to remove and retain adhering fat when removed. The crops must have a consistency suitable to produce a semihard surface from which exhausted flowers can be removed easily. Saturated fragrant fat extract is known as pomade. Enfleurage process of cold extraction is carried out in Bulgaria, Egypt, Algeria, Sicily (Italy), and Grasse (France). France remains the main centre of production of highly prized so-called natural flower oil. Natural flower oil does not include the distilled essential oil but applies only to flower oils obtained by enfleurage, maceration or solvent extraction. The whole process is carried out in cold atmosphere cellars (cold rooms). The mixture of one part highly purified tallow and two parts lard is best suited for enfleurage.

Different bases have been used in the preparation of crops. For example, vegetable fat, mineral oil, esters of polyhydric aliphatic alcohol (ester of glycol, glycerol, mannitol, hexitol), and fatty acids of high molecular weight have been tried, but the best results were from the old-fashioned mixture of lard and tallow. To purify tallow, it is melted after cleaning, washing and removing blood and muscles; the skin is beaten and cleaned, and benzoin (0.6%) and alum (0.15%-0.30%) are added. Benzoin is a preservative while alum causes impurities to coagulate during heating. Warm melted fat is filtered through cloth and left to cool.

The vehicle for holding the fat crops during the process is a specially designed “chassis”. These are rectangular wooden frames (2 in. high, 20 in. long and 16 in. wide) that hold a glass plate (Figure 3). The fat crops is applied to both sides of the glass with a spatula at the beginning of enfleurage: 360 g crops on each side is required. When piled one above the other, the chassis form an air-tight compartment with a layer of fat on the upper and lower sides of each glass (Figure 4). Every morning, freshly picked flowers are cleaned by removing the leaves and stalks and eliminating the moisture from dew or rain. The flowers are strewn (Figure 3) by hand on top of the fat layer of each glass plate. Traces of moisture will cause the crops to go rancid, so precaution must be taken.

Figure 4: Pile of chassis

When chassis are piled one above the other, flowers remain in contact with the lower fat crops which acts as direct solvent, whereas the upper fat layer (beneath the glass plate of the chassis) absorbs only the volatile perfume given off by the flowers. After 24 h, the flowers are removed from the chassis. The time of removal, however, depends on type of the flowers. Removal of the flowers from the crops is known as defleurage. Immediately following defleurage, the chassis are recharged with fresh flowers. For this purpose, the chassis are turned over, thus the fat layer which in the previous operation formed on top (ceiling) of the small chamber is now directly charged with fresh flowers. When the fat crops becomes saturated with fragrance (pomade), the crops is removed from the chassis with a spatula. The pomades are extracted with absolute alcohol in a process called *extrait*. At the beginning and several times during the harvest, the fat on the chassis is scratched over with a metal comb and tiny furrows are drawn in order to change and increase the surface area available for maximum absorption. The most highly saturated pomade is pomade no. 36, meaning the crops on the chassis has been treated with fresh flowers 36 times during the whole process of enfleurage. In the case of jasmine, every kilogram of fat is charged with 3 kg flowers during the entire period. The alcohol washing of pomade no. 36 are called extrait no. 36, which reproduces the natural flower oil to a remarkable degree. Sometimes a fatty note is present in the extrait, which can be removed by freezing and filtering the alcoholic washes.

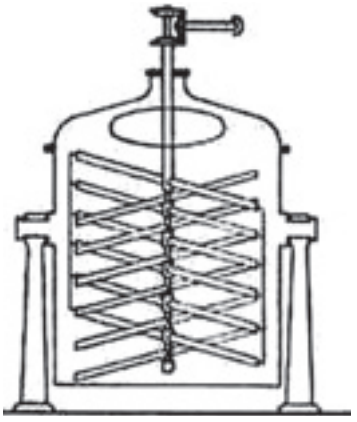

Figure 5: Batteuses

Pomades are processed during winter in cool cellars called batteuses (Figure 5) made of copper and equipped with stirrers. Several batteuses are arranged serially. The alcohol driven from one batteuse is poured in second, third and first washings of successive batteuses. For the last washing, fresh alcohol is used. Extrait no. 36 is cooled in a refrigerator to separate the fat. The exhausted fat is odorless and used for the manufacture of soap. Complete concentration of extrait is done under vacuum at low temperature to remove alcohol, producing absolute of enfleurage which is semisolid in nature. The flowers removed from the chassis are extracted with solvents (petroleum ether) and the concentrated residue is dissolved in absolute alcohol. The fat is removed by freezing. This preparation is known as absolute of chassis. Absolute of enfleurage and absolute of chassis logically supplement one another because each represents only part of the total oil present in the flowers but, due to cost difference, they are kept separately.

4.5 Conclusions

In spite of recent technological development in the field of extraction, hydrolytic maceration, expression and cold fat extraction techniques are inevitable for certain types of raw material. For these materials, no substitutes are available at present, although hydrodistillation or solvent extraction can be used for essential oils of citrus and flower oils or other perfumery materials. Nonetheless, the real replication of essential oil or the true natural fragrances present in these materials can only be produced by expression and cold fat extraction. Only technological improvements for easy extraction can be made without affecting the basic principles of these processes.

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5 Decoction and Hot Continuous Extraction Techniques

S. Tandon and S. Rane

Abstract

The chapter describes the techniques, parameters and equipment used for the extraction of plants by decoction and hot continuous extraction. Principles, mechanisms, merits and demerits of conventional solvent extraction and accelerated solvent extraction are also discussed.

5.1 Introduction

Of the traditional methods of extraction of medicinal plant material for making an aqueous extract, decoction is one of the most described. Decoction is a water-based preparation to extract active compounds from medicinal plant materials. In this process, the liquid preparation is made by boiling the plant material with water. Decoction differs from infusion in that the latter is not actively boiled. Decoction is the method of choice when working with tough and fibrous plants, barks and roots and with plants that have water-soluble chemicals. The plant material is generally broken into small pieces or powdered. Different methods have been described for the preparation of decoctions. In the Ayurvedic method, traditionally known as *kwatha*, the crude drug in form of *yavakuta* (small pieces) is placed in earthen pots or tinned copper vessels with clay on the outside. Water is added and the pot is heated on a fire. If the material is soft, four times water is used per 1 part drug; if the drug is moderately hard, eight times water is used and if the drug is very hard, sixteen times water is recommended. The mixture is then boiled on low flame until it is reduced to one-fourth starting volume, in case of soft drugs, and one-eighth in case of moderately or very hard drugs. The extract is then cooled and strained, and the filtrate is collected in clean vessels.

5.2 Solid-liquid Extraction Process

Solid-liquid extraction is one of the most widely used unit operations in the medicinal and aromatic plant industry. One example of solid-liquid extraction is the solvent extraction of herbs. This process, also referred to as leaching, is a separation technique that is often employed to remove a solute from a solid mixture with the help of a solvent. The insoluble solid may be colossal and permeable; more often it is particulate and the particles may be openly porous, cellular with selectively permeable cell walls, or surface-activated. The stream of solids being leached and the accompanying liquid are known as the underflow. The solid content of the

stream is called marc. The stream of liquid containing the leached solute is known as the overflow.

5.3 Process Parameters Affecting Solid-liquid Extraction

The following parameters generally affect the rate of solid-liquid extraction (leaching):

- Post-harvest processing
- Matrix characteristics
- Choice of solvent
- Method of contact
- Temperature of extraction
- Number of washes
- Condition for extraction (e.g. agitation)

5.3.1 Post-harvest Processing

After harvesting, most herbs have a moisture content of 60%-80% and cannot be stored without drying. Otherwise, important compounds can breakdown or micro-organisms may contaminate the material. Drying of the herbs in shade in a thin layer is generally preferred. Some medicinal plants, like pyrethrum, lose their active constituents if exposed to direct sun light for a long period. For drying large quantities of plant material, a hot air drying oven is used where material can be placed on a large number of trays stacked over each other. Oven temperature must be kept at a safe level so as not to damage the active constituents of the medicinal plant.

5.3.2 Matrix Characteristics

Knowledge of the matrix characteristics of the carrier solid is important to determine whether it needs prior treatment to make the solute more associable to the solvent. Grinding of plant material means mechanically breaking down leaves, roots, seeds, or other parts of a plant into small units, ranging from large course fragments to fine powder.

Solute may exist in the inert solids in a variety of ways:

1. On the surface of the solid,
2. Surrounded by a matrix of inert material, Chemically combined, or inside the cells.

Solute adhering to the solid surface is readily removable by solvent. When the solute exists in pores surrounded by a matrix of inert material, the solvent has to diffuse to the interior of the solid to capture the

solute. In the medicinal plant industry, generally ball mills and fluid mills are employed for powdering, and the optimum particle size is established prior to the large-scale extraction.

5.3.3 Choice of Solvent

The following factors should be considered when selecting a solvent for commercial use:

- *Solvent power (selectivity)*. Only the active, desired constituents should be extracted from the plant material, which means that a high selectivity is required.
- *Boiling temperature*. The boiling point of the solvent is as low as possible in order to facilitate removal of the solvent from the product.
- *Reactivity*. The solvent should not react chemically with the extract, nor should it readily decompose.
- *Viscosity*. A low viscosity of the solvent leads to low pressure drop and good heat and mass transfer.
- *Safety*. The solvent should be non-flammable and non-corrosive, and should not present a toxic hazard; its disposal should not imperil the environment.
- *Cost*. The solvent should be readily available at low cost.
- *Vapor pressure*. To prevent loss of solvent by evaporation, a low vapor pressure at operating temperature is required.
- *Recovery*. The solvent has to be separated easily from the extract to produce a solvent-free extract.

5.3.4 Conditions for Extraction

Too fine particle size may result in problems with packing of solids for extraction, preventing free flow of solvent through the solid bed. In such a case, extraction is more difficult, especially when finely divided solids are treated in an un-agitated state. Dispersion of the particles in liquid solvent by agitation facilitates contact of the solid with the solvent. Agitation, while giving good extraction, may cause suspension of fine particles in overflowing solution.

5.4 Method of Solid-liquid Extraction

The three principle types of flow used in leaching systems are:

1. Single-stage system
2. Multistage counter-current system
3. Multistage co-current system

Figure 1: Single-stage solid-liquid extraction

The single-stage system represents the complete operation of contacting the solid feed and fresh solvent. This is rarely encountered in industrial practice because of the low recovery of solute obtained and relatively dilute solution produced. Efficiency of extraction is somewhat improved by dividing the solvent into a number of smaller portions and then carrying out multiple successive extractions instead of only one contact of the entire amount of solvent with the solid.

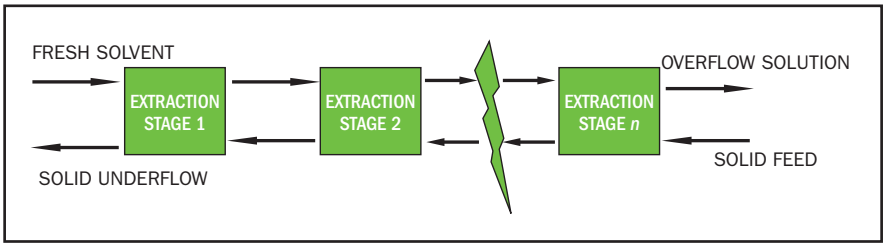

Figure 2: Multistage counter-current solid-liquid extraction

In the continuous counter-current multistage system shown in Figure 2, the underflow and overflow streams flow counter-current to each other. This system allows high recovery of solute with a highly concentrated product because the concentrated solution leaves the system after contact with fresh solid.

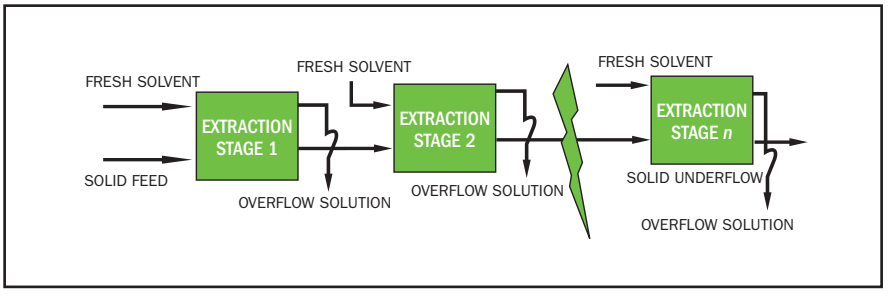

Figure 3: Multistage co-current solid-liquid extraction

In the multistage co-current (parallel) system shown in Figure 3, fresh solvent and solid feeds are contacted in the first stage. Underflow

from the first stage is sent to the second stage, where it comes in contact with more fresh solvent. This scheme is repeated in all succeeding stages.

5.5 Solid-liquid Extraction Equipment

Equipment for solid-liquid extraction is of two types:

a) Batch solid-liquid extractor
b) Continuous solid liquid extractor

The most common batch extractors employed for solid-liquid extraction of medicinal plants are:

- *Pot extractor*. The extractor has a volume of 2-10 m^3 and a mixer is necessary to guarantee good mixing for treatment of fine materials. For structured materials, the mixer is only used for evaporation of the solvent and for emptying the extractor.
- *Rotating extractor*. The extractor is filled with extraction material and solvent and starts then to rotate. The installation of heating coils and the use of a double jacket make it possible to evaporate the solvent at the end of the extraction cycle. A special form of heating coil can act as mixer during the extraction period.

The advantage of batch extractors is that they are simple to operate and are robustly constructed. Disadvantages of batch extractors are the limited capacity and the discontinuous output of the product.

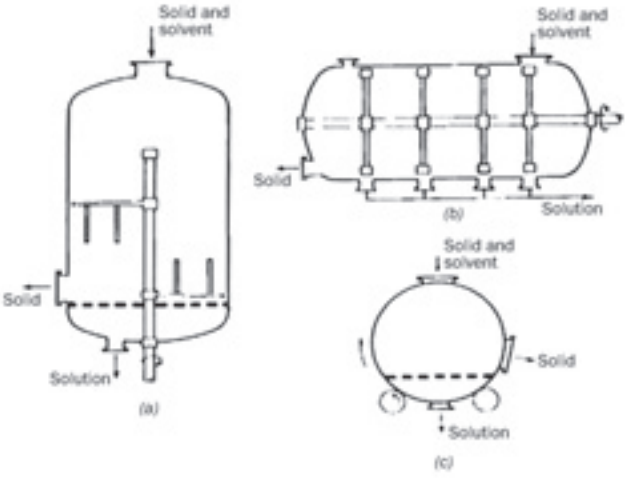

Figure 4: Pot extractor and rotating extractor

5.5.1 Continuous Extraction

For continuously operating extraction, percolation and immersion are used.

5.5.1.1 Percolation

The solvent passes through the non-moving solid material and extracts the soluble active constituents. One advantage of this method is that the solid material requires little mechanical treatment because it does not need to move in the percolator while the product passes in the solution. Moreover, since self-filtration takes place, there is minimum content of fine solid particles in the extract.

5.5.1.2 Immersion

In this process, the solid material dips completely into the solvent and is mixed with it. Therefore, no special percolation properties of the solid material are necessary. The disadvantage is that no self-filtration of the extract solution takes place. Therefore, a filtration step has to be added.

5.5.2 Continuous Extraction Equipment

5.5.2.1 Continuous Horizontal Extractor

The solid material is placed in baskets and comes in contact with the solvent by percolation. The flow of solvent through the extractor is counter-current to the flow of solid material.

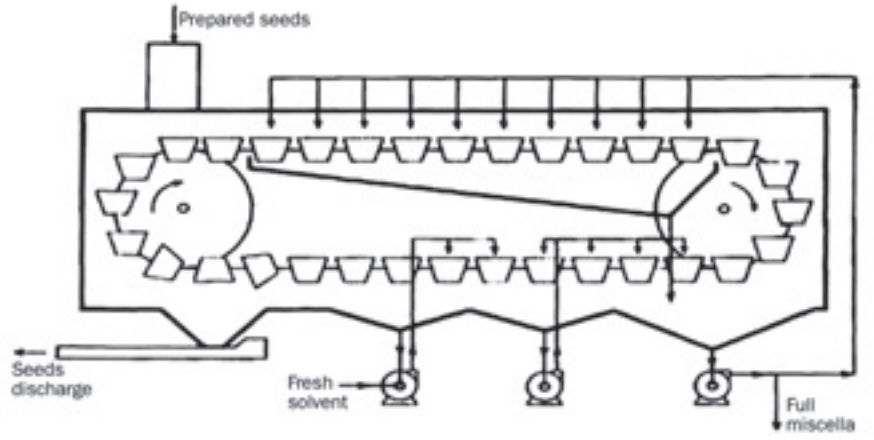

Figure 5: Continuous horizontal extractor

5.5.2.2 Hildebrandt Extractor

The solid material is extracted according to the immersion method. Screw conveyors are installed in the extractor for transporting the solid material. Again, the solvent flows counter-current to the solid materials through the extractor.

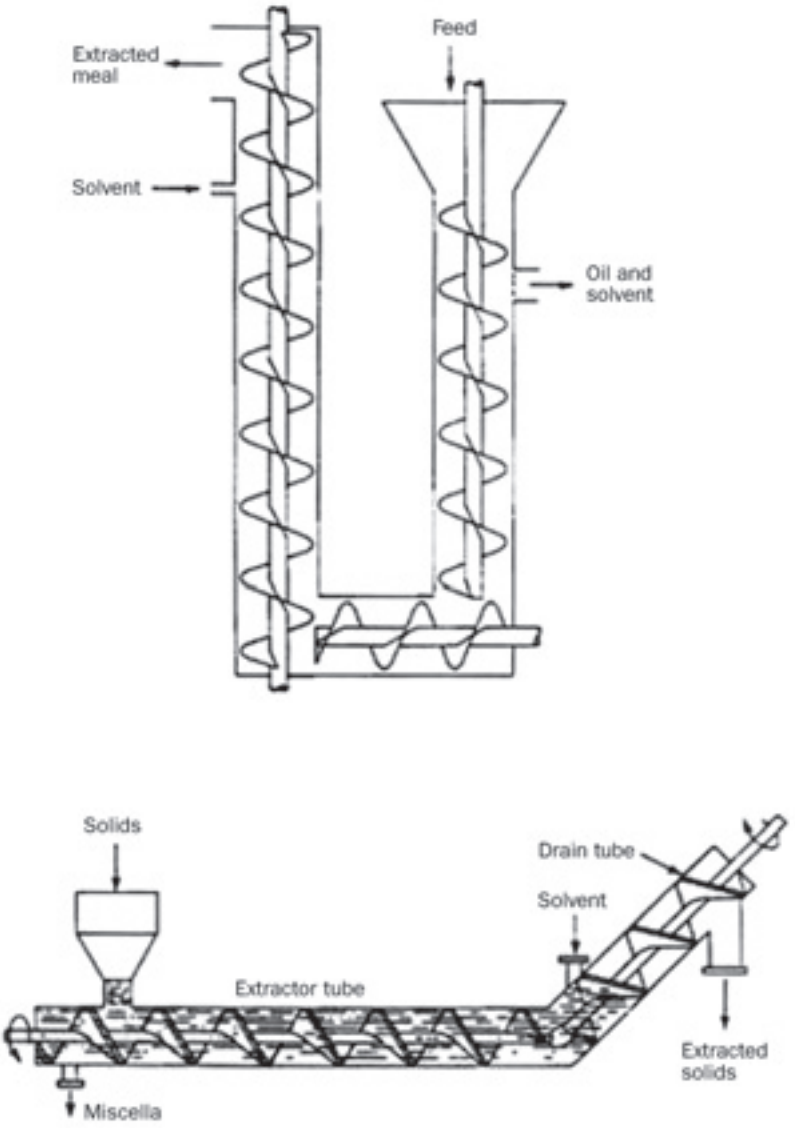

Figure 6: Hildebrandt extractor

5.5.2.3 Bonotto Extractor

The Bonotto extractor is used for counter-current extraction according to the immersion method. The solid material is transported by the mixer on a tray until it reaches the open sector where it falls onto the next tray. The screw conveyor at the outlet withdraws the extracted solid material (underflow) and prevents the solution from flowing out of the extractor.

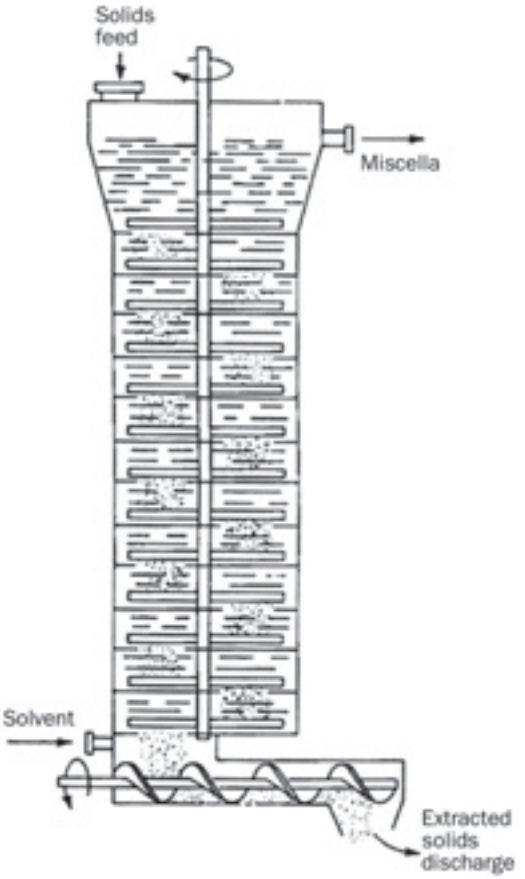

Figure 7: Bonotto extractor

5.5.2.4 Bollmann Extractor

The fresh solvent is added during the upward movement of the baskets so that this part operates in counter current. The already preloaded solution is withdrawn from the bottom of the extractor and enters the downward-moving baskets so that this part of the extractor operates in a co-current way. The full *miscella* is withdrawn at the bottom of the extractor.

In the baskets, self-filtration takes place, so that no further treatment of the *miscella* before distillation is necessary.

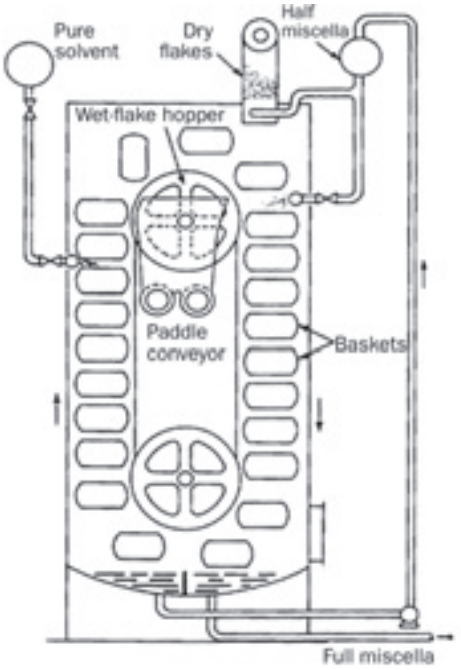

Figure 8: Bollmann extractor

5.5.2.5 Kennedy Extractor

The solid material is transported by paddles from one chamber to the next, in counter-current way to the solvent. The chamber where the *miscella* is withdrawn is used as a filtration unit where fine particles are separated from the extract solution.

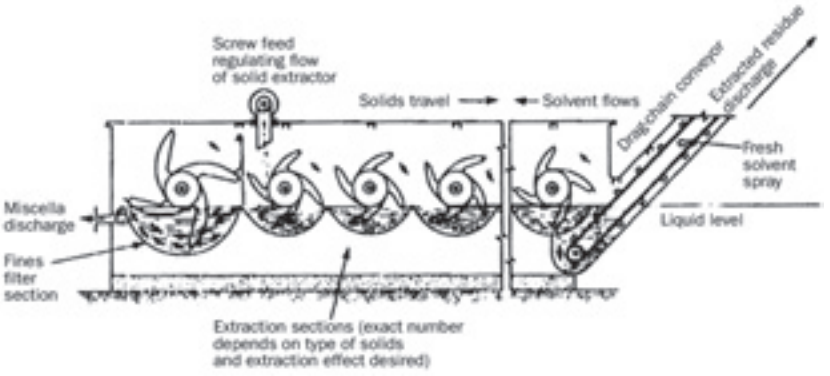

Figure 9: Kennedy extractor

5.6 Conventional Solvent Extraction

5.6.1 Principles and Mechanisms

Classic techniques for the solvent extraction of active constituents from medicinal plant matrices are based on the choice of solvent coupled with the use of heat or agitation. Existing classic techniques used to obtain active constituents from plants include: Soxhlet, hydrodistillation and maceration with an alcohol-water mixture or other organic solvents. Soxhlet extraction is a general and well-established technique, which surpasses in performance other conventional extraction techniques except for, in limited fields of application, the extraction of thermolabile compounds.

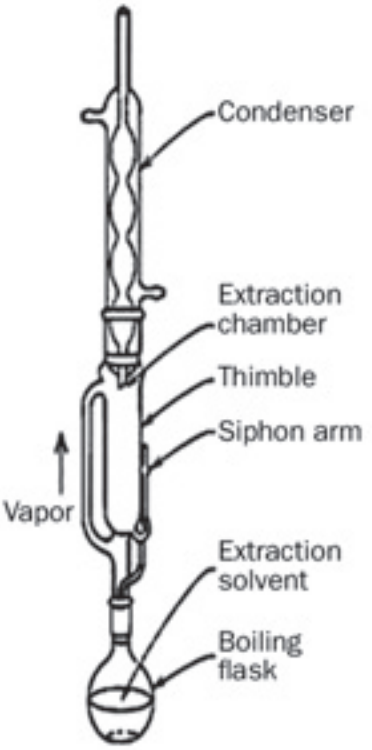

Figure 10: Soxhlet extractor

In a conventional Soxhlet system, as shown in Figure 10, plant material is placed in a thimble-holder, which is filled with condensed fresh solvent from a distillation flask. When the liquid reaches the overflow level, a siphon aspirates the solution of the thimble-holder and unloads it back into the distillation flask, carrying extracted solutes into the bulk liquid. Solute is left in the flask and fresh solvent passes back into the plant solid bed. The operation is repeated until complete extraction is achieved.

5.6.2 Advantages and Disadvantages of Soxhlet Extraction

Advantages:

1. The displacement of transfer equilibrium by repeatedly bringing fresh solvent into contact with the solid matrix.
2. Maintaining a relatively high extraction temperature with heat from the distillation flask.
3. No filtration of the extract is required.

Disadvantages:

1. Agitation is not possible in the Soxhlet device.
2. The possibility of thermal decomposition of the target compounds cannot be ignored as the extraction usually occurs at the boiling point of the solvent for a long time.

Worldwide, most of the solvent extraction units are based on the Soxhlet principle with recycling of solvents. Basic equipment for a solvent extraction unit consists of a drug holder-extractor, a solvent storage vessel, a reboiler kettle, a condenser, a breather system (to minimize solvent loss) and supporting structures like a boiler, a refrigerated chilling unit and a vacuum unit.

Table 1: Some common solvents used for the extraction of medicinal and aromatic plants

Solvent	Boiling point, °C	Miscibility with H_2O	Threshold limit values, ppm
Acetone	56	∞	1000
Acetic acid	116-117	∞	10
Ethyl acetate	77	80%	400
Benzene	80	<0.01%	25
2-Butanol	79.5	19%	2200
Cyclohexane	80.7	<0.01%	300
Dichloromethane	39.7	1.3%	2200
Chloroform	61	8%	50
Carbon tetrachloride	76.77	0.8%	10
Hexane	69	<0.01%	-
Ethanol	78	∞	1000
Ethyl ether	34.6	1.2%	400
Petrol ether	30-50	-	500
Propanetriole	290*	∞	-
Methanol	64.7	∞	200
1-Propanol	91	M	400
2-Propanol	82.4	M ?	400
Toluene	110.6	0.06	100

t = < 0.01%; * with decomposition; M miscible; ∞ completely miscible

5.7 Accelerated Solvent Extraction

5.7.1 Principles and Mechanisms

Accelerated solvent extraction (ASE) is a solid-liquid extraction process performed at elevated temperatures, usually between 50° and 200° C, and at pressures between 10 and 15 MPa. Therefore, accelerated solvent extraction is a form of pressurized solvent extraction. Increased temperature accelerates the extraction kinetics and elevated pressure keeps the solvent in the liquid state, thus achieving safe and rapid extraction. Also, high pressure allows the extraction cell to be filled faster and helps to force liquid into the solid matrix. A typical accelerated solvent extraction system is illustrated in Figure 11. Although the solvent used in ASE is usually organic, pressurized hot water can also be used. In these cases, one refers to pressurized hot water extraction or sub-critical water extraction.

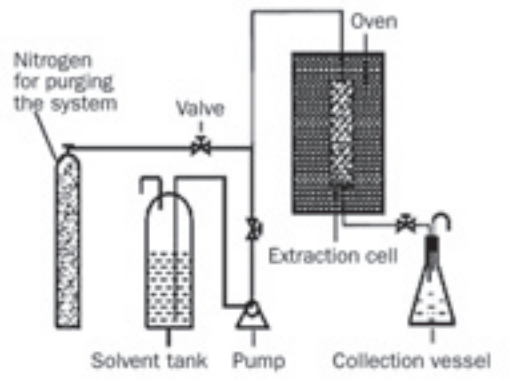

Figure 11: Accelerated solvent extraction

5.7.2 Advantages and Disadvantages of Accelerated Solvent Extraction

Compared with traditional Soxhlet extraction, ASE presents a dramatic reduction in the amount of solvent and extraction time. Particular attention should be paid to ASE performed at high temperature, which may lead to degradation of thermolabile compounds.

5.8 Important Factors for Designing a Solvent Extraction Plant for Medicinal Plants

- High efficiency of extraction
- Minimal solvent loss
- Facilities for cold and hot extractions
- Extraction with agitation

- Multiple solvent extraction systems
- Multiple fraction collection systems
- On-line filtration unit
- Solvent recycling and condensing unit
- Vent lines with breather for minimizing solvent loss and maximizing safety
- Brine circulation unit
- Fractionating column for separation of solvent mixtures
- Efficient evaporating systems like falling film, wiped film or rotary evaporators to work under low pressures
- Vacuum manifold system with cold traps
- GMP compatible
- Automation

Figure 12: Solvent extraction plant at CIMAP, India

5.9 Conclusions

Decoction, a water-based preparation, is one of the most used traditional methodologies for the extraction of active constituents of a medicinal plant. It is generally carried out by boiling the plant part for a fixed period. Hot continuous extraction or solvent extraction technique is one of the most widely used extraction techniques for the processing of medicinal plants. The solvent extraction method is simple, well established and economical. Important factors that can affect the efficiency of extraction, such as post-harvest processing, solid characteristics, choice of solvent, method of contact, and temperature, should be optimized for best yield. The choice of solvent especially for commercial plants and high efficiency usually de-

pends on many factors such as selectivity, polarity, boiling point, chemical and thermal stability, safety, flammability, and costs. Despite the economic advantages of solvent extraction, the use of volatile organic solvents such as hexane, acetone and methanol for processing medicinal plants has been limited due to environmental considerations. Hot continuous extraction technology shall always remain the method of choice for high efficiency, economical extraction and with less capital investment.

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6 Aqueous Alcoholic Extraction of Medicinal and Aromatic Plants by Fermentation

C. K. Katiyar

Abstract

The history of the development of pharmaceutical dosage forms can be traced back to the Vedic era. It provides insight into the innovations made during ancient times when the crude herbs were initially used in powder form and later on they were used as decoctions, self-fermented products, paste, pills and other advanced dosage forms. New drug development involves the search for novel and new pharmacophores. Combinatorial chemistry cannot solve the problem in a satisfactory manner nor can the reductionist approach be applied to medicinal plants. Asava arishta, a fermented Ayurvedic product, is a good source of novel pharmacophores for new drug discovery.

6.1 Introduction

The history of development of pharmaceutical dosage forms can be traced back to *Charak Samhita*, the first systematic documentation of Ayurveda. Ayurveda has recommended a comprehensive *Materia Medica* including medicinal plants, minerals, metals, and products of marine and animal origin. However, the use of herbs has been given priority.

Medicinal plants have been used for therapeutic purposes for centuries. Initially, these were used in fresh or dried powder form, which caused the problems of high dose, high volume and low shelf life. This led to the development of extraction processes. Extracts were found to be more useful as the necessary dose was less, the volume was low and shelf life was higher. Initially the solvents used for extraction were either water or alcohol, or their mixture. This evolutionary phase is continuing even today where solvents of all kinds of polarity are tried and extraction technologies have evolved from simple water decoction centuries ago to supercritical extraction. While this is true for some streams of products, Ayurveda did not exactly follow the same route but adopted a *sui generis* system of innovation.

6.2 Ayurvedic Dosage Forms

During ancient times, various dosage forms were developed. The number of dosage forms that developed over time is given below:

Charak Samhita (12th century BC)	128 dosage forms
Sushruta Samhita (10th century BC)	129 dosage forms
Ashtanga Hridaya (6th century AD)	90 dosage forms

Chakradutta (9th century AD)	90 dosage forms
Sarngadhara (14th century AD)	75 dosage forms
Bhaishajya Ratnavali (18th century AD)	98 dosage forms

The evolutionary phases of dosage forms followed by Ayurveda starting from crude plant material are mentioned in the following paragraphs.

6.2.1 *Swarasa* (Fresh Juice)

The evolution of liquid orals started from the administration of freshly obtained juices of plant material. To obtain fresh juices, green herbs are crushed and the juice is expressed by squeezing the crushed material. The product is referred to as *swarasa*.

6.2.2 *Kalka* (Wet Bolus)

In this method, the crushed fresh plant material is administered as such, without expressing the juices.

6.2.3 *Kwatha* (Decoction)

One part of coarsely powdered herb is boiled with 16 times its weight of water in an earthen pot over a mild fire until the liquid is reduced to one-fourth or one-eighth of the original quantity, depending upon the nature of the plant material.

6.2.4 *Hima* (Cold Infusion)

The plant material is dried and coarsely powdered. As and when required, the powder is soaked in plain water for a defined period. Then it is filtered, the marc is squeezed, and the combined filtrate is used.

6.2.5 *Phanta* (Hot Infusion)

As a further advancement of *hima*, the *phanta* method was adopted. This method uses boiled water for obtaining a hot infusion.

6.2.6 Solids

Anjana, Churna, Mansa potli, Utkarika, Kshara, Gutika, Guda, Dhumravarti, Puplika, Prithuka, Mandura, Modaka, Rasakriya, Vati, Varti, Shashkuli, Saktu, Bhasmas, Rasaushadhis.

6.2.7 Semisolids

Oral: o*dana* (rice preparation), *kalka, krishara, avaleha.*

Topical: *lepa, upnaha* (poultice), *tilapishta, patrasveda, madhucchishta* (beeswax).

6.2.8 Liquids

Oral: *taila, ghrita, asava/arishta, arka, kwatha, kshirapaka, takra, phanta, him, swarasa, peya, phanita, manda, manasa rasa, yusha, vesavara, vilepi, madya*

Topical: *ashchyotana, karna purana.*

6.2.9 Fumes

Dhumrapana, dhupana.

6.3 Shelf Life of Dosage Forms

Sarngadhara Samhita gave the shelf life of various dosage forms. In ancient times, Ayurvedic physicians themselves prepared the recipes for patients. In ancient times, Ayurvedic physicians themselves prepared the recipes for patients; during the fourteenth century AD, they became aware of the problem of poor shelf life of the botanicals in some dosage forms, such as powder and decoction. This led to the discovery of novel dosage forms termed *asava* and *arishtas,* which are self-fermented preparations having approximately 10%-12% alcohol. These are similar to medicated wines. In the preparation of *asava* cold infusion of unprocessed plant material is used, whereas for the preparation of *arishta* decoction of the plant material is used for fermentation.

6.4 *Asava* and *Arishta*: Self-fermented Products

This unique dosage form discovered by Ayurveda is supposed to have indefinite shelf life and it was said that the “older the better it is”. In terms of current understanding, this phrase assumes more importance because this dosage form has an inherent attribute of continuous hydro-alcoholic extraction and probably formation of natural analogues of the chemical compounds present in the medicinal plants. Some of the major self-fermented Ayurvedic preparations are given in Table 1.

Table 1: Major self-fermented Ayurvedic preparations

Product	No. of ingredients	Indication
Aravindasava	27	Pediatric tonic
Arjunarishta	5	Cardiotonic
Ashokarishta	14	Menstrual cycle regulator, especially to control excessive bleeding for prolonged periods during menstrual cycle
Ashwagandharishta	24	General tonic
Dashamularishta	67	Normalization of physiological processes after childbirth in women; also anti-inflammatory
Drakshasava	17	General tonic
Jeerakadyarishta	13	Galactogogue
Kumaryasava	46	Liver disorders
Kutajarishta	6	Diarrhea and dysentery
Lohasava	14	Anemia

6.4.1 Self-fermentation Process for Preparing *Asava Arishta*

Preparation of *asava arishtas* involves complicated processes where the following factors play important roles:

- Ingredients and their ratios
- Process
- Pot
- Season
- Place

The method of preparing *asava arishtas* is known as *sandhana kalpana* in Ayurveda. Briefly, a decoction or cold infusion of several herbs is taken and a defined amount of jaggery (dried juice of sugarcane) is added along with flowers of *Woodfordia fruticosa* as inoculum to initiate fermentation. It is kept for about four weeks for fermentation by anaerobic method to obtain a particular level of self-generated alcohol. The product is then kept for some time for maturation. Spices like cardamom and cinnamon are added as flavoring agents. A brief outline of the process of preparing *asava arishta* is given in Figure 1.

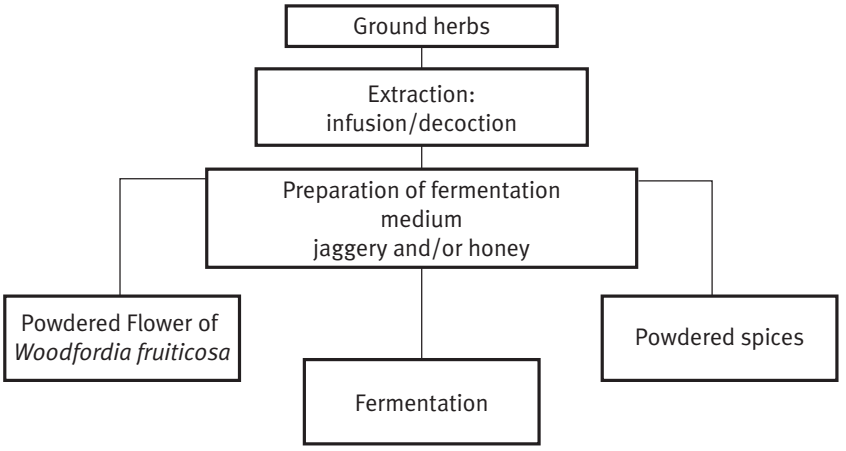

Figure 1: The traditional process of preparing asava-arishtas

A crude match-box method is applied to check whether fermentation has occurred. This method depends upon the release of carbon dioxide during the process. The major role in this dosage form is played by *Woodfordia fruticosa,* which is used as inoculum for fermentation but appears to play a role beyond that.

6.4.2 Merits of the Process

Prahst has mentioned some of the benefits of fermented herbal products which are reproduced below:

- Fermentation removes most of the undesirable sugars from plant material, makes the product more bio-available and eliminates side effects such as gas and bloating.
- Fermentation extracts a wider range of active ingredients from the herb than any extraction method since the menstruum undergoes a gradient of rising alcohol levels.
- Yeast cell walls naturally bind heavy metals and pesticide residues and, therefore, act as a natural cleansing system.
- Not only does fermentation remove contaminants, it can also lower the toxicity of some of the toxic components in plants.
- Fermentation actively ruptures the cells of the herb, exposing it openly to the menstruum and bacteria have enzymes that break down cell walls to further assist in the leaching process. Fermentation also creates an active transport system that moves the dissolved constituents from the herbal material to the menstruum.

6.5 Application of *Asava Arishta* Technology in New Drug Discovery

The Ayurvedic dictum with regard to *asava arishtas* that "older is better" needs to be scientifically evaluated. The process of preparing *asava arishtas* appears to involve:

1. Slow hydro-alcoholic extraction at room temperature of crude plant material particles floating in the liquid. Since the particle size of the plant material floating in the liquid is small, the effectiveness of extraction may be higher because of the larger surface area.
2. During the process, if the product is kept for a prolonged period, the probability of development of analogues of some of the pure chemical compounds of the plant material is high.

With a view to enhance the success rate of isolation of pure "druggable" compounds from medicinal plants, it is advised to start from 2- to 3-year-old self-fermented preparations than from solvent extracts. The chances of successful isolation of effective therapeutic compounds using this approach may be high and need to be evaluated.

6.6 Conclusions

Fermentation was applied hundreds of years ago in Ayurveda to develop *asava arishtas*, a multiherbal product, with a view to increase the shelf life and also to enhance the efficacy profile. The race for discovery of new molecules is getting increasingly competitive; at the same time, lack of new and novel pharmacophores is a big impediment which slows down drug discovery. Nature continues to be a source of pharmacophores, although the compounds isolated may not be "druggable" as such. They need to be derived, mostly to enhance their potency. In *asava arishtas*, the self-fermented products can undergo continuous chemical transformation which goes on beyond hydro-alcoholic extraction of the suspended material. This may result in novel natural molecules with enhanced therapeutic activity.

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7 Distillation Technology for Essential Oils

S. Tandon

Abstract

In many developing countries, the technology employed for extraction of essential oils from aromatic plants is primitive and obsolete. This results in low yield and poor quality of essential oils. Thus, there is great need for attaining adequate technological capability in the area of processing essential oil plants. This article deals with the different techniques of distillation, the principles of distillation, and important processing and design aspects which affect the yield and quality of the essential oils.

7.1 Introduction

Distillation is the most popular, widely used and cost-effective method for producing essential oils throughout the world.

Distillation of aromatic plants simply implies vaporizing or liberating the oils from the plant cellular membranes in the presence of moisture, by applying high temperature and then cooling the vapor mixture to separate the oil from the water on the basis of the immiscibility and density of the essential oil with respect to water.

7.2 Principles of Distillation

The choice of a particular process for the extraction of essential oil is generally dictated by the following considerations:

a) Sensitivity of the essential oil to the action of heat and water
b) Volatility of the essential oil
c) Water solubility of the essential oil

Essential oils with high solubility in water and those that are susceptible to damage by heat cannot be steam distilled. Also, the oil must be steam volatile for steam distillation to be feasible. Most of the essential oils in commerce are steam volatile, reasonably stable to heat and practically insoluble in water; hence they are suitable for processing by steam distillation.

Essential oils are a mixture of various aroma chemicals, basically monoterpenes, sesquiterpenes and their oxygenated derivatives, having a boiling point ranging from 150° to 300° C. When the plant material is subjected to heat in the presence of moisture from the steam, these oils are liberated from the plant. For the oil to change from the liquid to the vapor phase, it must receive latent heat that, within the tank, can only come from condensing steam. Consequently, the temperature of the steam within the

still must be higher than the temperature at which the oil boils in the presence of water on the surface of the plant material, otherwise there would not be a temperature gradient to take the latent heat from the condensing steam to vaporize the oil droplet. Thus, the energy from the steam in form of heat as latent heat of vaporization converts the oil into a vapor. But, as the boiling point of the oil is higher than that of water, the vaporization takes place with steam on the basis of their relative vapor pressures.

It is imperative to note that a liquid always boils at the temperature at which its vapor pressure equals the atmospheric or surrounding pressure. For any two immiscible liquids, the total vapor pressure of the mixture is always equal to the sum of their partial pressures. The composition of the mixture in the vapor phase (in this case, oil and water) is determined by the concentration of the individual components multiplied by their respective partial pressures. For example, if a sample of an essential oil comprised of component A (boiling point, 190° C) and water (boiling point, 100° C) is boiled, after some time, once their vapors reach saturation, the temperature will immediately drop to 99.5° C, which is the temperature at which the sum of the two vapor pressures equals 760 mmHg. In other words, the oil forms an azeotropic mixture with water. Thus, any essential oil having high boiling point can be evaporated with steam in a ratio such that their combined vapor pressures equal the atmospheric pressure; the essential oil can be recovered from the plant by the wet distillation process.

7.3 Methods for Distillation

The following four techniques for the distillation of essential oils from aromatic plants are employed:

1. Water distillation (or hydrodistillation)
2. Water and steam distillation
3. Direct steam distillation
4. Distillation with cohobation

7.3.1 Hydrodistillation

Hydrodistillation is the simplest and oldest process available for obtaining essential oils from plants. Hydrodistillation differs from steam distillation mainly in that the plant material is almost entirely covered with water in the still which is placed on a furnace. An important factor to consider in water distillation is that the water present in the tank must always be enough to last throughout the distillation process, otherwise the plant material may overheat and char. In this method, water is made to boil and the essential oil is carried over to condenser with the steam which is formed. Water-distilled oil is slightly darker in color and has much stronger still notes than oils produced by other methods. The stills based on this principle are

simple in design and are extensively used by small-scale producers of essential oils. Care should be taken during distillation of powdered herbs, as they tend to settle on the bottom of the still and get thermally degraded. Also, for plant material that tends to form mucilage and increase the viscosity of the water, the chances of charring are greater. For plant material that has a tendency to agglomerate or to agglutinate into an impenetrable mass when steam is passed through (like rose petals), water distillation is the preferred method of oil isolation.

The primitive, traditional Indian system of essential oil distillation, *bhapka* method, is also based on water distillation (Figure 1). In this process, the plant material is entirely covered with water in a distillation still, which is made of copper and is known as *deg*. This *deg* is placed in a brick furnace. Another copper vessel with a long neck is placed in a water tank or natural pond to serve as a condenser. A bamboo pipe is used as the vapor connection and mud is used to seal the various joints. The water is boiled, the oil vapors along with steam are condensed in the copper vessel, and oil is separated. The capacity of one *deg* is around 40 kg/batch. These types of units are still being used in Kannauj in Uttar Pradesh and in the Ganjam district of Orissa, India for the preparation of *rooh* and attars of *gulab, kewda, khus, rajnigandha*, and *bela*. These units can easily be transported from one place to another, but are not suitable for large-scale distillation of aromatic crops like grasses and mints.

Figure 1: Traditional Indian deg bhapka method

Although hydrodistillation (water distillation) is still being used, the process suffers from the following serious drawbacks:

a) As the plant material near the bottom of the still comes in direct contact with the fire from the furnace, it may char and thus impart an objectionable odor to the essential oil.
b) The prolonged action of hot water can cause hydrolysis of some constituents of the essential oil, such as esters.
c) Heat control is difficult, which may lead to variable rates of distillation.
d) The process is slow and distillation times are much longer than those of steam distillation.

7.3.2 Water and Steam Distillation

To eliminate some of the drawbacks of water distillation, some modifications were made to the distillation units. A perforated grid was introduced in the still, to support the plant material and to avoid its direct contact with the hot furnace bottom. When the water level is kept below the grid, the essential oil is distilled by the rising steam from the boiling water. This mode of distillation is generally termed water and steam distillation.

The field distillation unit (FDU), also known as a directly fired-type distillation unit, is designed according to the principle of water and steam distillation. The FDU consists of a still or tank made of mild stainless steel with a perforated grid and is fitted directly to a brick furnace. A chimney is connected to the furnace to minimize the pollution at the workplace and also to induce proper firing and draft. The plant material is loaded on the perforated grid of the tank and water is filled below it. The tank is connected to the condenser through a vapor line. The water is boiled and the steam vapors pass through the herb, vaporize the oil and get condensed, mostly in a coil condenser by cooling water. The condensate (oil-vapor mixture) is then separated in the oil separator.

These units are simple to fabricate and can be installed in the farmer's field. Due to their simple construction, low cost and easy operation, FDUs are extremely popular with essential oil producers in developing countries. The furnace is always fueled by locally available firewood or straw. This makes the unit suited for use in remote areas where the raw material is available. This also helps in reducing transportation costs in the production of essential oils. FDUs are currently finding application in distillation of patchouli oil in Indonesia, aromatic grass and mint oil in India, citronella oil in Taiwan and many more all over the world. A local FDU currently being used by rural farmers in India for the distillation of mint oils is shown in Figure 2. Such field units generally can hold 100-2000 kg plant material. Total time for distillation with these units is about 6-8 h.

Figure 2: Local type field distillation unit in India

7.3.2.1 Improved Field Distillation Units

Due to the limited heating surface available, the rate of steam production in the FDU is always insufficient. This results in prolonged distillation periods and sometimes lower oil yields. Refluxing of oil back into the still due to inadequate steam rate may lead to decomposition reactions and poorer oil quality. Experimental measurements made at the Central Institute of Medicinal and Aromatic Plants (CIMAP), India, have shown that firewood consumption in a conventional field still may be up to 2.5-times greater than that of a modern steam distillation unit operated by an external boiler. This factor may not be critical where fuel supplies are cheap and abundant but, in many developing countries, fuel supplies are getting scarce and costly and low thermal efficiency can directly affect the cost of production.

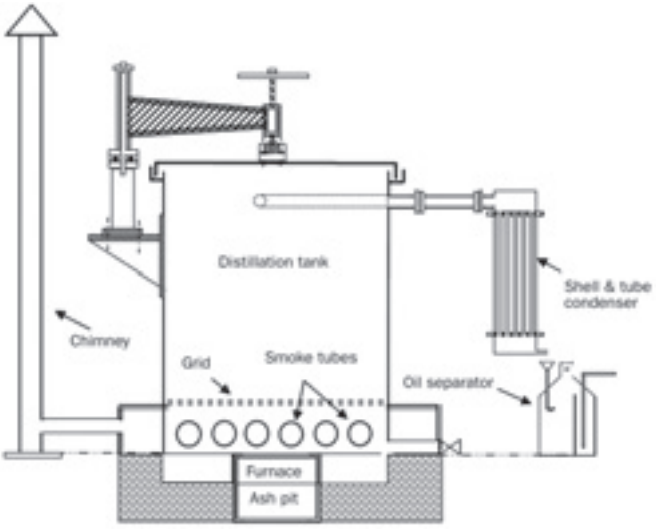

Figure 3: CIMAP's improved field distillation unit

Considering the previously mentioned demerits of FDUs, designs of economical and improved units with capacities 500-2000 kg per batch are now being preferred (Figure 3). The units are fabricated with high quality mild stainless steel, keeping in view the plant materials to be distilled. The improved distillation unit consists of a cylindrical distillation tank fitted on a square inbuilt boiler (calandria) having smoke pipes which reduces the heating time of the water, resulting in a high rate of steam generation and lower fuel consumption (20%-30%). Hot flue gasses of the furnace are led through the smoke tubes where they impart heat to the water, thus raising additional steam. The tank is fitted on a specially designed furnace having fire grate, flue ducts and fire door for proper controlling of the firing and draft. The furnace is connected to a chimney of optimum height to maximize the air draft and control the pollution by smoke in the workplace. A similarly designed stainless steel shell and tube-type condenser having higher condensation capacity are used for cooling the vapors. It prevents loss of oil due to improper condensation. The condensed oil-water mixture is then allowed to pass through a specially designed stainless steel oil separator. The separator has an inbuilt baffle to maximize the retention time of the mixture, thereby resulting in no loss of oil with the outgoing water from the separator. The unit also has a chain pulley hoist system with a support structure that makes work easier and saves time during discharge of the distillation waste material from the tank. CIMAP has designed, fabricated and supplied these improved units to entrepreneurs and farmers in different parts of India.

7.3.3 Direct Steam Distillation

In direct steam distillation, plant material is distilled with steam generated outside the tank in a steam generator or boiler. As in water and steam distillation, the plant material is supported on a perforated grid above the steam inlet. As already noted, the steam in an FDU is at atmospheric pressure and hence its maximum temperature is 100° C. But, steam in a modern pressure boiler operating at, for example, 50 psi pressure will have a temperature correspondingly higher. Moreover, there is no limitation to the steam generation when an external boiler is used as a source of steam. The use of high-pressure steam in modern steam distillation units permits much more rapid and complete distillation of essential oils.

Steam distillation is preferred when a lot of area is under cultivation and more than one unit is to be installed. Also, for distillation of high boiling oils and hardy materials such as roots and woods like sandalwood, cedar wood and nagarmotha, steam distillation is more efficient. Steam distillation also reduces the time required for the extraction of oils. A charge of Java citronella, which takes up to 5 h in an FDU, is processed within two to 3 h in a steam distillation still. In this method of distillation, steam is generated separately in a steam boiler and is passed through the distillation tank through a steam coil (Figure 4). The plant material is tightly packed above the perforated grid. Steam, containing the oil vapor, is condensed in a tube condenser and is separated in

the oil receiver. Fuel costs are generally lower in modern steam distillation units due to higher thermal efficiency at which most of the boilers operate. Capital cost is higher, thus only bigger producers can afford to own such units. Still capacities range from 1 to 3 tonne plant material per batch.

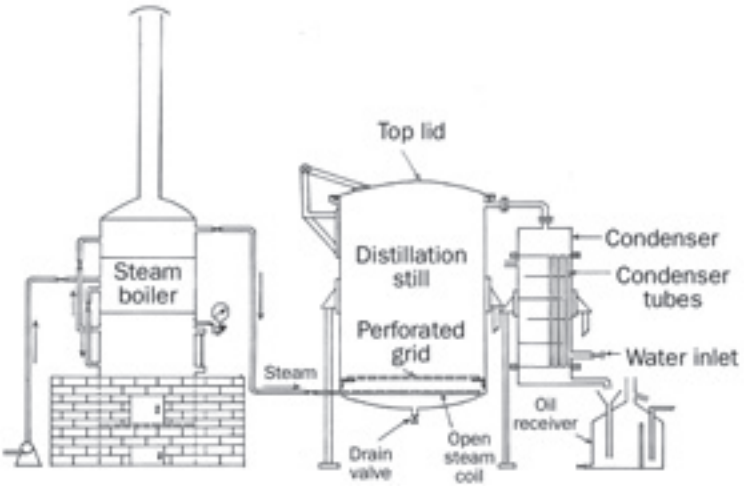

Figure 4: Boiler-operated steam distillation unit

7.3.3.1 Comparison of Boiler-operated Unit with Directly Fired Type

a) It is possible to run a large number of distillation units by a single steam boiler. Hence a boiler system is ideal for large-scale production of essential oils. An FDU is more suited for small and medium-sized farmers.
b) The efficiency of extraction of essential oil in a well-designed FDU can equal that obtained by a boiler unit. But in a poorly designed FDU, oil recovery may be low and fuel wastage may be heavy accompanied by smoke pollution.
c) Steam injection rate in a boiler-operated unit can be adjusted with ease but steam generation rate in an FDU is limited by the heat transfer area provided in the unit. Insufficient steam generation in an FDU can result in low oil yield.
d) Boiler-operated units require a skilled boiler man for operation but an FDU can be operated by relatively low-skilled workers.

7.3.4 Distillation with Cohobation

Cohobation is a technique that can be used for water distillation or for water and steam distillation. It uses the process of returning the distillate water to the still after the oil has been separated from it so that it can be re-boiled. This is basically an improvised methodology of the directly fired type

steam and water distillation units for oils which have partial solubility in water. Although most of the essential oils have finite solubility in water, some oils like those of rose, lavender and geranium have comparatively higher solubility. In such extractions, the loss of oil with the outgoing water of distillation can become alarmingly high. This problem can be solved by returning the condensate water from the separator back to the still; this is known as cohobation. It is evident that this cannot be done with steam distillation as the water level in the still will keep building up due to continuous steam injection.

In a further improved version, a packed column is placed on top of the column for providing mass transfer to the oil-water vapors, so as to increase the concentration of the outgoing condensate and to coalesce the oil droplets in the oil separator (Figure 5). The condenser is placed above the column so that the condensate water from the separator can be recycled back to the still by means of gravity. Additional heat, if required, can be provided by a closed steam coil immersed in the tank bottom. The condenser is moved above the distillation still so that condensed water from the separator can flow by means of gravity to the still. By limiting the total quantity of water in this closed cycle operation, it is possible to obtain increased yields of essential oils that are more water soluble. It is relevant to point out here that prolonged recirculation of the distillation water allows the various impurities and plant decomposition products to build up in the system. This may sometimes affect the quality of the oil. One must always keep this in mind when considering a cohobation distillation system for any application.

Figure 5: Distillation unit with cohobation

7.4 Hydrodiffusion

This system was first described in 1983. Unlike traditional steam distillation, hydrodiffusion works on the diffusion principle of allowing steam to enter the top of the plant charge and diffuse through the charge by gravity. The process uses the principle of osmotic pressure to diffuse oil from the oil glands. The system is connected to a steam source, and low pressure steam is passed into the plant material from a boiler. The condenser, which is directly under the basket within the still, is of the tube type. The oil and water are collected below the condenser in a typical oil separator. Hydrodiffusion is an efficient process that is easy to use, especially regarding the processes of loading and unloading the plant material. The yield of oil is higher and the process is advantageous because of the reduced steam consumption, shorter distillation time and absence of hydrolysis, as the raw material does not come in contact with boiling water. However, because of the downward flow of steam and condensate, co-extraction of other non-volatile compounds (such as lipids, chlorophyll and fatty acids) and polar components makes the process complicated. Although it may seem that hydrodiffusion is a better alternative to conventional distillation processes, the fact remains that commercial ventures based on hydrodiffusion have not been able to take off successfully.

7.5 Parameters Affecting Yield and Quality of Essential Oils

The yield and quality of essential oil from steam distillation is affected by the various process parameters. It is advisable to keep them in mind while designing such systems. Some of the important parameters are being listed below.

7.5.1 Mode of Distillation

The technique for distillation should be chosen considering the boiling point of the essential oil and the nature of the herb, as the heat content and temperature of steam can alter the distillation characteristics. For high boiling oils such as woody oils (e.g. sandalwood, cedar wood) and roots (e.g. *Cyperus*), the oil should be extracted using boiler-operated steam distillation. Since the heat content and temperature of steam depend upon its pressure, a change in steam pressure can alter the distillation characteristics. High-boiling constituents of essential oils normally require high-pressure steam to distill over. For oil of rose and other florals, the material is generally immersed in water, i.e. hydrodistillation, as flowers tend to aggregate and form lumps which cannot be distilled using water and steam distillation or direct steam distillation.

7.5.2 Proper Design of Equipment

Improper designing of tank, condenser or separators can lead to loss of oil and high capital investments. The design of the furnace and chimney affects the firing and heat control of the distillation rates. Tank height:diameter ratio is important. Similarly the use of a condenser with an improper design and without calculating the heat transfer areas based on the steam generation areas will lead to improper condensation and loss of oil.

7.5.3 Material of Fabrication of Equipment

Essential oils which are corrosive in nature should be preferably distilled in stills made of resistant materials like aluminum, copper or stainless steel. The tank still can be made from a cheaper metal like mild steel or galvanized iron, and the condenser and separator can be made from a resistant material like stainless steel. As only vapor is present in the tank still, the rust and other products of corrosion may not be carried over into the oil. This can result in considerable savings in the capital cost of the equipment. Expensive, high-value essential oils like rose, agarwood, *kewda*, sandalwood and lavender should be distilled in stainless steel systems. Although copper was the most common material of fabrication of distillation stills since ancient times, its availability is getting reduced and with the arrival of superior alloys like stainless steel, it is slowly disappearing from the scene.

7.5.4 Condition of Raw Material

The condition of the raw material is important because some materials like roots and seeds will not yield essential oil easily if distilled in their natural state. These materials have to be crushed, powdered or soaked in water to expose their oil cells. Chopping of plants will also change the packing density of the material when placed in the distillation still. One can pack up to 50% more plant material in the same still after chopping of some aromatic herbs like mint. Air drying and wilting the herb prior to distillation also has considerable effect on distillation. If required, drying of the herbs prior to distillation should be done in shaded areas and the dried material should not be kept in heaps.

7.5.5 Time for Distillation

Different constituents of the essential oil get distilled in the order of their boiling points. Thus, the highest boiling fractions will be last to come over when, generally, very little oil is distilling. If the distillation is terminated too soon, the high-boiling constituents will be lost. In many aromatic plants, like vetiver, patchouli, chamomile, sandalwood and agarwood,

these high-boiling fractions are valuable due to the quality of their aromas. Thus, the time of distillation must be chosen with due care.

7.5.6 Loading of Raw Material and Steam Distribution

Improper loading of the herb may result in steam channeling, causing incomplete distillation. The herb should be evenly and uniformly loaded in the tank without leaving any voids. Excessive filling of plant material may also lead to formation of "rat holes" which may allow steam to escape without vaporizing the oil. For powdered herbs, a proper stainless steel wire mesh or muslin cloth should be put at the false bottom to prevent plant material from falling into the tank base.

7.5.7 Operating Parameters

Proper control of injection rates and pressure in boiler-operated units is necessary to optimize the temperature of extraction for maximal yield. Generally, high-pressure steam is not advisable for the distillation of essential oils. The temperature of the condensate should not be high, as it can result in oil loss due to evaporation. In directly fired-type FDUs, the firing of the furnace should be well controlled as it can result in high flow rates and high condensate temperatures.

7.5.8 Condition of Tank and Equipment

The tank and other equipment should not be rusted. If rusted, the tank should be cleaned with dilute caustic solutions. The perforated grids should not be corroded or have large gaps permitting the plant material to settle to the bottom of the tank and emit a burnt odor. The distillation tanks should be well steamed prior to distillation for multiple crop distillation.

7.6 Purification of Crude Essential Oils

Essential oil as obtained from the oil separator is in crude form. It may have suspended impurities and appreciable moisture content. It might even contain some objectionable constituents which degrade its flavor quality. The presence of moisture and impurities adversely affects the keeping quality of oil and accelerates polymerization and other undesirable reactions. Addition of a drying agent like anhydrous sodium sulphate to the oil, standing overnight followed by filtration will remove the moisture and free the oil of suspended impurities. Use of high-speed centrifugation to clarify the essential oils is common.

Essential oils are frequently rectified or re-distilled to remove objectionable constituents. In order to keep the temperature of re-distillation

within permissible limits, the process is carried out under vacuum or with the help of steam distillation.

7.6.1 Continuous Steam Distillation

Steam distillation units involve manual charging and discharging of plant material from the tank still. These operations are labor intensive and time consuming. To overcome these problems, continuous steam distillation plants have been developed in the Soviet Union and have been in operation since the last couple of decades. These units are being used for distillation of lavender and require negligible manual handing. Capacities of 2 tonnes per hour are quite common. Incoming plant material is first chopped with special ensilage cutters and then conveyed to the top of a tall distillation column by means of a belt conveyor. The movement of material inside the column is by gravity or by special helical screw conveyors. Sometimes two columns in series are used for complete removal of oil. Steam is injected at multiple points in the column. Spent material is continuously ejected out of the bottom of the distillation column by special screw conveyors with a vapor lock which does not allow steam to escape.

Fabrication and operation of continuous distillation columns is rather complicated and these have not yet gained acceptance and popularity outside the former Soviet Union. In another development, containerized distillation is also being used for the distillation of *Mentha piperita* and lavender in some parts of the United States. In this method, large capacity containers mounted on wheels are attached to a harvester which directly loads the plant material into the containers from the fields (these containers have inbuilt steam coils); these are then taken to the distillation area where steam is directly connected to the coils and the top is closed and connected through a vapor line to the condenser and subsequently to the oil separator.

7.7 Conclusions

Distillation is the most widely used method for the extraction of essential oils. Proper selection of the distillation technique, design and material of fabrication of the equipment, and processing parameters all play vital roles in determining the quality and yield of an essential oil.

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8 Microdistillation,Thermomicrodistillation and Molecular Distillation Techniques

V. G. Pangarkar

Abstract

Medicinal and aromatic plants (MAPs) have assumed considerable significance in view of their special attributes. There are many compounds of great therapeutic value which can be obtained only from the plant kingdom. Most of the ingredients in such extracts and oils are large bulky molecules highly sensitive to processing conditions. The processes for such extractions have been mostly based on "recipes". In the recent past, significant advances have been made in the unit operations which are part of the recipe-based processes. It is imperative that these advances, which are essentially aimed at achieving better yields at lower costs and are termed "process intensification", are incorporated into the processing of MAPs. This paper introduces the theme of process intensification as applied to the processing of MAPs and presents an overview of several new technologies which allow rapid, cost-effective extraction. The application of such innovative technologies can yield significant benefits in terms of the quality of the product and its yield per unit weight of the plant material processed. Recovery of dissolved essential oil components from steam distillation condensates is also addressed and the two available techniques are discussed in detail.

8.1 Introduction

Phytochemicals derived from medicinal and aromatic plants (MAPs) have been important to humans for centuries. Before the advent of modern synthetic chemistry, many aroma and flavor chemicals were derived from sources of natural origin such as flowers, roots and stems. The contemporary system of allopathic medicine, which has gained tremendous importance in the treatment of various diseases, is mainly based on active pharmaceutical compounds made synthetically. However, in recent years increasing attention has been paid to the traditional systems of treatments followed in Asia and Africa. The variety of medicinal plants and their constituents are being discovered only recently. There are many compounds of great therapeutic value which can be obtained only from the plant kingdom. Thus, vincristine, perhaps better known as the chemotherapy agent Oncovin, is only synthesized in the periwinkle plant *Catharanthus roseus*, and the sole source of the compound is this plant species. There are many other compounds which are equally valuable in other sectors such as food flavors, fragrances and cosmetics.

The processing of MAPs for obtaining the required extracts and oils has been based on traditionally established "recipes". Most of the ingredients in such extracts and oils are large bulky molecules highly sensitive to processing conditions. Generally, relatively mild conditions are used in such processes to protect the integrity of the valuable components. The

recipe-based methods are time-tested but arguably not the most efficient in terms of yield, energy consumption per unit of product, etc. With the advent of modern processing techniques, there is an urgent need to revisit the recipe-based processing, understand the science underlying them and develop modern, cost-effective processes. This article deals with some important advances made in the extraction of MAPs and the post-extraction treatment of the products and their byproducts.

8.2 Process Intensification

The modern chemical industry is undergoing drastic changes driven mainly by economic considerations. There is an upsurge of interest in clean, energy-efficient and material-conserving processes. An entirely new discipline, "process intensification" (PI), has become the focus of a large and sustained effort all over the world. Stankiewicz and Moulijn have given a precise definition of PI as "Any chemical engineering development that leads to a substantially smaller, cleaner and more energy efficient technology". India has not been lagging behind in developing innovative PI concepts.

PI can be broadly divided into two categories, with specific reference to processing of MAPs as per the definition of Stankiewicz and Moulijn. These are processes that employ multifunctional equipment (MF) and those that use process-intensifying equipment.

8.2.1 Multifunctional Equipment

This category of PI employs equipment that can perform multiple functions simultaneously. Thus, earlier process plants that required a number of different instruments devoted to individual tasks are being replaced by such MF equipment. A brilliant example of the use of MF equipment is the conversion of a slow and polluting process for the enzymatic hydrolysis of penicillin G to 6-amino penicillanic acid (important intermediate for semisynthetic antibiotics) into an intensified and sustainable process. Since MF equipment-based plants are smaller and consume less energy, they have become popular for globally competitive and sustainable processes.

8.2.2 Process-intensifying Equipment

This category of PI employs equipment that specifically focuses on intensifying the rates of the various steps. In the case of MAP processing, the main resistance in the overall extraction process is the diffusion of the active molecules through the plant cell membrane to the surface before extraction by the fluid. Microwave-assisted extraction (MAE) is highly useful in obtaining rapid and complete extraction without significant damage to the active molecules; this technique is discussed in some detail later. Ultrasound-assisted extraction is also an alternative. However, considering

that ultrasound waves can produce free radicals and that many active molecules are susceptible to such highly reactive species, this approach does not seem feasible.

8.3 Solvent Extraction of MAPs

This process entails the extraction of solid MAPs by liquid solvents. This is a typical solid-liquid extraction process. Two factors that affect the extent and rate of extraction are the thermodynamics and kinetics (rate of mass transfer) of the process.

8.3.1 Thermodynamics of Solvent Extraction and Choice of Solvent

Relative sorption of solutes in the solvent depends on the interactions between the solutes and the solvent. Solubility or miscibility of a component with the solvent depends on their relative solubility parameters. For mutual solubility of two components, their free energy of mixing, ΔG_m should be negative. ΔG_m is defined as

$$\Delta G_m = \Delta H_m - T\,\Delta S_m \tag{3.1}$$

Enthalpy of mixing, ΔH_m can be correlated to cohesive energy density, i.e. solubility parameter (δ) as:

$$\Delta H_m = n_1\, n_2\, V_1\, (\delta_1 - \delta_2)^2 \tag{3.2}$$

In equation (3.2), the solubility parameter is that due to only dispersive forces between structural units of the concerned solute and solvent, since the original regular solution theory of Scatchard and Hildebrand was restricted to non-polar, non-hydrogen bonding solute-solvent systems. However, for many liquids and solutes, contributions from polar and hydrogen bonding forces need to be considered. Accordingly, equation (3.2) becomes:

$$\Delta H_m = n_1\, n_2\, V_1\, [(\Delta\delta_d)^2 + (\Delta\delta_p)^2 + (\Delta\delta_h)^2] \tag{3.3}$$

From equations (3.2) and (3.3), it is clear that to make ΔG_m negative, the difference between δ_i (solvent) and δ_i (solute), i.e. ($\Delta\delta$) for all the three forces of interactions, should be as small as possible. It implies that the solvent and the desired solute to be extracted should have comparable polarity and hydrogen bonding capabilities to achieve similar solubility parameter values. Grulke has given an exhaustive tabulation of solubility parameters for the most common chemical compounds. The in-

dividual δ_h, δ_d and δ_p values for compounds not listed in the tabulation can be obtained using the group contributions due to various different groups given by Grulke. When the individual δ_i (solvent) and δ_i (solute) values are very close, a high solubility of the solute in the solvent is obtained. For instance, it is well known that non-polar solvents dissolve terpene fractions more than oxygenated compounds because both are non-polar. On the other hand, mixed solvents of polar and non-polar compounds can yield better results for oxygenated compounds. Bio-ethanol is a good solvent for such oxygenated compounds on two accounts: (i) it is natural, and (ii) it is "green" (renewable). However, most MAPs contain water and the complete miscibility of ethanol with water implies dilution of the solvent after each use. This is further complicated by the fact that ethanol forms an azeotrope at high concentration (~95 wt%). As a result, ingress of small quantities of water is sufficient to reach the azeotropic composition. Implementation of the Montreal Protocol, the Clean Air Act, and the Pollution Prevention Act of 1990 has resulted in increased awareness of organic solvent use in chemical processing.

8.3.2 Solid-liquid Mass Transfer

The MAPs to be processed are in solid form. Solid-liquid extraction is a typical heterogeneous mass transfer process. In such processes, the rate of extraction depends upon: (i) the interface area, and (ii) the mass transfer coefficient. Both should be high. High effective interface area can be obtained by comminuting the solid material to be processed. During comminution, the ensuing friction can increase the temperature of the solid and thereby possibly lead to degradation of thermally labile components. To avoid this, special water-cooled roll crushers are used.

The mass transfer coefficient depends on the diffusivity of the solute in the solid matrix (main resistance) and the level of turbulence in the extractor. Traditional extraction has relied upon percolation or extraction in stirred vessels. In the case of percolation, the solid is packed in a vessel which is filled with solvent. The latter is allowed to percolate in the solid matrix under stagnant conditions. In the case of extraction in stirred vessels, different types of agitators are used to suspend the solid in the solvent and accelerate the mass transfer process. In both percolation and extraction in stirred vessels, the solvent is first sorbed by the matrix of the solid. This sorption, which causes swelling of the matrix, is a relatively slow process. However, once the matrix is swollen, the diffusion coefficient increases several fold or even by an order of magnitude as compared to the dry matrix. Evidently the controlling step is the diffusion of the solute through the solid matrix to the surface of the solid. Once the solute is available at the surface, the solvent can dissolve it depending upon the rate of transport from the solid surface into the bulk of the solvent. In percolation vessels, this latter transport is predominantly by molecular diffusion and hence is slow, although not as slow as the transport through the solid matrix. The

stirred vessels, on the other hand, provide a high level of turbulence and hence facilitate transport into the bulk solvent phase. In both percolation and stirred vessels, the dominant resistance is diffusion through the solid matrix. It is then clear that even stirred vessels with high power inputs may not intensify the mass transfer process. Therefore, instead of focusing on the transport at the solid surface, it is desirable to increase the rate of transport through the solid matrix by rupturing the cells which contain the solute or oil and consequently bring the same in direct contact with the solvent.

8.3.3 Microwave-assisted Extraction

8.3.3.1 Principle of Microwave Heating

Microwave radiation interacts with dipoles of polar and polarizable materials. The coupled forces of electric and magnetic components change direction rapidly (2450 MHz). Polar molecules try to orient in the changing field direction and hence get heated. In non-polar solvents without polarizable groups, the heating is poor (dielectric absorption only because of atomic and electronic polarizations). This thermal effect is practically instantaneous at the molecular level but limited to a small area and depth near the surface of the material. The rest of the material is heated by conduction. Thus, large particles or agglomerates of small particles cannot be heated uniformly, which is a major drawback of microwave heating. It may be possible to use high power sources to increase the depth of penetration. However, microwave radiation exhibits an exponential decay once inside a microwave-absorbing solid.

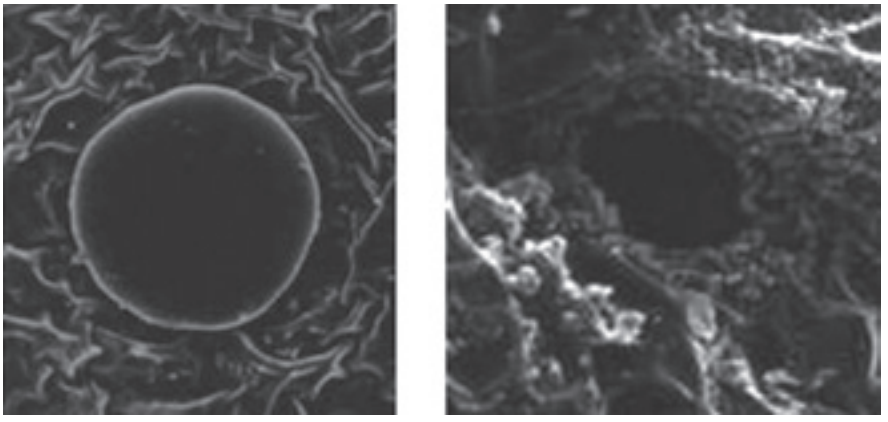
The various industrial techniques used for heating are listed in Table 1, which shows that microwaves have the highest efficiency when compared with the other competitive techniques.

8.3.3.2 Mechanism of MAE

In microwave-assisted extraction (MAE): 1) the heat of the microwave irradiation is directly transferred to the solid without absorption by the microwave-transparent solvent; 2) the intense heating of step 1 causes instantaneous heating of the residual microwave-absorbing moisture in the solid; 3) the heated moisture evaporates, creating a high vapor pressure; 4) the vapor pressure generated by the moisture breaks the cell; and 5) breakage of cell walls releases the oil trapped within it (Figure 1).

Table 1: Relative efficiencies of common heating devices

Appliance	Temperature, °C	Rating, W	Time	Energy used, kWh	Energy cost, US$
Electric oven	177	2000	1 h	2	0.17
Convection oven	163	1853	45 min	1.39	0.12
Gas oven	177	36	1 h	3.57	0.07
Microwave oven	High	1440	15 min	0.36	0.03



A B

Figure 1: Mint gland: (A) before and (B) after microwave irradiation (Microphotographs courtesy of Radient Technologies Inc.)

It is evident then that the main resistance to solid-liquid mass transfer, the transport of the solute through cell membrane, is eliminated because of the rupture of the cells. Besides cell breakage, the other advantages of microwave heating are:

1. Improved “existing” products
2. Increased marker recovery
3. Increased purity of the extract
4. Reduced heat degradation
5. Reduced processing costs
6. Significantly faster extraction
7. Much lower energy usage
8. Much lower (order of magnitude) solvent usage
9. Potential for “new” products

8.3.3.3 Literature on MAE

Some interesting results on MAE have recently been published. For example, the extraction of vanillin from *V. planifolia* pods using MAE and ultrasound-assisted extraction has been described. Using absolute ethanol as the solvent at room temperature, the yield of vanillin was 1.25 wt% at each of 3 conventional extractions performed over 24 h. Using ultrasound-assisted extraction, the yield was 0.99 wt%, while it was 1.86 wt% using MAE. These investigations clearly showed that vanillin extraction by MAE is superior to other techniques in terms of yield, purity of vanillin, and the time taken to extract the same percentage of the vanillin from the pods. The extraction of vanillin and *p*-hydroxy benzaldehyde (PHB) from vanilla beans using MAE has also been studied: MAE was superior to the conventional, official method of extraction in Mexico, which involves maceration of the beans with ethanol for 12 h. Specifically, extraction time decreased 62-fold and vanillin and PHB concentrations increased between 40% and 50% with respect to the Mexican extraction method. This study also showed that extraction of commercial samples was superior to extraction of dried and lyophilized beans. This observation illustrates the role played by moisture in aiding extraction, as discussed in Section 8.3.3.2. Several other investigations have shown that MAE has gained acceptance as a mild and controllable processing tool. MAE is a simple, rapid and low-solvent-consuming process.

8.3.3.4 Industrial-scale MAE

As mentioned earlier, microwave radiation decays exponentially inside a solid matrix. This aspect must be carefully weighed while designing industrial-scale MAE. The major requirements that must be met are:

1. Free distribution of particles allows uniform heating of all the particles in the solid bed. This criterion also enhances the extent and probability of proximity of the substrate to the wall of the sample holder where the microwave exposure is highest. Most comminuted samples of MAPs which are used for commercial extraction are not of the same shape and size. Therefore, there is a strong tendency to "segregate", which must be curbed by regular renewal of the layer.
2. Thin and uniform spreading of the substrate layers. This permits complete and uniform penetration of microwave radiation even at large water contents.
3. Low depth of the layers. Since microwaves have low penetration depth (~1.5 cm in H_2O at 2.45 GHz), the layers should be <1.5 cm thick.

Large-scale commercial (3 tonne/hour) MAE is available for industrial use (www.radientinc.com). In view of the advantages of MAE and the development of equipment for large-scale commercial operation, MAE has a bright future. Figure 2 shows a flowsheet for industrial-scale MAE.

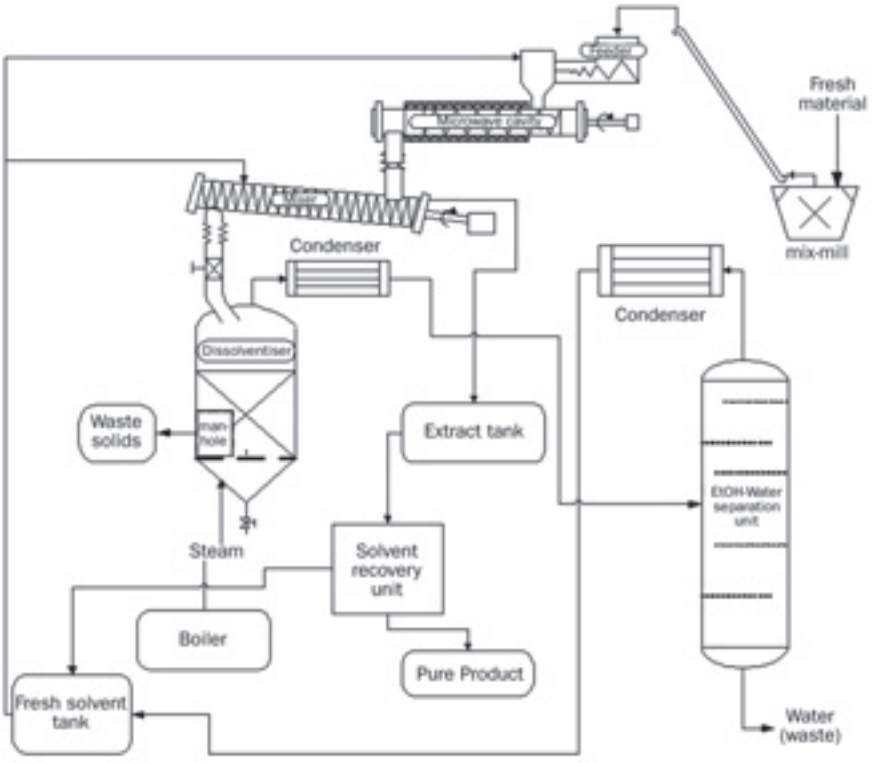


Figure 2: Flowsheet of microwave-assisted extraction (courtesy, Radient Technologies Inc).

8.4 Microwave-assisted Hydrodistillation

The ability of microwave radiation to heat solid material effectively can also be used for obtaining essential oils. Thus, the herb is placed in a microwave cavity and irradiated with microwaves. This process yields essential oils consisting of relatively low volatile fractions as compared to hydrodistillation. For instance, in coriander oil, the percentage of tetradecanoic and hexadecanoic acid increased whereas that of linalool decreased. This is possibly due to the poor stability of linalool, a tertiary alcohol.

Dill seed oil obtained by microwave-assisted hydrodistillation (MWAHD) contained greater quantities of compounds with higher boiling points and lesser quantities of compounds of lower stability. These and other findings indicate that MWAHD is better for extracting stable, high-boiling point components, whereas it is not suitable for recovering chemically unstable compounds.

8.5 Molecular Distillation or Short Path Distillation

Molecular distillation (MD), also known as short path distillation, is a fairly well established technique. In view of this, the discussion of MD is restricted to its principle, advantages and applications in the processing of MAPs.

8.5.1 Principle of MD

The term MD refers to a non-equilibrium process. The still used has an evaporating surface very close to a condensing surface. Under very low pressures, this results in a situation where the distance traveled by the evaporating molecules is comparable to the mean free path of the molecules. The nomenclature MD is derived from this particular condition under which the so-called distillation is carried out.

8.5.2 Advantages of MD

1. Operating pressures as low as 0.001 mbar can yield relatively low processing temperatures, thereby reducing thermal degradation.
2. Agitated film MD units can process high viscosity feeds with very good turndown.
3. Combination of low pressures and high temperatures (up to 300° C) allows processing of extremely high-boiling materials without degradation.
4. Short exposure to high temperature (low residence time) prevents degradation.
5. Very low liquid hold-up allowing use in applications involving low volume, high value materials.
6. Available in low (laboratory scale) to high heat transfer areas to suit the requirements.

8.5.3 Separation Efficiency of MD

For high viscosity liquid films falling under gravity, agitated film MD units perform far better than those without agitation of the film. This is due to the fact that, particularly for high viscosity liquids, the agitation of the film renews the surface more frequently than when there is no agitation. The surface renewal model is useful for predicting the efficiency η of MD stills without mechanical control.

8.5.4 Parameters that Affect the MD Process

The amount of low-boiling volatiles as well as dissolved air, moisture, or other gasses in the feed material has a deleterious effect on the efficiency of MD. This is due to the fact that the non-condensable components cover the condensing surface.

Higher temperature difference between the condensing and evaporating surfaces yields higher efficiency. High viscosity liquids (without mechanical agitation) yield high liquid film thicknesses and hence lower efficiency. As a rule, the relative volatility of organics increases with decreasing pressure, particularly in the very low pressure range common to MD. Therefore, low operating pressure generally yields higher efficiency.

8.5.5 Typical Applications of MD

1. Concretes obtained by solid-liquid extraction are conventionally converted to the absolutes by dissolving in aqueous alcohol solvents and then precipitating the waxes by chilling to sub-zero temperatures. This process is highly energy intensive due to the electrical energy required for refrigeration.
2. Red palm oil (high vitamin E content).
3. Separation of tocopherols from vegetable oil deodorization residues.
4. Natural vitamins A, E, K-1 and K-2 (replacing synthetics in the pharmaceutical industry).
5. Purification and separation of natural extracts into crude fractions.
6. Recovery of lanolin from wool grease, the soft wax from hair of sheep (cosmetic industry).
7. Fragrances derived from fatty acids.

8.6 Recovery of Dissolved Essential Oils from Steam Distillation Condensates

The major prerequisite of the process used for production of essential oils is that the product obtained must resemble the natural aroma and flavor of the original source, which is a combination of different compounds of varying organoleptic characteristics. Oxygenated organic compounds like aldehydes, ketones, alcohols and esters are the dominant contributors to the overall aroma and flavor. The essential oil produced should ideally have all these components in the same proportions as in the original natural product in order to match the natural aroma and flavor. For example, steam-distilled rose oil contains less than 1 wt% phenyl ethyl alcohol (PEA) whereas the solvent-extracted rose oil contains greater than 60 wt% PEA. It is a common experience that the steam distillation condensate has an

odor similar to that of the oil. Thus, this condensate has some value. The sale of rose water (otto of rose) for use in weddings in the sub-continent is probably the only way by which the distiller realizes the value of the condensate. In practically all other cases, the condensate is wasted. Table 2 gives estimates of the values of wasted oil in the condensate in India for some essential oils. The estimate is conservative because it does not include oil physically carried with the condensate. Even this conservative estimate is a mind-boggling number and is particularly important in the social context of developing countries like India where marginal farmers are main contributors to the overall produce. The value of the recovered oil from central distillation facilities and pro rata distribution of the value will be a big bonus to the small farmer and can certainly stop the downslide. Table 2 shows a major contribution from *Mentha arvensis*. If the relatively high value flavor sector is included, for India alone the value of wasted oil can easily reach US$ 100 million. On a rough estimate, the combined number for the South East Asian countries can be upwards of US$ 160 million. It must be noted that these numbers are simple statistics. They do not reflect in any way the high value that can be gained when the recovered oil is blended with the main distilled oil fraction to obtain a premium grade of the respective essential oil.

Table 2: Loss of essential oils in distillation condensate water. Production figures are for India only

Essential oil	Production, 2006, million tonnes	Unit price, 2006	Volume, 2007	Oil lost in water, kg*	Value of oil lost in water, $/yr
Arvensis	28,000	$ 14/kg	35,000 MT	$5.6\text{-}7\times10^{6}$	$46\text{-}58\times10^{6}$
Basil	100	$ 8/kg	100 MT	1×10^{4}	8.2×10^{4}
Citrodora	100	$ 8/kg	100 MT	1×10^{4}	7.4×10^{4}
Citronella	300	$ 8/kg	300 MT	3×10^{4}	2.52×10^{5}
Peppermint	450	$ 23/kg	450 MT	4.5×10^{4}	1.3×10^{5}
Spearmint	250	$ 23/kg	300 MT	2.5×10^{4}	7.2×10^{4}
Total	29,200		36,200 MT	$5.72\text{-}7.72\times10^{6}$	$47\text{-}59\times10^{6}$

** Data refer to 100 kg water per kilogram oil. Solubility of oil in water is 1000 ppm.*

8.6.1 Polymeric Adsorption Process

Various techniques, such as cohobation, poroplast extraction and adsorption, which can be used to recover the dissolved substances, have been discussed in the literature. Polymeric adsorbents can be advantageously used to recover dissolved essential oil components. Several investigators have established the utility of adsorption in this context beyond doubt. One study showed that although cis-rose oxide could not be detected in the condensate, this valuable component was found in the recovered oil

in significant proportions. These investigations show that more than 95% of the oil in the condensate can be recovered. The polymeric adsorbents used are hard cross-linked macroreticular beads which can be used in adsorption-regeneration cycles practically indefinitely. The technique is simple to use and does not require sophisticated instrumentation as the breakthrough can be judged from the smell of the water coming out of the adsorbent bed. The regeneration of the spent bed can be done using low-boiling alcohols or ketones, and the eluate can be distilled in a relatively short distillation column to obtain a relatively high boiling oil fraction.

8.6.2 Pervaporation Process

Membrane separation processes have been receiving increasing attention particularly for situations involving recovery from relatively dilute (~1000 ppm) aqueous solutions. Pervaporation is one such process which yields very high (~1000 ppm or more) selectivity in the very dilute solution range. Essential oil components which have high affinity for organophilic polymers can be recovered at very high selectivities. One study showed that silicone rubber membranes yielded bold menthol crystals when the *Mentha* condensate water was studied under the pervaporation mode. Similar results were also obtained for basil water. Subsequent studies showed that the high selectivity of properly selected membranes results in a permeate concentration far exceeding the solubility limit of the organics, resulting in phase separation.

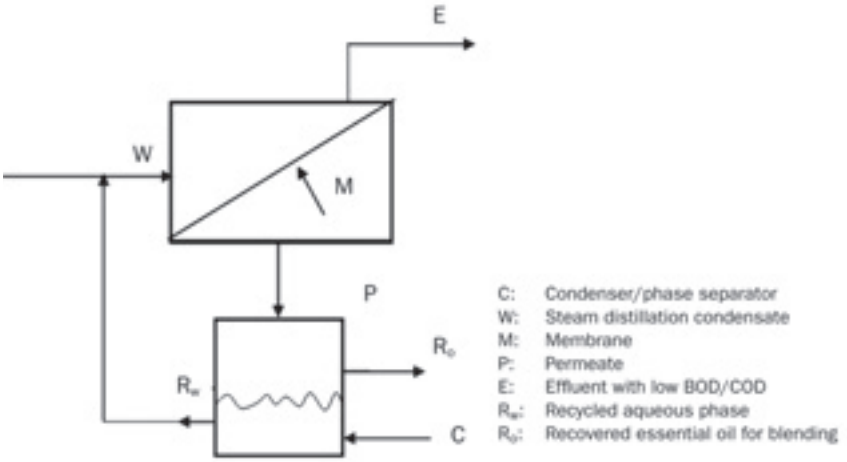


Figure 3: Recovery of dissolved organics using pervaporation

The separate oil layer can be directly recovered to blend with the main oil fraction to obtain premium grade oil. Figure 3 shows a schematic of pervaporation-based recovery of dissolved essential oils in the condensate. It is evident that this technique consists of a closed-loop operation

with only treated water going out of the battery limits. This treated water has very low biochemical oxygen demand (BOD) and chemical oxygen demand (COD), which is another bonus for the processor.

8.7 Conclusions

Various new technologies for efficient and cost-effective extraction of medicinal and aromatic plants have been discussed. Microwave-assisted extraction (MAE) is highly efficient for obtaining extracts under mild conditions. MAE is particularly important since the active components which are thermally labile can be recovered without any damage. The loss of valuable aroma components in steam distillation condensates is estimated to be of the order of US$ 50 million per year from aroma oils for India alone. Two types of separation processes – adsorptive and membrane-based pervaporation – are useful in recovering practically all the oil that is lost with the condensate water. The recovered oil can be sold as such or blended with the main oil fraction to yield a much more natural aroma and hence a high value. This recovered oil will be a big bonus even for the marginal farmer and hence this approach needs to be seriously considered.

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9 Solid Phase Micro-extraction and Headspace Trapping Extraction

R. Harlalka

Abstract

Solid phase micro-extraction (SPME) is a technique used in the quantitative analysis of analytes in aqueous and gaseous phases. This novel technology captures aroma molecules surrounding flower petals without touching the flower or other part of the plant. SPME has gained widespread acceptance as the technique of choice in many fields of application, including forensics, toxicology, and the analysis of flavors, fragrances, and environmental and biological matrices. SPME is ideal for field monitoring. SPME sampling can be performed in three basic modes: direct extraction, headspace trapping and extraction with membrane protection. Headspace trapping is essentially a gas extraction technique permitting the direct analysis of volatile compounds present in a non-volatile matrix. This technique is needed because the aromas of living plant materials are different from those of the extracted oil. Headspace trapping permits getting closer to the natural aroma of the living plant, and gives a clearer view of the differences in volatile constituents between the living plant and the extracted phase. There are two types of headspace trapping: static and dynamic, which is also called the purge-and-trap method. A few examples of headspace trapping of well known aromatic flowers, fruits and leaves, in comparison to the analyses of the extracted oil, are presented in this paper. Some classical perfumes are also discussed.

9.1 Introduction

Solid phase micro-extraction (SPME) was developed in the 1990s by Professor J. Pawliszyn to provide a quick and solventless technique for the isolation of analytes from a sample matrix. The traditional methods by which the analytes of interest were isolated are typically time- and labor-intensive and involve multistep procedures, which could reduce sensitivity. Also, the use of solvents can be hazardous to the operators' health and can damage the environment.

SPME was developed from the technique of solid phase extraction, but the sorbing material is permanently attached to the fiber, allowing reuse of the extracting phase. SPME uses a small volume of sorbent, typically dispersed on the surface of small fibers, to isolate and concentrate analytes from the sample matrix. After contact with the sample, analytes are absorbed or adsorbed by the fiber phase (depending on the nature of the coating). After the extraction step, the fibers are transferred, with a syringe-like handling device, to the analytical instrument, for separation and quantification of the analytes. This technique integrates sampling, extraction and sample introduction, and is a simple way of performing on-site monitoring. Applications of this technique include environmental monitoring, fragrance drug analysis, and in-laboratory and on-site analyses.

SPME was introduced in 1990 as a solvent-free sample preparation technique. The basic principal of this approach is to use a small amount of the extracting phase, usually less than 1 microliter. Sample volume can be large when the investigated material is sampled directly, e.g. the air in a room. The extracting phase can be either a high molecular weight polymeric liquid, similar in nature to stationary phases in chromatography, or a solid sorbent, typically of a high porosity, to increase the surface area available for adsorption.

The configuration of SPME is a small, fused silica fiber, usually coated with a polymeric phase. The fiber is mounted for protection in syringe-like device. The analytes are absorbed or adsorbed by the fiber phase until equilibrium is reached in the system. The amount of an analyte extracted by the coating at equilibrium is determined by the magnitude of the partition coefficient of the analyte between the sample matrix and the coating material.

In SPME, analytes typically are not extracted quantitatively from the matrix. Equilibrium methods are more selective because they take full advantage of the difference between extracting phase and matrix distribution constants to separate target analytes from interferences. Exhaustive extraction can be achieved in SPME, and this can be accomplished for most compounds by the application of an internally cooled fiber. In exhaustive extraction, selectivity is sacrificed to obtain quantitative transfer of target analytes to the extracting phase.

SPME is ideal for field monitoring. It is unnecessary to measure the volume of the extracted sample, and therefore the SPME device can be exposed directly to the investigated material for quantification of analytes of interest. In addition, extracted analytes are introduced into the instrument by simply placing the fiber in the desorbtion unit. This convenient, solvent-free process results in sharp injection bands and rapid separations.

9.2 The SPME Device

The commercial SPME device manufactured by Supelco (Bellefonte, USA) is presented in Figure 1. The fiber glued into a piece of stainless steel tubing is mounted on a special holder. The holder is equipped with an adjustable depth gauge, which makes it possible to control repeatedly, how far the needle of the device penetrates the sample container or the injector. This is important, as the fiber can break if it hits an obstacle. The movement of the plunger is limited by a small screw that moves in the z-shaped slot of the device. For protection during storage or septum piercing, the fiber is withdrawn into the needle of the device, with the screw in the uppermost position. During extraction or desorption, the fiber is exposed by depressing the plunger. The plunger is moved to its lowermost position only for replacement of the fiber assembly. Each type of fiber has a hub of a different color.

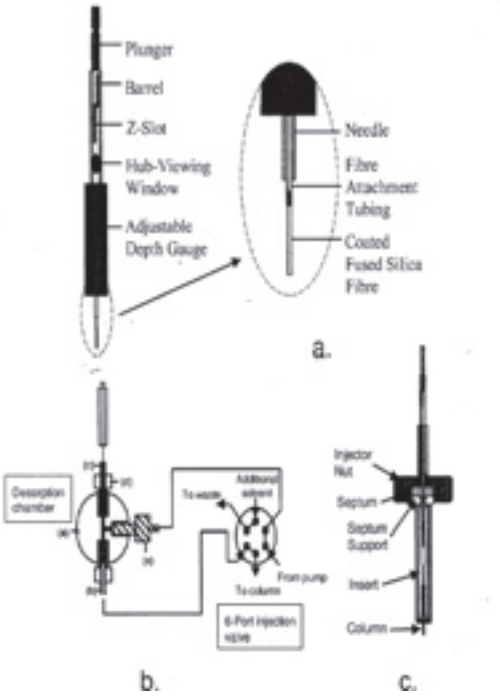

Figure 1: The SPME device

If the sample is in a vial, the septum of the vial is first pierced with the needle (with the fiber in the retracted position), and the plunger is lowered, which exposes the fiber to sample. The analytes are allowed to partition into the coating for a pre-determined time, and the fiber is then retracted back to the needle. The device is then transferred to the SPME instrument. When gas chromatography is used for analyte separation and quantification, the fiber is inserted into a hot injector, where thermal desorption of the trapped analyte takes place.

For spot sampling, the fiber is exposed to a sample matrix until partitioning equilibrium is reached between sample matrix and the coating material. In the time average approach, on the other hand, the fiber remains in the needle during exposure of the SPME device to the sample. The coating works as a trap for the analytes that diffuse into the needle, resulting in integral concentration over time measurements.

SPME sampling can be performed in three basic modes: direct extraction, headspace trapping, and extraction with membrane protection.

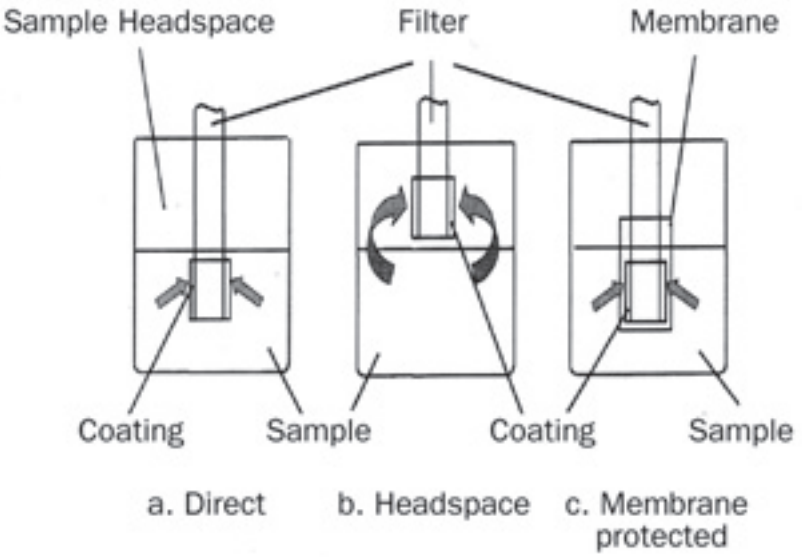

Figure 2: Modes of SPME operation: direct extraction (a), headspace trapping (b) and membrane-protected SPME (c)

In direct extraction, the coated fiber is inserted into the sample and the analytes are transported directly from the sample matrix to the extracting phase. To facilitate rapid extraction, some agitation is required to transport the analytes from the bulk of the sample to the vicinity of the fiber. For gaseous samples, natural flow of air (e.g. convection) is usually sufficient to facilitate rapid equilibrium for volatile analytes.

In headspace mode, the analytes are extracted from the gas phase equilibrated with the sample. The primary reason for this modification is to protect the fiber from the adverse effects caused by non-volatile, high molecular weight substances present in the sample matrix (e.g. human acids or proteins). Here, the amount of an analyte extracted by the fiber coating does not depend on the location of the fiber, in the liquid or gas phase; therefore, the sensitivity of headspace trapping is the same as that of direct sampling as long as the volumes of the two phases are the same in both sampling modes. When no headspace is used in direct extraction, a significant sensitivity difference between direct and headspace trapping can occur only for very volatile analytes. However, the choice of sampling mode has a significant impact on the extraction kinetics. When the fiber is in the headspace, the analytes are removed from the headspace first, followed by indirect extraction from the matrix.

In general, the equilibration times for volatile compounds are shorter for headspace SPME than for direct extraction under similar agitation conditions, because of the following reasons: a substantial portion of the analytes is present in the headspace prior to the beginning of the ex-

traction process; there is typically a large interface between sample matrix and headspace; and the diffusion coefficients in the gas phase are typically higher by four orders of magnitude than in liquids. The concentration of semivolatile compounds in the gaseous phase at room temperature is small, and headspace extraction rates for these compounds are substantially lower. They can be improved by using efficient agitation or by increasing the extraction temperature.

In the third mode (SPME extraction with membrane protection), the fiber is separated from the sample with a selective membrane, which lets the analytes through while blocking the interferences. The main purpose for the use of the membrane barrier is to protect the fiber against adverse effects caused by high molecular weight compounds when dirty samples are analyzed. While headspace trapping serves the same purpose, membrane protection enables the analysis of less volatile compounds. Use of thin membranes and an increase in extraction temperature result in shorter extraction times.

9.3 Calibration, Optimization, Precision and Suitability of SPME

9.3.1 Selection of Fiber Coating

The chemical nature of the analyte of interest determines the type of coating used. A simple general rule, “like dissolves like”, applies very well for liquid coatings. Selection of the coating is based primarily on the polarity and volatility of the analyte. Poly(dimethylsiloxane) (PDMS) is the most useful coating and should be considered first. It is rugged and able to withstand high injector temperatures, up to about 300° C. PDMS is a non-polar liquid, thus it extracts non-polar analytes very well with a wide linear dynamic range. However, it can also be applied successfully to more polar compounds, particularly after optimizing extraction conditions.

Both the coating thickness and the distribution constant determine the sensitivity of the method and the extraction time. Thick coatings offer increased sensitivity, but require much longer equilibration times. As a general rule, to speed up the sampling process, the thinnest coating offering the sensitivity required should be used.

9.3.2 Selection of the Extraction Mode

Extraction mode selection is based on the sample matrix composition, analyte volatility, and its affinity to the matrix. For dirty samples, the headspace or fiber-protection mode should be selected. For clean matrices, both direct and headspace trapping can be used. The latter is applicable for analytes of medium to high volatility. Headspace trapping is always preferen-

tial for volatile analytes because the equilibration times are shorter in this mode than in direct extraction. Fiber protection should be used only for dirty samples in cases where neither of the first two modes can be applied.

9.3.3 Selection of the Agitation Technique

Equilibration times in the gaseous samples are short and frequently limited only by the rate of diffusion of the analytes in the coating. When the aqueous and gaseous phases are at equilibrium prior to the beginning of the sampling process, most of the analytes are in the headspace. As a result, the extraction times are short even when no agitation is used. However, for aqueous samples, agitation is required in most cases to facilitate mass transport between the bulk of the aqueous sample and the fiber.

Magnetic stirring is most commonly used in manual SPME experiments. Care must be taken when using this technique to ensure that the rotational speed of the stirring bar is constant and that the base plate does not change temperature during stirring. This usually implies the use of high quality digital stirrers. Alternatively, with cheaper stirrers, the base plate should be thermally insulated from the vial containing the sample to eliminate variations in sample temperature during extraction. Magnetic stirring is efficient when fast rotational speeds are applied.

9.3.4 Selection of Separation or Detection Technique

Most SPME applications have been developed for gas chromatography (GC), but other separation techniques, including high performance liquid chromatography, capillary electrophoresis (CE) and supercritical fluid chromatography, can be used in conjunction with this technique. The complexity of the extraction mixture determines the proper quantitative device. Regular chromatographic and CE detectors can normally be used for all but the most complex samples, for which mass spectrometry (MS) should be applied.

9.3.5 Optimization of Desorption Conditions

Standard gas chromatographic injectors, such as the popular split-splitless types, are equipped with large volume inserts to accommodate the vapors of the solvent introduced during liquid injections. As a result, the linear flow rates of the carrier gas in those injectors are very low in splitless mode, and the transfer of the volatilized analytes onto the front of the GC column is also slow. No solvent is introduced during SPME injection; therefore, the large insert volume is unnecessary. Opening the split line during SPME injection is not practical, since it results in reduced sensitivity. Efficient desorption and rapid transfer of the analytes from the injector to the column require high linear flow rates of the carrier gas around the coating. This can be accomplished by reducing the internal diameter of the injector insert, matching it as closely as possible to the outside diameter of the coated fiber.

9.3.6 Optimization of Sample Volume

The volume of the sample should be selected based on the estimated distribution constant. The distribution constant can be estimated by using published values for the analyte or a related compound, with the coating selected. The distribution constant can also be calculated or determined experimentally by equilibrating the sample with the fiber and measuring the amount of analyte extracted by the coating.

9.3.7 Determination of the Extraction Time

The equilibration time is defined as the time after which the amount of analyte extracted remains constant and corresponds within the limits of experimental error to the amount extracted after infinite time. Care should be taken when determining the equilibration time, since in some cases a substantial reduction of the slope of the curve might be wrongly taken as the point at which equilibrium is reached. Determination of the amount extracted at equilibrium allows calculation of the distribution constants.

When equilibrium times are excessively long, shorter extraction times can be used. However, in such cases the extraction time and mass transfer conditions have to be strictly controlled to assure good precision. At equilibrium, small variations in the extraction time do not affect the amount of the analyte extracted by the fiber.

On the other hand, at the steep part of the curve, even small variations in extraction time may result in significant variations of the amount extracted. Shorter is the extraction time, larger is the relative error. Autosamplers can measure the time precisely, and the precision of analyte determination can be good, even when equilibrium is not reached in the system. However, this requires that the mass transfer conditions and the temperature remain constant during all experiments.

9.3.8 Optimization of Extraction Conditions

An increase in extraction temperature increases the extraction rate but simultaneously decreases the distribution constants. In general, if the extraction rate is of major concern, the highest temperature that still provides satisfactory sensitivity should be used.

Adjustment of the pH of the sample can improve the sensitivity of the method for basic and acidic analytes. This is related to the fact that unless ion exchange coatings are used, SPME can extract only neutral (non-ionic) species from water. By properly adjusting the pH, weak acids and bases can be converted to their neutral forms, so that they can be extracted by the SPME fiber.

9.3.9 Determination of the Linear Dynamic Range of the Method

Modification of the extraction conditions affects both the sensitivity and the equilibration time. It is advisable, therefore, to check the previously determined extraction time before proceeding to the determination of the linear dynamic range. This step is required if substantial changes in sensitivity occur during the optimization process.

SPME coating includes polymeric liquids, such as PDMS, which by definition have a broad linear range. For solid sorbents, such as Carbowax/DVB or PDMS/DVB, the linear range is narrower because of the limited number of sorption sites on the surface, but it still can span over several orders of magnitude for typical analytes in pure matrices. In some rare cases when the analyte has extremely high affinity to the surface, saturation can occur at low analyte concentrations. In such cases, the linear range can be expanded by shortening the extraction time.

9.3.10 Selection of the Calibration Method

Standard calibration procedures such as external calibration can be used with SPME. The fiber blank should first be checked to ensure that neither the fiber nor the instrument causes interference with the determination. The fiber should be conditioned prior to the first use by desorption in a GC injector or in a specially designed conditioning device. This process ensures that the fiber coating itself does not introduce interference. Fiber conditioning may have to be repeated after analysis of samples containing large amounts of high molecular weight compounds, since such compounds may require longer desorption times than the analytes of interest.

A special calibration procedure, such as isotopic dilution or standard addition, should be used for more complex samples. In these methods, it is assumed that the target analytes behave similarly to spikes during the extraction. This is usually a valid assumption when analyzing homogeneous samples.

9.3.11 Precision of the Method

The most important factors affecting precision in SPME are:

- Agitation conditions
- Sampling time (if non-equilibrium conditions are used)
- Temperature
- Condition of the fiber coating (cracks, adsorption of high molecular weight species)
- Geometry of the fiber (thickness and length of the coating)
- Sample matrix components (salt, organic material, humidity, etc.)

- Time between extraction and analysis
- Analyte loss (adsorption on the walls, permeation of Teflon, absorption by septa)

9.3.12 Suitability

SPME is well suited to the analysis of flavor and fragrance compounds. The typically small, volatile compounds are easily extracted by the fibers, and the simplicity of the method allows easy coupling to analytical instruments. Headspace trapping can reduce the potential for interference peaks and prevent contamination of both the needle and the instrument. Loss of these volatile compounds during sample preparation steps is minimized or eliminated compared to conventional methods, and the method is amenable to field sampling and analysis.

SPME has been shown to be useful for semivolatile compounds, even though these appeared more challenging in the early years. With appropriate matrix modification, one can take advantage of headspace trapping for these as well. SPME provides significant convenience for field and air analysis. Quantification is relatively straightforward, even in the presence of varying air temperature. Finally, the use of SPME for time-weighted average sampling provides simplicity in monitoring flavor and fragrance concentrations over time.

9.4 Headspace Trapping Extraction and GC-FID/MS Analysis

Orange juice volatiles were extracted from the juice headspace using a syringe-like SPME device equipped with a 75 μm Carboxen-PDMS fiber (Supelco). Aliquots (25 ml) of juice were placed in 40-ml glass vials with plastic screw caps and Teflon-coated septa, warmed to 40° C, and gently swirled to coat the walls of the vial. Juices were allowed to equilibrate for at least 15 min prior to fiber insertion and were maintained at 40° C throughout the 35-min extraction period. The fiber was then removed from the headspace and inserted into the heated GC injector, where the volatile compounds were thermally desorbed. Flavor extract was separated using an HP 5890 GC instrument equipped with a 30 m x 0.32 mm i.d. DB5 capillary column. Column temperature was initially 32° C, with a 3-min hold, and was then increased at 6° C/min to 200° C. Helium carrier gas linear velocity was 29 cm/s. A special narrow boar (0.75 mm) injector liner was used to improve peak shape and chromatographic efficiency; the entire separation was conducted in the splitless mode.

9.4.1 History of Headspace

In November 1986, at the 10th International Congress on Essential Oils, in Washington DC, USA, Dr. B. D. Mookherjee presented a paper on the impact of "live vs. dead" on headspace trapping extraction, using as example jasmine flowers.

The SPME needle which is 2- to 3-mm solid glass fiber coated with a high-boiling liquid adsorbent, is placed in close proximity to a flower without touching it and is kept there for a period of 30-60 min depending on the odor strength of the blossom. The aroma molecules around the petals are absorbed onto the fiber. Then with GC/MS, the fiber is analyzed to determine the aroma profile of that particular flower. The aroma of the living flower was brought into space by NASA in 1998.

9.4.2 The Aura

When the Sun is totally eclipsed by the moon, the surrounding glow is called an aura. Similarly, if we consider a drop of fragrance, the molecules surrounding the drop form an aura of that particular fragrance.

It is a common belief that one smells a fragrance, layer by layer, from the top note of the volatile components, to the middle note of components with boiling points in the middle range, and finally to the bottom note of components with the highest boiling point.

In reality, when a drop of fragrance is placed on the skin, several different molecules, from the lowest to the highest boiling types, irrespective of their molecular weights, boiling points and vapor pressures, form an aura, which eventually reaches our nose and gives us our first impression of the particular fragrance. The composition of this aura depends on a characteristic property of each fragrance molecule, knows as its diffusivity.

9.4.3 What is Diffusivity?

Diffusivity is the inherent property of a compound to emit its molecules into the air. One compound is said to be more diffusive than another if its molecules tend to pass into the air to a greater extent than those of other compounds. Diffusivity is independent of boiling point, molecular weight, odor threshold or odor value.

9.4.4 Application of Headspace Trapping

Some examples of headspace trapping are discussed here.

9.4.4.1 Jasmine

The headspace constituents of living and picked *Jasminum grandiflorum* flowers are:

Compound	Living flowers, %	Picked flowers, %
Benzyl acetate	60.0	40.0
Linalool	3.0	30.0
Indole	11.0	2.0
Cis-jasmone	3.0	–
3,5-Dimethyl-2-ethyl pyrazine	–	0.5
Epi-methyl jasmonate	0.5	–
Methyl jasmonate	0.3	–

Differences in the volatile compounds of living flowers from *Jasminum grandiflorum* and *Jasminum sambac* are:

Compound	*J. grandiflorum*, %	*J. sambac*, %
Methyl benzoate	–	5.0
Benzyl acetate	60.0	37.0
Indole	11.0	5.0
Linalool	3.0	9.0
Epi-methyl jasmonate	0.5	–
Methyl jasmonate	0.3	–

9.4.4.2 Yellow Tea Rose

The differences in headspace constituents between living and picked yellow tea rose flowers are:

Compound	Living flowers, %	Picked flowers, %
Cis-3-hexenyl acetate	20.67	5.39
Hexyl acetate	8.40	4.26
Phenylethyl alcohol	5.73	3.30
3,5-Dimethoxy toluene	9.96	18.58
Alpha-elemene	–	4.07
Geranyl acetone	2.17	–
Dihydro-beta-ionol	–	2.62
Isocaryophyllene	0.30	2.12
Alpha-farnesene	5.83	2.96

9.4.4.3 Passion Flower

Volatile constituents of living passion flower (*Passiflora* spp.) are:

Compound	Living flowers, %
Methyl benzoate	90.3
Methyl salicylate	1.1
Methyl cinnamate	1.6

9.4.4.4 Lotus

The major differences in headspace constituents of living and picked lotus (*Nelumbo nucifera*) are:

Compound	Living flowers, %	Picked flowers, %
Sabinene	6.0	12.0
p-Dimethoxy benzene	18.0	8.0
4-Terpineol	3.0	1.5
Alpha terpineol	9.0	1.0
Cis-jasmone	0.1	–
C15 hydrocarbons	20.0	30.0

9.4.4.5 Lavender

The volatile constituents of living French lavender (*Lavandula dentata)* and English lavender (*Lavandula angustifolia*) are:

Compound	French lavender, %	English lavender, %
Limonene	18	6
1-Octen-3-ol	–	7
Hexyl acetate	11	2
Eucalyptol	9	3
Linalool	7	–
Cis-3-hexenyl acetate	17	13
Borneol	–	2
Cryptone	–	6

9.4.4.6 Chamomile

The volatile constituents of living Roman and German chamomile are:

Compound	Roman chamomile, %	German chamomile, %
Ethyl 2-methyl butyrate	–	12
Cis-3-hexenyl acetate	3	22
Isobutyl methacrylate	7	–
Isobutyl angelate	18	–
Ocimene	–	1
Iso-amyl angelate	10	–
Isohexyl angelate	10	–

9.4.4.7 Shefali

The volatile constituents of living shefali (*Nycanthus arbortristis*) are:

Compound	Living flower, %
Benzyl alcohol	11.2
Phenyl acetaldehyde	9.4
Phenyl ethyl alcohol	6.3
Methyl anthranilate	10.7

9.4.4.8 Spearmint

The major differences between living and picked spearmint are:

Compound	Living plant, %	Picked plant, %
Hexanal	0.5	–
Hexanol	–	–
Beta-pinene	0.8	2.0
Sabinene	0.5	–
Myrcene	8.8	4.0
Alpha-phellandrene	0.7	–
Limonene	18.0	2.0
Cis- and trans-ocimene	1.0	–
Dihydrocarvone	0.6	2.6
Carvone	24.0	70.0
Alpha- and beta-elemene	5.0	0.1
Beta-caryophyllene	4.0	0.1

9.4.4.9 Cinnamon Bark

Comparative analysis of fresh cinnamon bark headspace and commercial oil has revealed:

Compound	Fresh cinnamon bark, %	Commercial oil, %
Cis- and trans-cinnamic aldehyde	80.3	71.7
Eugenol	–	12.7
Ortho-methoxy cinnamaldehyde	0.3	–
Eugenyl acetate	–	0.5

9.4.4.10 Ginger

Comparative analysis of the headspace of fresh ginger root and commercial oil has revealed:

Compound	Fresh ginger root, %	Commercial oil, %
Citral	15.3	1.2
Beta-bisabolene	3.3	6.2
Alpha zingiberene	15.2	34.4
Cis- and trans-alpha-farnesene	13.7	6.0
ar-Curcumene	11.3	4.8
Beta-sesquiphellandrene	8.0	11.8

9.4.4.11 Peach

Volatile constituents of living and picked peach (*Prunus persica*) are:

Compound	Living peach, %	Picked peach, %
Ethyl acetate	6.2	–
Dimethyl disulfide	0.6	–
Cis-3-hexenyl acetate	9.7	–
Methyl octanoate	34.2	7.1
Ethyl octanoate	7.4	11.0
6-Pentyl alpha pyrone	Trace	10.6
Gamma decalactone	2.5	39.2

9.4.4.12 Pineapple

Volatile constituents of the interior and exterior of living pineapple are:

Compound	Exterior, %	Interior, %
Methyl hexanoate	13.3	24.6
Ethyl hexanoate	25.6	4.7
Methyl 3-methylthiopropionate	0.9	0.5
Ethyl 3-methylthiopropionate	1.1	–

9.4.5 Classical Perfumes

Almost all successful classical perfumes are based on floral aromas. Perfumers created them using natural flower oil such as rose and jasmine. Few persons are aware of the fact that fruit and flower oils that are made by extraction of picked material exhibit different aromas from those of the living entities. Examples of classical perfumes based on floral aromas are Amarige (Givenchy), Joy (Jean Patou), White Linen (Estée Lauder), Aura (Hugo Boss), Anais Anais (Cacharel) and Beautiful (Estee Lauder). The difference in composition between the oil and the aura of Amarige is as follows:

Compound	Oil, %	Aura on skin after 60 min, %
Linalool	1.7	17.9
Benzyl acetate	4.9	22.7
Styrallyl acetate	1.2	9.7
Cashmeran	–	0.5
Bacdanol	0.2	0.5
Hedione	29.9	4.9
Cedramber	1.5	4.9
Iso E super	7.1	12.1
Ambrox	0.2	0.1
Benzyl salicylate	32.5	1.1
Muskalactone	0.9	0.4

The examples include Joy by Jean Patou; White Linen by Estée Lauder; Aura of Hugo BOSS; Anais Anais by Cacharel; and Beautiful by Estee Lauder.

9.4.6 Need for Headspace

When we go to a rose-field full of bloomed roses, we detect a pleasant smell in the atmosphere and expect the same fragrance when we use the bottled perfume or 100% genuine essential oil extracted from the same roses. However, this is not true. The fragrance we detect in the field is completely different from the bottled perfume or essential oil, for the following reasons:

a) When a flower or herb is processed to obtain the essential oils, the low volatile compounds cannot always be recovered and often evaporate. These low volatiles are important for aroma.
b) During the processing of an herb, many chemical reactions take place, such as saponification, trans-esterification, polymerization and condensation. These reactions actually change the character of the oil, so that its aroma no longer resembles that of the actual plant and the product is different in composition. Many stereoterpenes, which are highly volatile in nature, cannot be extracted and remain in the herbs. These stereoterpenes do not contribute directly to the odor but, in combination with other ingredients, impart a synergic effect to the overall odor quality.

9.5 Types of Headspace Trapping

Headspace trapping can be static or dynamic, which is generally called the purge-and-trap method. In static headspace trapping, gas extraction is carried out in a single step or in a limited number of steps. On the other hand, the purge-and-trap technique consists of two or three separate steps, the first of which is continuous gas extraction.

9.5.1 Static Headspace Trapping

This is a single-step gas extraction procedure (Figure 3). By thermosetting the sample for a certain time at a preselected temperature, equilibrium is reached between the sample phase and the gas phase of the sample vial. Subsequently, a single aliquot of the headspace is introduced into the carrier gas flow, which then carries it to the column where the volatile compounds are separated in the usual way.

The equilibrium of the two phases in the sample vial is characterized by a partition coefficient (Ki) representing the ratio of the analyte's concentration in the sample phase and in the gas phase.

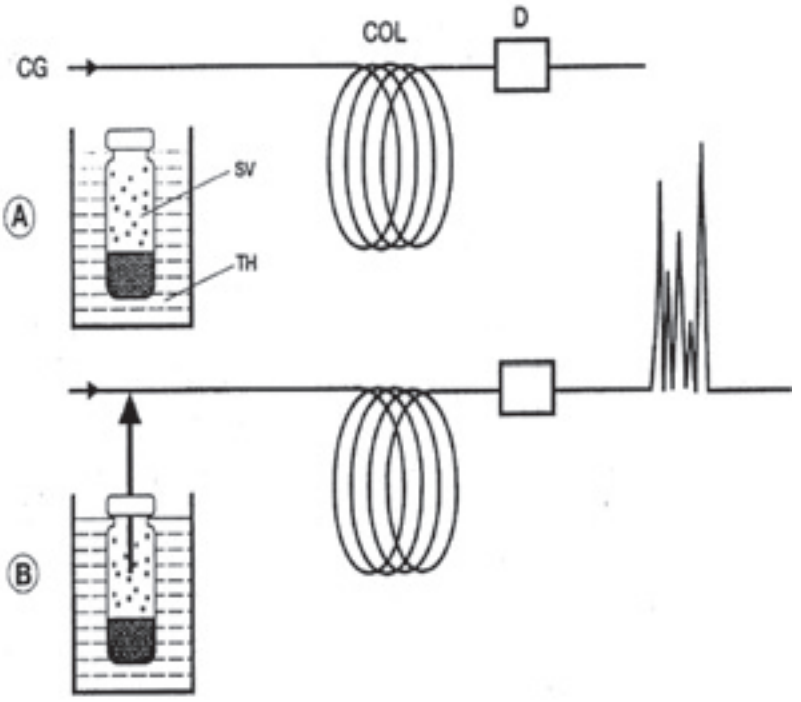

Figure 3: Static headspace trapping technique

9.5.2 Dynamic Headspace Trapping

In this technique (Figure 4), the sample is continuously purged with an inert gas (the purge gas), until all volatile compounds are removed. During this step, the gas effluent leaving the sample vessel is conducted through a trap, either cooled to low temperature or containing an adsorbent. This trap retards the volatile analytes purged from the sample. When gas extraction is complete, the condensed or adsorbed analytes by rapid heating of the trap now get purged with the carrier gas.

In Figure 4, the desorbed analytes are conducted directly into the gas chromatograph. Thermal desorption from an adsorbent is not instantaneous: thus, the sample "slug" might be too long, creating broad peaks, with tailing. This is particularly the case when a capillary column is used in the gas chromatograph. For this reason, usually a second, small trap, cooled to low temperature, is placed in the carrier gas line between the primary trap and the column. When desorption is finished, this small trap is then heated very rapidly: in this way, a sharp band of the analytes enters the column.

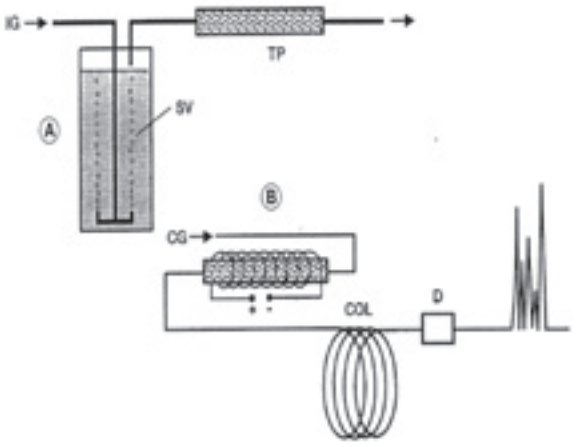

Figure 4: Dynamic headspace trapping technique

This technique is being used more generally after the introduction of Tenax (poly(2,6-diphenyl-*p*-phenylene oxide)) as a universal adsorbent for dynamic headspace GC by Zlatkis and his group, at the University of Houston, in 1973. They used the technique for the investigation of biological fluids and demonstrated the reproducibility of the purge-and-trap method.

9.6 Principles of Static Headspace-GC Systems

Gas from the headspace of a closed vessel can be sampled simply with a gas-tight syringe. However, with such a manual method, it is difficult to reproduce all the conditions necessary for reliable quantitative analysis. Therefore, today, headspace-gas chromatography (HS-GC) is carried out almost exclusively with automated instruments, in which thermosetting, aliquoting the headspace and introducing it into the gas chromatograph are fully automated. In this way and using the proper calibration methods, the required precision, accuracy and reliability are assured.

Present-day HS-GC instruments are of two types. In the first, the headspace aliquot is taken by an automated syringe which then is moved above the injection port of the gas chromatograph and the sample is injected. In essence, such systems are similar to the autosamplers used in GC. In the second case, the aliquot from the vial's headspace is not withdrawn by suction as in the case of a syringe: instead, after equilibrium is reached, the vial is pressurized by the carrier gas. After pressurization there are two possibilities. The carrier gas flow can be temporary interrupted while the pressurized gas in the vial is allowed to expand onto the column; the transferred volume of headspace can be accurately controlled by controlling the time of transfer and the pressure. The second possibility is to have a gas introduced between the sample vial and the column, and fill the sample

loop of the valve by the pressurized headspace gas. Today, automated instruments based on these principles are commercially available.

9.6.1 Trace Analysis by HS-GC

HS-GC in both its dynamic and static versions permits the determination of analytes at low concentrations. Usually the dynamic technique is considered to be more sensitive; however, this is not necessarily true. For example, trace impurities in a water sample, at the parts-per-billion level, can be determined relatively easily by static HS-GC.

9.7 Headspace Trapping Techniques

9.7.1 Static Headspace Trapping

Using the static method (Figure 5), a food sample is normally placed in a heated vessel, which is sealed gas-tight by a septum. The food sample stays inside the vessel for a certain period of time, so that the volatile compounds evaporate to a certain concentration in the air or to certain equilibrium. In order to determine the best conditions for the experiment, the odor of the headspace can be checked by sniffing the vessel. Subsequently, a distinct volume is taken out of the vessel by a gas-tight syringe and directly injected into a gas chromatographic column, with or sometimes without prior concentration (e.g. cryofocussing). The advantage of this method is that it accurately assesses the composition of the odorants. An application of this technique, called GC olfactometry of static headspace samples, has been widely used to identify the highly volatile compounds causing the first odor impression of foods.

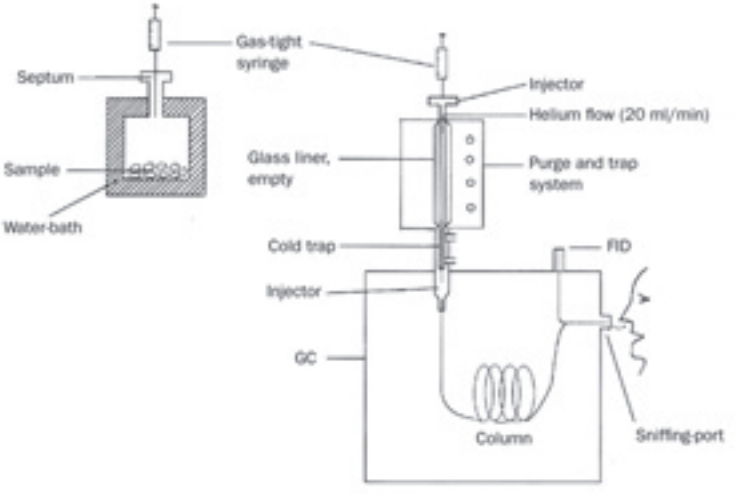

Figure 5: Static headspace trapping technique

However, static headspace samples are normally too small to quantify odorants that are present only at low concentrations in the vapor phase. In other words, one can smell them, but in many cases it is not possible to obtain a signal in a mass spectrometer.

9.7.2 Dynamic Headspace Trapping

To overcome the disadvantages of headspace trapping method, dynamic headspace trapping can be used (Figure 6). Again, the food sample is placed in a heated vessel but the evaporating compounds are continuously swept by a stream of inert gas into a trap containing a porous polymer, which adsorbs more or less the organic constituents. This method yields a much higher amount of trapped volatiles so that, after desorption, it is no longer problematic to obtain an MS signal.

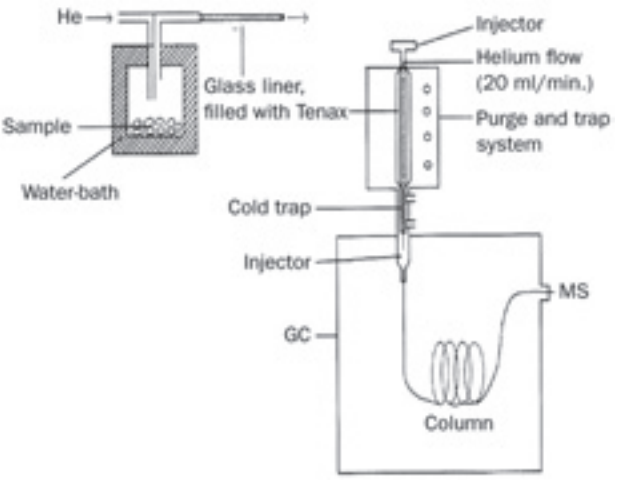

Figure 6: Dynamic headspace trapping technique

However, the disadvantage of this procedure is the strong dependence on the yield of the odorants, on the velocity of the carrier gas and on the selectivity of the adsorption and desorption process for different compounds. It is very difficult to control these parameters precisely and therefore, the results of such quantitative measurements might be inaccurate.

9.7.3 Recovering the Adsorbed Volatiles by Thermal or Liquid Solvent Desorption

Several studies have reported methods of desorption using organic solvents. Drawbacks of the use of solvent desorption include the loss of volatile compounds during removal of excess solvent before GC analysis, solvent selectivity and solvent impurities. We recently developed a sensitive and highly reproducible dynamic headspace (DHS) protocol with thermal

desorption (using injector glass liners packed with Tenax-TA as adsorbent traps for aroma collection at ambient room temperature) and desorption at the interior of a GC injector. This DHS-type protocol was used to characterize fresh tomato flavor compounds; the results were compared with published data from a static headspace method (Table 1).

Table 1: Concentration of selected tomato aromas from heat-processed tomato juice by static headspace trapping (SHT) and dynamic headspace trapping (DHT), expressed in parts per billion (ppb)

Compound	SHT, ppb	DHT, ppb
(E)-2-hexanal	5	340
1-Penten-3-one	61	100
2-Isobutylthiazole	2	450
2-Methylfuran	97	1,060
2-Pentylfuran	26	700
3-Methybutanal	17	750
3-Methylfuran	717	3,200
6-Methyl-5-hepten-2-one	21	1,330
Acetone	325	-
Benzaldehyde	3	30
Dimethyl disulfide	16	630
Dimethyl sulfide	5,205	2,974
Ethanol	311	-
Geranial	2	130
Hexanal	188	6,210
Pentanal	48	470

In the present study, this DHT-type protocol was used to characterize fresh tomato flavour compounds for comparison with related literature methods.

9.7.4 Some Practical Examples of Headspace Technique Use

9.7.4.1 Tomato Juice

Fresh tomato juice was made from vine-ripe fruit by Campbell Soup Company's R&D centre in Davis, USA. Chemicals were reagent grade, supplied from reliable sources.

9.7.4.1.1 Preparation of Traps

Traps were prepared using silane-treated glass tubing (79 mm x 6 mm) packed with 13 mg 60/80 mesh Tenax-TA (2,6-diphenyl-*p*-phenylene oxide) polymers held in place by silanized glass wool. The traps were initially conditioned at 330° C for 2 h under nitrogen gas at a 20 ml/min flow rate. The traps were regenerated at 250° C for 1 h immediately before each purge-and-trap experiment.

9.7.4.1.2 Thermal Desorption

Adsorbed volatile compounds were recovered from the trap directly inside the GC injector. The desorption time and temperature were previously determined. The injector temperature was 200° C and a loop of the analytical column at the injector end was immersed in a liquid nitrogen-filled Dewar flask to cryogenically trap the desorbed volatiles. Subsequently, the injector glass liner (insert) was replaced with the trap to desorb volatiles. Thermal desorption was carried out for 5 min with the split vent and septum purge closed.

9.7.4.2 Headspace of *Hedychium coronium*

Hedychium coronium flower has a delicate, pleasant fragrance, but the essential oil and concrete extracted by traditional methods usually lose this fragrance. Thus, the headspace of the *H. coronium* flower was analyzed. The essential oil of *H. coronium* flowers, which was absorbed by XAD-4 resin, eluted by organic solvent and concentrated, had a fragrance similar to the natural fragrance of *H. coronarium* flowers.

9.7.4.3 Volatiles of White Hyacinths Isolated by Dynamic Headspace Trapping

More than 70 constituents of white hyacinths can be identified by GC and GC-MS. The principal constituents are benzyl acetate and (E,E)-α-farnesene. Beside these, sensorily important substances like indole, oct-1-en-3-ol and phenylacetaldehyde were identified. Minor traces of three substituted pyrazines were detected by GC-sniffing. The advantages of the simultaneous closed-loop stripping technique using various adsorbing agents at the same time were demonstrated. By this method, artifact formation and discrimination of individual components can be determined

9.7.4.4 Medical Materials Testing by Headspace Trapping-GC-MS

The new technology provided by the HS-40 Trap coupled with a sensitive detection method such as GC-MS allows volatile organic compounds in medical sutures to be analyzed easily at trace levels. Individual compounds present in the sutures can be analyzed by GC-MS and identified

by a NIST library search of the acquired mass spectral data. The innovative, patent-pending, headspace trapping technology used in this application provides sensitivity beyond the capability of traditional static headspace. This presents a new level of detection capability for the evaluation of materials used in medical applications, as well as in other types of material testing, including pharmaceutical formulations and food-packaging film.

9.8 Conclusions

Advanced technologies such as SPME and headspace trapping extraction are well suited for the analysis of flavor and fragrance compounds. The typically small, volatile compounds are easily extracted. The simplicity of the method allows easy coupling to analytical instruments. Loss of volatile compounds during sample preparation steps is minimized or eliminated, compared to conventional methods.

These techniques are useful for semivolatile compounds, even though these were more challenging in the early years. With appropriate matrix modification, one can take advantage of this analytical method, which provides significant convenience for field and air analyses.

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10 Supercritical Fluid Extraction of Medicinal and Aromatic Plants: Fundamentals and Applications

A. Bertucco and G. Franceschin

Abstract

The main issues related to supercritical fluid extraction of medicinal and aromatic plants are discussed in view of the development of this separation technique at industrial scale. After an introduction to supercritical fluid extraction, the roles of thermodynamics and mass transfer properties are emphasized, and the effects of the main operating variables on product recovery are briefly examined. Fundamental concepts about the equipment needed and basic technology are presented, including economical evaluation. Finally, a short literature survey of successful supercritical extraction processes of medicinal and aromatic plants is reported and a future outlook is given.

10.1 Introduction

In the second half of last century, an increasing interest has been paid to supercritical fluids as alternate solvents for the extraction of natural bioactive molecules from plants. The main reason for the interest in supercritical fluid extraction (SFE) was the possibility of carrying out extractions at temperature near to ambient, thus preventing the substance of interest from incurring in thermal denaturation.

A thorough review of the results achieved up to the early 1980s is presented in a book by Stahl *et al.*, published in 1986. Clearly, by that time, the fundamentals of this new extraction process were already understood, even though the technical-economical assessment and the design criteria for large-scale application of SFE were still missing. After twenty years of research and development, it is now possible to say that such achievements are at hand, so SFE is currently a well-established unit operation for extraction and separation. Moreover, its design and operating criteria are fully understood, so that it can profitably be applied in the extraction of medicinal and aromatic plants (MAPs).

10.2 Supercritical Fluids

A fluid at supercritical condition, also referred to as a dense gas, is a fluid above its critical temperature (T_C) and critical pressure (P_C) to a certain extent: to be supercritical, the reduced temperature T_r (i.e. T/T_C) must not exceed 1.2 or 1.3, whereas the reduced pressure P_r (i.e. P/P_C) may be as high as allowed by technological limits.

At suitable conditions, any fluid can reach its supercritical state. However, only those having a critical temperature not far from ambient temperature can be used as alternative solvents for the extraction of MAPs. Carbon dioxide (CO_2), with T_C=31.06° C and P_C=73.81 bar, is the most attractive solvent, because of its proprieties regarding toxicity, flammability and cost.

The possibility of using supercritical fluids (SFs) as extraction solvents is directly linked to their density. In fact, according to an empirical correlation proposed by Chrastil in 1982,

$$s = \rho^a \exp\left(\frac{b}{T} + c\right) \quad (1)$$

where *s* is the solute solubility, ρ is the solvent density and *T* is the absolute temperature; *a*, *b* and *c* are correlation parameters to be adjusted to experimental solubility data in supercritical CO_2.

When a fluid approaches the critical conditions, its density gets closer and closer to that of the liquid state. This can be seen, for CO_2, in Figure 1, where density isotherms are plotted against the reduced pressure. For example, at T = 35° C and P = 200 bar, ρ = 866 kg/m$^{3.}$

It is also clear from Figure 1 that, close to the critical point, both the compressibility and expansion coefficient of the fluid are high, so slight changes in the operating conditions can significantly modify the density, i.e. the supercritical fluid solvent power. The importance of the Chrastil equation (Eq. 1) lies in the fact that solvent density is identified as the key factor in a successful SFE process.

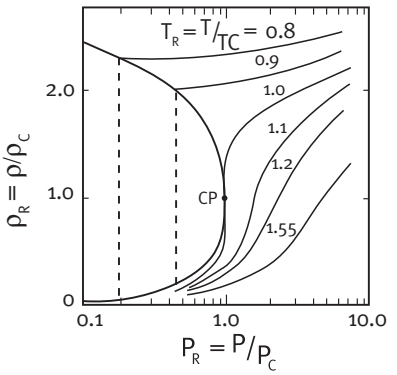

Figure 1: Density vs. pressure diagram for carbon dioxide

When plotted against solvent density, solubility data for supercritical CO_2 always display a regular trend such as that in Figure 2a, whereas a more complex behavior is seen when pressure is improperly used as the independent process variable (Figure 2b).

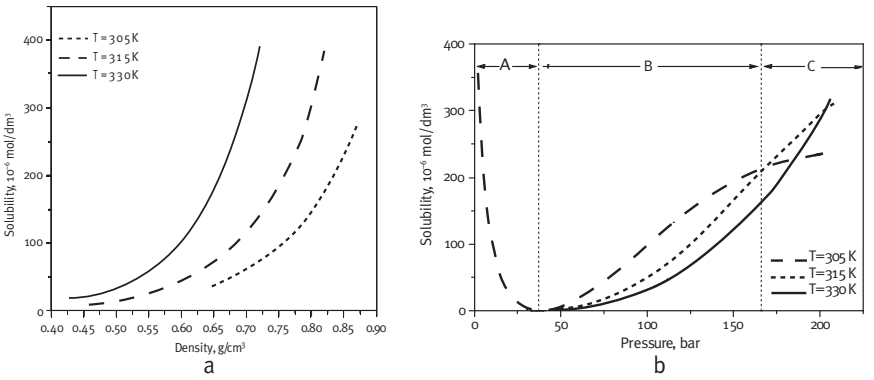

Figure 2: Solubility of 1,4-bis-(n-propylamino)-9,10-anthraquinone in supercritical CO_2: as a function of solvent density (2a) and of pressure (2b)

Coming back to Eq. 1, is important to point out that it is not theoretically correct and must be applied within restricted temperature ranges only.

More importantly, the exponential form of Eq. 1 does not guarantee that the solubility of a solute in SF is high. The solubility depends on parameters *a*, *b* and *c*; in fact, in the case of CO_2, the solubility of a solute of interest for MAP applications is at best in the range of 1 to 1000 by weight or often 1 to 10,000 (Figure 2a illustrates this). This is because CO_2 is a poor solvent, even at supercritical conditions. In addition, this holds for non-polar substances only, as supercritical CO_2 does not dissolve polar molecules at all. Actually, CO_2 is a good solvent only for low molecular weight solutes.

The limit on solubility is dictated by thermodynamics. According to the iso-fugacity criterion applied to the substance to be extracted, between the two phases at equilibrium (the condensed one – either solid or liquid – and the supercritical one), we have:

$$y_i = \frac{P^{sat}}{P} E_i \tag{2}$$

$$E_i = \frac{\varphi_i^{o,v}}{\varphi_i^{v}} \exp\left(v^{S/L} \frac{P - P^{sat}}{RT}\right) \tag{3}$$

where y_i is the mole fraction of *i* in the supercritical phase, $\varphi_i^{o,v}$ and φ_i^{v} are the fugacity coefficients of *i* in the standard state and in the mixture, respectively, at the process conditions, P^{sat} is the solute saturation (or sublimation) partial pressure (i.e. the component volatility), and $v^{S/L}$ is the molar volume of the condensed phase (either solid or liquid). *T* is the absolute temperature and *R* is the universal gas constant. E_i is the so-called enhancement factor, which accounts for the increasing solubility due to system nonidealities with respect of the ideal behavior (given by Eq. 2).

According to Eq. 2, the solubility of *i* in the SF can be calculated at the process condition, provided that the fugacity coefficients φ_i can be evaluated accurately by means of an equation of state. However, the substance vapor pressure directly influences the solubility when P^{sat} is as usual for MAPs, very low. Only an equally low value of the fugacity coefficient, i.e. a high system nonideality, can partially counteract the lack of volatility of the pure component.

Regardless of the way its value has been obtained, i.e. from Eq. 1 or Eqs. 2-3, the solubility is only one of two fundamental pieces of information that must be known in order to assess the feasibility of an SFE process for MAPs. The second one is selectivity, which is defined as the ratio of the solubility of the substance *i* of interest with respect to a reference substance *j*:

$$\alpha_{ij} = \frac{s_i}{s_j} \tag{4}$$

If on one hand high solubility is desirable, to reduce the solvent consumption per unit product extracted, on the other hand selectivity must be as far as possible from 1, to ensure that the substance of interest is extracted as pure rather than in mixture with other components. In summary, to develop a successful SFE process for MAPs, both solubility and selectivity issues must be fulfilled properly.

Coming back to CO_2, it must be kept in mind that this solvent is rather non-selective: when it is able to dissolve a group of similar substances (for example, in terms of carbon atoms), all of them are extracted to a similar extent, provided they have similar polarities.

Therefore, it can be stated that CO_2 alone is not as selective as a good and pure solvent. It is also noteworthy that CO_2 capacity and selectivity may be improved by using an organic solvent as the entrainer, also called the co-solvent, with the function of modifying chemical interactions between CO_2 and the substance to be dissolved in it.

But by doing this, the SFE process becomes more complicated, as an extra chemical component needs to be introduced into the process. However, the co-solvent can be easily separated from the product downstream, due to the high selectivity displayed by supercritical CO_2 in this respect.

10.3 SFE Processes

An SFE process for extracting MAPs is basically composed of two main sections (Figure 3a). The feed, containing the substance of interest, indicated by A, comes in contact with supercritical CO_2, at suitable temperature

and pressure, in an extraction device. In this simple scheme, component A is selectively extracted and must be recovered from the supercritical solution, which is usually a dilute one for the reason explained in the previous section. Product recovery occurs in the separation section, whose temperature and pressure can be adjusted in order to optimize the amount of A produced.

Note that, due to the low solubility in supercritical CO_2, after recovery of the product of interest the solvent must be recycled and pumped back to the extractor, in order to minimize operating costs. It is also noteworthy that the separator can be operated either at the same temperature or at the same pressure of the extractor, the best condition resulting from an economical analysis of the overall production costs.

If the temperature is kept constant, product separation is achieved by depressurization (Figure 3a), and mechanical energy has to be provided to the system to raise the CO_2 pressure from the separator to the extractor conditions. On the other hand, extracted products can be separated from CO_2 by increasing the temperature, and thermal energy must be supplied in this case (Figure 3b), where the circulation of the solvent can be done at nearly isobaric conditions. Of course, the way the separation of products from CO_2 is achieved can be more complex: for instance, both temperature and pressure can be varied when passing from the extractor to the separator sectors, or a solid can be used to promote separation by adsorption.

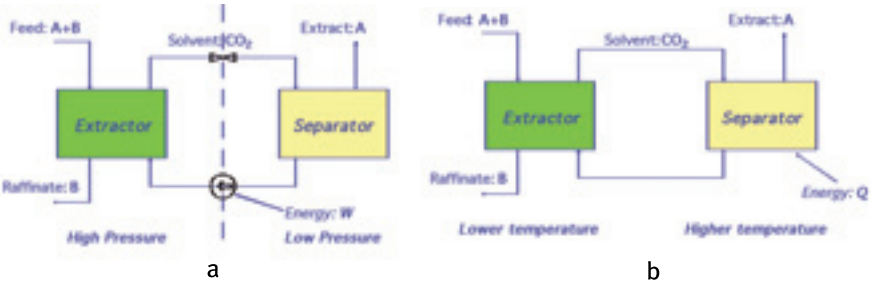

Figure 3: Block flow diagrams of simple SFE processes: with separation obtained by pressure change (a) and by temperature change (b)

If, as it often occurs, many substances are extracted by CO_2 at the extraction conditions because of lower CO_2 selectivity, their fractionation can also be achieved in the separation section, by simply using more than one separator, operated at different conditions. As shown in Figure 4, multiple fractions with different properties can be recovered from the same extraction.

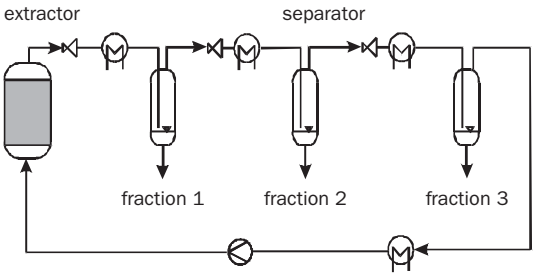

Figure 4: Single extractor, multiple separator scheme

Finally, a multiple extractor scheme can also be envisaged, as represented in Figure 5 with only one separation step, for sake of clarity. This configuration is particularly useful when, as in the case of SFE of MAPs, the substances to be extracted are embedded in a solid matrix, which is initially loaded in the extraction vessel as a fixed bed. In this case, the extractors can be connected either in parallel or in series, depending on specific requirements.

More details on the development of SFE processes are provided in a book by Brunner listed in the bibliography. SFE of solids is a semibatch operation, which can also be operated in a simulated moving bed configuration to obtain a continuous production.

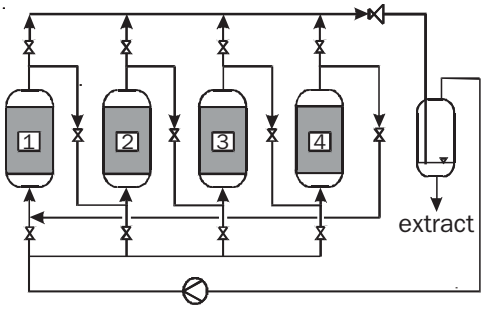

Figure 5: Multiple extractor, single separator scheme

Typical extraction profiles from solid materials (single vessel) are shown in Figure 6 where the extractor yield, i.e. the amount of substance of interest extracted with respect to the total amount initially contained in the solid, is plotted against extraction time. The profiles, which are steeper if the temperature is higher, have two parts: a straight line corresponding to the extraction of the substance "readily available" to supercritical CO_2, and an asymptotic curve representing the extraction of the part attached to the solid matrix. In the first case, the extraction is limited by solubility; in the second, mass transport (diffusion) properties are important and can be limiting and crucial for the success of SFE.

The effects of operating temperature are also clear in Figure 6. Other important operating variables are pressure, CO_2 flow rate and humidity of the material to be extracted.

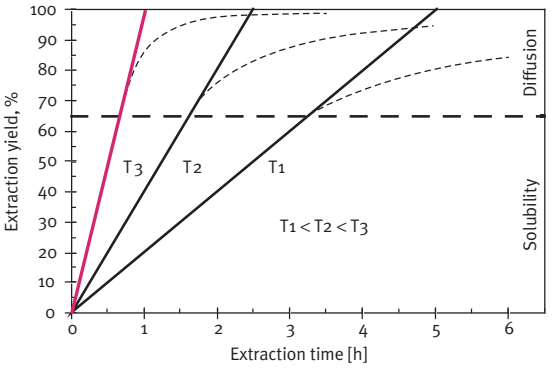

Figure 6: Extraction yield versus time at different temperatures

10.4 The SFE Process and Equipment Development

In order to design and develop an SFE process for MAPs with CO_2 (possibly assisted by ethanol or water as entrainers), we need to know and optimize:

1. The solubility of the substance of interest
2. The selectivity of this substance with respect to others that are extracted simultaneously
3. The extraction profiles (such as those in Figure 6)
4. The way to separate the substance of interest from the total extract

All this information can be obtained by simple measurements performed in a laboratory-scale apparatus of minimum volume such as that illustrated in Figure 7.

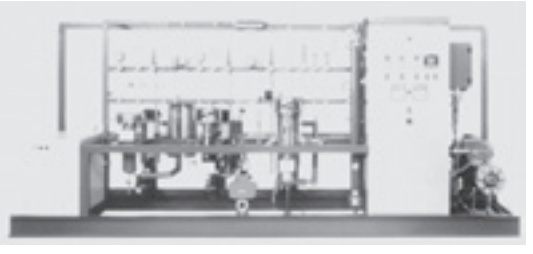

Figure 7: Laboratory-scale apparatus for SFE process design

To measure extraction profiles, a small pilot-scale apparatus can be used. Extractor and separator volumes do not need to exceed 1 liter each. The analytical system must be suitable to measure the concentration and purity of the products of interest. Basic requirements in terms of equipment are:

1. A liquid CO_2 storage tank
2. A pump for liquid CO_2
3. A cooler to prevent CO_2 from evaporating in the pump
4. A heat exchanger to control the temperature of CO_2 entering the extractor
5. An extraction vessel
6. A heat exchanger to control the CO_2 plus solute mixture entering the separator
7. A separation vessel

Note that condensing and recycling of CO_2 after separation is not needed at the laboratory-scale developmental level, whereas these are essential requirements at the industrial production level.

All parts of the SFE laboratory-scale plant must be designed in order to resist the maximum operating pressure. If this does not exceed 300-350 bar, the entire equipment (e.g. vessels, valves, fittings) is pretty much of standard type and relatively inexpensive. If, as usual, stainless steel is used, the thickness of any part of the plant can be easily calculated by applying the Von-Mises equation:

$$P_i \frac{k^2}{k^2 - 1} \sqrt{3} < \sigma_{am} \tag{5}$$

with:

$$\sigma_{am} = \frac{\sigma_s}{S_f} \tag{6}$$

where P_i is the internal pressure, k is the external to internal diameter ratio, σ_s is the yield stress, and S_f is a suitable safety factor (usually S_f=1.5).

From Eq. 5, it can be seen that the thickness of a cylindrical vessel depends on its diameter. Examples are given in Table 1 for a vessel of 0.2 m internal diameter, with both stainless steel and carbon steel construction materials.

Table 1: Thickness (s) of a thick-wall cylindrical vessel of 200 mm internal diameter as a function of pressure (SS=stainless steel, CS=carbon steel)

P [atm]		**50**	**100**	**150**	**200**	**250**	**300**
s [mm]	SS	3.8	8.1	12.9	18.5	25.0	32.7
	CS	2.2	4.6	7.2	10.0	13.0	16.2

Special care must be paid to closures and seals. SFE of MAPs is mostly an extraction operation from solid materials, which is carried out in batch or semibatch mode. Therefore, extraction vessels need to be pressurized, depressurized, opened, filled, and closed again several times per day. In order to ensure fast and safe operation procedures and reliable seals, gaskets like O-rings are useful and closure devices have been specifically designed. Again, the technology needed is already fully developed. We refer to chapter 4 of the book by Bertucco and Vetter for details. The book also describes the machinery for moving fluids under pressure, i.e. pumps and compressors. We conclude that setting up a laboratory-scale apparatus with which to perform feasibility studies concerning the possibility of applying SFE to MAPs is not really an issue, and can be done with a relatively small capital cost.

However, this does not mean that SFE of MAPs is in itself an economically convenient operation. An accurate evaluation of production costs, including both capital and utility costs, must be performed before scaling up a process whose technical feasibility has been demonstrated at the laboratory level. Costs are also discussed in the book by Bertucco and Vetter (chapter 8), but are only indicative. The reader should remember that capital costs have been steadily decreasing in the last years must be taken into account.

10.5 SFE Applied to Medicinal and Aromatic Plants

A large number of MAPs has been considered for possible extraction by supercritical CO_2. The most recent developments suitable to have industrial relevance are listed in Table 2. These examples illustrate the great potential of SFE in this field.

Table 2: Medicinal and aromatic plants extracted by SFE

Plant name (part used)	Product(s) extracted	Reference
Calendula officinalis (flowers)	Oleoresin	Campos *et al.,* 2005, Experimental data and modeling the supercritical fluid extraction of marigold (*Calendula officinalis*) oleoresin, *J Supercritical Fluids*, 34: 163-170 Danielski *et al.,* 2007, Marigold (*Calendula officinalis* L.) oleoresin: solubility in SC-CO_2 and composition profile, *Chem Eng Proc*, 46: 99–106

Plant name (part used)	Product(s) extracted	Reference
Echinacea purpurea (whole herb)	Alkamides, polyphenolics including chichoric acid, carbohydrates	Catchpole *et al.,* 2002, Supercritical extraction of herbs I: saw palmetto, St John's wort, kava root, and Echinacea, *J Supercritical Fluids*, 22: 129-138
Eucalyptus spp. (leaves)	Essential oil	Della Porta, *et al.,* 1999, Isolation of eucalyptus oil by supercritical fluid extraction, *Flavour Fragr J,* 14: 214–218
Ginkgo biloba (leaves)	Flavonol glycosides (flavonoids) and terpenoids	Chun Yang *et al.,* 2002, Extraction of pharmaceutical components from *Ginkgo biloba* leaves using supercritical carbon dioxide, *J Agric Food Chem,* 50: 846-849
Hypericum perforatum (herb)	Naphthodianthones, hypericin and pseudohypericin	Catchpole *et al.,* 2002
Levisticum officinale (dry rhyzomes, roots)	Essential oil	Daukšas *et al.,* 1999, Supercritical CO_2 extraction of the main constituents of lovage (*Levisticum officinale* Koch.) essential oil in model systems and overground botanical parts of the plant, *J Supercritical Fluids,* 15: 51–62
Matricaria chamomilla (flowers)	Oleoresin	Kotnik *et al.,* 2007, Supercritical fluid extraction of chamomile flower heads: comparison with conventional extraction, kinetics and scale-up, *J Supercritical Fluids*, Available online 13 February 2007 (in print)
Mentha spp. (leaves)	Essential oil	Marongiu *et al.,* 2001, Extraction and isolation of *Salvia desoleana* and *Mentha spicata* subsp. insularis essential oils by supercritical CO_2, *Flavour Fragr J,* 16: 384–388
Origanum spp. (herb)	Essential oil	Leeke *et al.,* 2002, *Eng Chem Res,* 41: 2033–2039
Piper methysticum (roots, rhizomes)	Kava lactones	Catchpole *et al.,* 2002
Piper nigrum (fruit)	Oleoresin	Ferreira *et al.,* 1999, Supercritical fluid extraction of black pepper (*Piper nigrum* L.) essential oil, *J Supercritical Fluids*, 14: 235–245.

Plant name (part used)	Product(s) extracted	Reference
Saccharum spp. (crude wax)	Long chain n-alcohols	De Lucas *et al*., 2005, Supercritical extraction of long chain n-alcohols from sugar cane crude wax, *J Supercritical Fluids*, 34: 163-170
Salvia desoleana (leaves)	Essential oil	Marongiu *et al.,* 2001
Serenoa repens (fruit)	Free fatty acids, phytosterols (low concentrations), fatty alcohols and triglycerides	Catchpole *et al.,* 2002
Solanum lycopersicum (fruit)	Carotenoids, tocopherols and sitosterols	Vagi *et al.*, 2007, Supercritical carbon dioxide extraction of carotenoids, tocopherols and sitosterols from industrial tomato by-products, *J Supercritical Fluids*, 40: 218–226
Taxus brevifolia (bark)	Taxol	Jennings *et al.,* 1992, Supercritical extraction of taxol from the bark of Taxus, *J Supercritical Fluids*, 5: 1-6
*Taxus cuspidate (*needles)	Paclitaxel and baccatin III	Moon-Kyoon Chun *et al.,* 1996, Supercritical fluid extraction of paclitaxel and baccatin III from needles of *Taxus cuspidate*, *J Supercritical Fluids*, 9: 192-198
Vitis vinifera (seeds)	Procyanidins	Cao *et al.,* 2003, Supercritical fluid extraction of grape seed oil and subsequent separation of free fatty acids by high-speed counter-current chromatography, *J Chromatogr* A 1021: 117–124
Zingiber officinale (rhizome)	Oleoresin	Badalyan *et al.,* 1998, Extraction of Australian ginger root with carbon dioxide and ethanol entrainer, *J Supercritical Fluids*, 13: 319-324

10.6 Conclusions

SFE with CO_2 is a technically and possibly economically valid technique to extract bioactive components from MAPs. Organic solvent-free products can be obtained and the low operating temperature makes it possible to preserve all their natural properties. The feasibility study on specific products can be performed rather easily at laboratory scale. However, accurate evaluation of production costs, including both capital and operating ones, must be done in order to exploit SFE at the industrial level.

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11 Process-scale High Performance Liquid Chromatography for Medicinal and Aromatic Plants

M. M. Gupta and K. Shanker

Abstract

High performance liquid chromatography (HPLC) is widely used by chromatographers and by the pharmaceutical industry for the accurate and precise analysis of chemicals and drugs of diverse nature. The systematic scale-up from analytical to preparative and process scale and further scale-up to industrial scale can be used in the medicinal and aromatic plant industry for the isolation and purification of phytomolecules of therapeutic and commercial interest. Due to the gradual increase in the demand for phytomolecules, the importance of process-scale HPLC as a purification tool has been increasing. In this article, we discuss the practical aspects of process-scale HPLC and focus on terminology, operational problems, advantages and applications of this technology to medicinal and aromatic plants.

11.1 Introduction

The term liquid chromatography (LC) refers to a range of chromatographic systems, indicating liquid-solid, liquid-liquid, ion-exchange and size exclusion chromatography. Glass column chromatography is an example of classic liquid column chromatography in which the mobile phase percolates under gravity through a glass column filled with a finely divided stationary phase. Liquid chromatography has overtaken gas chromatography, as high performance liquid chromatography (HPLC) systems now provide features such as:

i) High resolving power
ii) Fast separation
iii) Continuous monitoring of column effluent
iv) Qualitative and quantitative measurements and isolation
v) Automation of analytical procedures and data handling

There has been tremendous growth in this technique since 1964 when the first HPLC instrument was constructed by Csaba Horvath at Yale University. For the isolation of compounds, preparative mode HPLC (prep-HPLC) can be used in pharmaceutical development for trouble-shooting purposes or as part of a systematic scale-up process. The importance of prep-HPLC in pharmaceutical production as a purification tool has been increasing. Chromatographic separation can remove impurities of different polarity and can reduce the content of an enantiomer in a racemic mixture. In both of these instances, crystallization may be used to prepare the pure product. Bench to pilot scale production of natural products needs some

form of automation: thus, developing well-automated preparative chromatographic methods is a necessary but demanding task.

Innovations in micro-analytical to preparative HPLC played an important role in the progress of natural product chemistry. HPLC is used routinely in phytochemistry to pilot the preparative-scale isolation of natural products and to control the final purity of the isolated compounds. The development of hyphenated techniques related to this efficient separation technique in the past 20 years has provided powerful new tools such as LC/UV-photodiode array detection, LC/mass spectrometry (LC/MS) and LC/NMR. The combination of high separation efficiency of HPLC with these different detectors has made possible the acquisition of data on an LC peak of interest within a complex mixture.

11.2 Theoretical Aspects of HPLC

Separation of chemical compounds is carried out by passing the mobile phase, containing the mixture of the components, through the stationary phase, which consists of a column packed with solid particles. The cause for retention is physical and chemical forces acting between the solute and the two phases, on the chromatographic column. The reason for retention is the difference in the magnitude of forces; this results in the resolution and hence separation of the individual solutes. The separation of compounds occurs by distribution of solutes between the two phases.

11.2.1 Chromatography Classification

Chromatography can be classified according to mechanism of separation as: adsorption chromatography, partition chromatography, ion exchange chromatography, size exclusion chromatography and affinity chromatography. In HPLC, separation is mainly governed by adsorption and partition chromatography. In adsorption chromatography, separation is based on the difference between the adsorption affinities of the sample components on the surface of an active site, whereas in partition chromatography separation is mainly based on the difference between the solubility of sample components in the stationary phase and the mobile phase.

There are two modes of analysis depending on the operation techniques viz. isocratic and gradient. Isocratic analysis is the procedure in which the composition of the mobile phase remains constant during the elution process. In gradient elution, the composition of the mobile phase changes continuously or stepwise during the elution process. HPLC can also be classified according to special techniques, such as reverse phase (RP) and normal phase chromatography. Reverse phase is an elution procedure used in liquid chromatography where the mobile phase is significantly more polar than the stationary phase. On the other hand, in the normal phase

procedure, the stationary phase is more polar than the mobile phase. Lipophilic substances like oils, fats and lipids are separated by normal phase chromatography. Commonly used mobile solvents are n-hexane, heptane, chloroform, and alcohols. Most biomedical substances are separated by reverse phase chromatography using aqueous mixture with methanol, acetonitrile and additives (buffers, ion-pairs).

11.2.2 Important Factors that Influence HPLC Separation

HPLC separation is influenced by dead volume, capacity factor, theoretical plate count and selectivity:

- ***Dead volume*** (V_0) is the volume at which an un-retained component elutes.
- ***Capacity factor*** (K') is a measurement of the retention time of a sample molecule, relative to column dead volume. It changes with variations in mobile phase composition, column surface chemistry or operating temperature. Capacity factor is calculated as follows:

  $$K' = \frac{V_1 - V_0}{V_0} E_i$$

  V_1 = Retention volume of peak 1
- ***Theoretical plate count*** (N) is a measure of column efficiency in terms of band-spreading of a peak. The smaller the band-spread, the higher the number of theoretical plates, which indicates good column and system performances.
- ***Resolution*** (R_s) is the distance between the peak centres of two component peaks divided by the average base of the peaks, as follows:

  $$R_S = \frac{V_2 - V_1}{\sqrt{W_1 + W_2}}$$

  W_1 = width of peak 1
  W_2 = width of peak 2
- ***Selectivity*** (α) is the relative retention of two peaks in a chromatogram.

  $$\alpha = \frac{K'_2}{K'_1} = \frac{V_2 - V_0}{V_1 - V_0}$$

  K_1 and K_2 = capacity factors for retention volume of peak 1 and peak 2 respectively.

Capacity factor (K'), selectivity (α) and column efficiency (N) are three fundamental parameters that influence the resolution of a chromatographic separation, as follows:

$$R_s = \frac{1}{4}\left(\frac{\alpha - 1}{\alpha}\right)\sqrt{N}\left(\frac{K'}{1 + K'}\right)$$

11.2.3 Main Components of HPLC

An HPLC system contains the following components:

a) *Reservoir.* This is meant for the mobile solvents. Acetonitrile, methanol, heptane, isopropanol and cyclohexane are the organic modifiers most commonly used. Trifluoroacetic acid, heptafluorobutyric acid, phosphoric acid and triethylamine phosphate are ion-pairing reagents for better chromatographic results. All tubing and fittings should be chemically inert. Solvent must be filtered through a 0.45-µm filter unit.
b) *Degasser.* In analytical operations, the mobile phase should be free of air bubbles. For this purpose, a degasser is used.
c) *Pumps.* These are devices that deliver the mobile solvent at a controlled flow rate to the separation system. HPLC uses reciprocating pumps: a pump with a single or multiple chambers, from which the mobile phase is displaced by reciprocating pistons or diaphragms. Binary gradients are created by the selected mixing of two solvents, on a single-headed two-pump system. Accurate gradient is maintained by microprocessor control.
d) *Injector/autosampler.* This device introduces a liquid sample into the mobile phase or onto the chromatographic bed. An autosampler can perform repeated functions without operator attendance, and thus is a labor-saving device.
e) *Column.* Silica and modified silica columns are available for various applications. Examples are octyl (C_8), octadecyl (C_{18}), phenyl (C_6H_5), and cyno (CN) columns.
f) *Guard column.* This is used to protect the main column.
g) *Detectors.* No universal detector is available for all molecules. However, according to the characteristic of the molecules investigated, various detectors are used (Table 1).
h) *Fraction collector.* This device collects the fractions containing the molecules of interest during the chromatographic run.
i) *Records.* A computer is used for chromatographic data acquisition.

Table 1: Characteristics of various HPLC detectors

Detector	Application	Advantages and limitations
Electrochemical	Responds to substances that are oxidizable or reducible	Commercially available. Non-specific. High LOD

Detector	Application	Advantages and limitations
Fluorescence	Detects trace-level analytes such as aflatoxins, carbamates and polycyclic aromatic hydrocarbons	Very specific. Low LOD. Not everything fluoresces
Infrared	Works for all molecules	Many solvents are infrared-active
Mass spectrometry	Analyte identification	Ability to ionize analyte. Low LOD
Photodiode array	Works for wavelengths 190-800 nm	High LOD
Refractive index	Works for nearly all molecules	Temperature sensitive. High LOD
Scattering	Uniform response	Non-specific. LOD, 5 ng per 25 mL. Interference from solvent
Ultraviolet and visible	Works for molecules with chromophores and for complex samples	Non-specific. All molecules that absorb UV and visible light can be detected.

LOD, level of detection

11.2.4 HPLC Classification

HPLC may be characterized depending on column diameter, which is the governing factor for flow rate from microscale to industrial scale chromatography. Column internal diameter (i.d.) defines the sample load and flow rates (Figure 1).

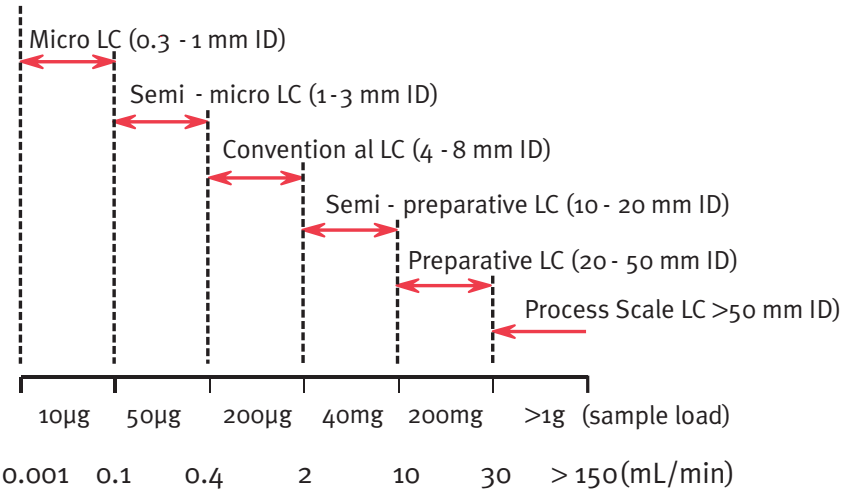

Figure 1: Classification of HPLC according to column diameter

11.2.5 Advantages of HPLC

The use of HPLC in the isolation and purification of complex compounds is increasing tremendously due to its flexibility and efficiency. It has several advantages over traditional methods of isolation and purification:

i) Variety of separating techniques.
ii) Variety of column packings for different techniques.
iii) Separation optimized by alteration of the mobile phase.
iv) Mobile phase easily manipulated in gradient systems.
v) RP technique separates very similar and very different compounds simultaneously.
vi) HPLC can be used as a preparative method.
vii) HPCL can be used as a purification technique. More than one detector can be connected in series (e.g. UV and evaporative light scattering detector).
viii) Most sample analysis is carried out at room temperature.
ix) Short analysis runs. More than 70% of HPLC separations are performed on UV detectors and 15% rely on fluorescence without any derivatization.

11.3 Preparative HPLC

Preparative chromatography is the most powerful and versatile method for isolation as well as purification of complex compounds used in drug development studies. Prior to performing preparative HPLC, the following points must be taken into account to optimize the separation and maximize the sample load on a small column:

- Prior to pilot-plant scale, a systematic method for development is required
- Validated robust analytical methods are required
- Scale-up of parameters from analytical method to prep-HPLC
- In prep-HPLC, buffer is not used
- Stationary phase with large particle sizes to decrease costs for prep-HPLC

11.3.1 Strategy for Preparative Separation

Selection of the appropriate mode of chromatography is followed by the optimization of the separation, i.e. stationary phase, mobile phase, temperature, additives. The next step is optimization of the throughput, i.e. sample amount and column overloading. In the final step, stepwise scale-up of separation is performed to obtain the desired compound.

It is always important to optimize the small-scale separation (which will significantly impact on throughput), the size of packing material and the column needed to obtain the desired throughput, the mobile solvent and the instrument capability. Normal-phase methods are the first choice because: direct transfer from normal-phase thin-layer LC or HPLC to prep-LC is possible; costs of RP packing materials are still high; cleaning normal-phase silica is easier because the material is more robust; removing organic solvents typically used in normal-phase chromatography from the final product solution is easier than removing water from an RP chromatographic fraction and can be achieved at lower temperatures and provides higher product quality and lower energy costs. Particle size of the stationary phase material also plays an important role in the isolation of the desired compounds. The choice of 5-µm particle size in a preparative column is not practical because it not only increases the column pressure but also is extremely expensive. Moreover, when the sample amount is increased, resolution performances of 5-µm and 15-µm particles are not different.

11.4 Practical Consideration in Preparative HPLC Scale-up

11.4.1 Sample Loading

If the tests on analytical columns with analytical loadings show good separations, a scale-up to a larger column diameter can be performed on prep-HPLC. Instead of jumping directly to the largest column diameter, stepwise scale-up should be done. The first step in the scale-up process is the transfer of the analytical separation procedure to a 5 cm i.d. preparative LC column. Optimization in a preparative column is required. The sample injected onto the column usually starts at 1 g and increases to as much as 20 g, depending upon the quality (resolution) of the separation achieved, the quality of the initial material, and the specifications for the pure product. Start with the 1-g injection, collect fractions and re-analyze them for purity using the analytical method, because with an increase in sample loading there is a decrease in resolution.

$$\text{(A) Scale up factor for column size} = \frac{(\text{Diameter}_{prep})^2 \times \text{Length}_{prep}}{(\text{Diameter}_{anal})^2 \times \text{Length}_{anal}}$$

$$\text{(B) Flow rate (prep)} = \text{Flow rate (analytical)} \times \frac{(\text{Diameter}_{prep})^2}{(\text{Diameter}_{anal})^2}$$

$$\text{(C) Gradient duration (prep)} = \text{Gradient duration (anal)} \times = \frac{\text{Length}_{anal}}{\text{Length}_{prep}}$$

11.4.2 Separation Time

In preparative separations, the stationary phase is usually recovered and used again to purify the next batch of the same substance. Often the major operating cost in preparative LC is the solvent rather than the packing material. Therefore, the choice of solvent is important in method development and scale-up. As per the need for separation, isocratic mixture or gradient elution of water with organic solvent (methanol or acetonitrile) is used. Gradient elution has a shorter run time than isocratic elution, but sometimes purity of the isolates is compromised.

11.4.3 Solvent Composition

Methanol is often used in preparative separations. It is an inexpensive and strongly polar solvent commonly used in combination with water as a mobile phase in RP separations. Methanol can be used for flushing normal-phase silica columns to remove adsorbed polar contaminants. It can also be recovered easily from many mixtures. In RP applications, acetonitrile yields better peaks but is too expensive in most situations for process-scale separations.

An initial goal of the scale-up process is to find an acceptable separation. If analysts find more than one set of valid conditions, then the cost of solvents becomes a major criterion. Solvent selection is usually determined during the initial method development with the 4-mm i.d. analytical-scale columns. Sometimes, when the overall costs of the goods is important to a final product, one can perform a systematic solvent selection even at later stages of development.

11.4.4 Washing Steps

The accumulation of impurities on the column can decrease the resolution of the subsequent separation, and late-eluted impurities can spoil the collected fractions of the subsequent separation. Therefore, washing steps are often implemented between chromatographic runs. Solvent gradients and recycling steps are sometimes necessary to increase the resolution for difficult separation problems. Sometimes temperature programming is used to remove strongly held impurities.

11.4.5 Recycling

Sometimes gradients and recycling steps are required for better preparative separation of complex mixture of compounds.

11.5 Stepwise Operations in Process-scale HPLC

Various aspects should be taken into consideration for operating process-scale HPLC and stepwise scale-up, as follows.

- Develop a robust analytical method and scale up the method for process scale using the same stationary phase.
- The main objective of method development is a simple, well-automated and robust separation process able to run 24 h per day.
- Optimize sample loading, flow rate and column pressure.
- Select the best solvent for both sample preparation and elution.
- The injection solvent should be optimized because sharp peaks and high loadability are important goals.
- In process-scale separations, control the first two runs manually and observe the process. If no technical problems occur, subsequent runs can be performed automatically.
- Collect fractions and re-analyze them for purity using the analytical method.
- Peak purity at three points (i.e. up slope, apex and down slope) should be confirmed.

11.6 Problems Encountered in Preparative Scale-up

11.6.1 Purity of Crude Extract

A typical problem encountered in process scale-up is that the plant material or enriched fraction used during method development had been produced in analytical scale and differs in solubility and impurity from the material that is being processed in pilot scale. The process-scale plant material can be either of a different quality or show larger amounts of the same impurities, and, in some instances, even new impurities can arise. If an impurity profile shows larger amounts of the same impurities or new impurities, the chromatographer must retest the separation method at analytical scale before starting the process-scale separation.

If during a process scale up, a compound shows higher purity, the solubility in the weak solvent chosen during optimization may not be good enough. In this instance, productivity can be lower than expected because the amount separated in each run will be less. Because scale-up is linear, the chromatographic run takes the same time in preparative scale as in analytical scale, and the substance is not stressed longer in the separation equipment.

11.6.2 Removal of Chromatographic Solvent

The final work-up after the separation step is removal of the chromatographic solvent. The desired fraction collected is a solution which contains the substance of interest in the range of a small percent by weight and, therefore, large amounts of solvent must be removed. The evaporation of solvents, especially water, takes time, so the purified drug substance can be changed or even destroyed during the concentration process. This step should be performed with care considering the thermal stability of the compound of interest.

11.6.3 Temperature Variation from Laboratory to Pilot Scale

It is useful to test the temperature stability of the substance during analytical method development. Temperature also influences the separation performance. For example, the mixing of organic solvents before they enter the column can result in a strong increase or decrease in solvent temperature and can influence the operating temperatures of the mixing unit and column. Temperature effects at the centre of the column caused by heat dissipation can also influence the separation and ultimately the purity of isolates.

11.6.4 Increase in Pump Pressure Due to Accumulation of Impurities on the Column

Another problem that often occurs during the first separation in process scale is that some impurities accumulate on the column during a series of sequential runs. The quality of the separation deteriorates during the sequence. Because the fraction collection is commonly controlled by peak height, a UV detector does not detect this problem and the purity of the fractions decreases. When impurities accumulate on the column, the peak shapes or the retention times of the components of interest might change, so the chromatographer can see quality problems. Unfortunately, sometimes peak shapes and retention times show no changes. An additional indicator that impurities have accumulated on the column is a pressure increase; therefore, it is helpful to monitor column pressure. The increase in pressure is commonly related to instrument failure.

11.7 Summary: Scale-up Strategy

- Define the problem
- Find the chromatographic mode
- Develop and optimize the separation
- Maximize throughput
- Increase sample mass and volume to the maximum while meeting purity objectives
- Determine recovery

- Scale up to desired column size to meet throughput and load objectives
- Pool fractions of comparable purity and rerun if necessary
- Check fraction purity using an analytical column

11.8 Applications: Natural Products Isolation

A few examples of prep-HPLC for the isolation of natural products are summarized herein. First, tannins from *Guiera seregalensis* can be isolated using the following conditions:

Column	RP-18e (250 x 10 mm i.d.)
Mobile phase	Water:methanol:THF (90:10:0.25) and (80:20:0.25)
Flow rate	2.5 ml/min
Detection	280 nm
Compounds isolated	Galloylquinic acids, namely, 3-*O*-, 5-*O*-, 1,3-di-*O*-, 3,4-di-*O*-, 3,5-di-*O*-, 4,5-di-*O*-, 1,3,4-tri-*O*-, 3,4,5-tri-*O*- and 1,3,4,5-tetra-*O*-galloylquinic acid

Flavonoids from *Lychnophora ericoides* require the following conditions:

Columns	Silica (250 x 10 mm i.d.) and ODS (250 x 10 mm i.d.)
Mobile phase	Water:methanol:THF
Flow rate	2.5 ml/min
Detection	280/225 nm
Compounds isolated	7,4'-dihydroxy-flavonol; 5,7-dihydroxy-3-methoxy-flavonol; galangine, 7,4'-dihydroxy-dihydroflavono l,5,7,4'-trihydroxy-dihydroflavonol,7-hydroxy-4'-methoxy-dihydroflavonol; pinobanksin, 5,7'-dihydroxy-4'-hydroxy-flavanone; 7-hydroxy-4'-methoxy-flavanone; 5,7-dihydroxy-flavone; acacetin; 7-hydroxy-3',4'-dihydroxy-isoflavone; 15-desoxigoiazensolide, 2',3'-dihydro-15-desoxygoyazensolide; eremantholides A and C; 4,5-dihydroeremantholide A and lychnopholide

For the isolation of peptide components of bacitracin, use:

Column	C_8 (250 x 16 mm i.d.)
Mobile phase	Gradient acetonitrile, methanol and phosphate buffer
Flow rate	9.0 ml/min
Detection	254 nm
Compounds isolated	Peptides

The isolation and purification of β-carotene from carrot involves:

Column	Shim-pack PREP-ODS (H) kit (250 x 20 mm i.d.)
Mobile phase	Ethanol (99.5%)
Flow rate	10 ml /min
Detection	480 nm monitor with photodiode array (PDA)
Compound isolated	β-carotene

The isolation of anti-HIV compounds from *Gleditsia japonica* and *Gymnocladus chinensis* requires the following conditions:

Column	C18-μBondpak (300 x 24.4 mm i.d.)
Mobile phase	Methanol-water (varying percentages)
Detection	Refractive index detector
Compound isolated	Saponins

Isolation of procyanidins from *Vicia faba* requires these conditions:

Column	Sephadex LH-20 column (580 × 25 mm i.d.)
Mobile phase	Sequential elution with ethanol, ethanol:methanol, methanol and finally methanol:acetone
Flow rate	Varying
Detection	280 nm
Compounds isolated	(+)-gallocatechin-4-phloroglucinol; (−)-epigallocatechin-4-phloroglucinol; (+)-gallocatechin; (−)-epicatechin-4-phloroglucinol; (+)-catechin-4-phloroglucinol; (+)-catechin and (−)-epicatechin

Isolation of the anticancer compound taxol from *Taxus yunnanensis* is done with:

Column	D1 (4000 x 200 mm i.d.) packed with 956 polymeric resin
Mobile phase	Acetone:water (58:42)
Flow rate	79 ml/min
Detection	228 nm
Compound isolated	Taxol

11.9 Conclusions

Stepwise scale-up starting with analytical scale to process scale is an important issue that needs to be considered. Optimization of operating conditions is always useful for getting high purity phytomolecules. Thus, process-scale HPLC is the choice for isolating valuable molecules with desired purity for commercialization. Its significance will continue to grow because of the increasing requirements for high-purity molecules.

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12 Flash Chromatography and Low Pressure Chromatographic Techniques for Separation of Phytomolecules

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Abstract

Flash chromatography is a rapid form of preparative column chromatography that employs prepacked columns through which a solvent is pumped at high flow rate. Two types of solvent systems are used in flash chromatography: isocratic and gradient. In the isocratic system, a single-strength mobile phase brings about the desired separation. The gradient system, in which the solvent composition changes during the course of elution, is suited for complex samples containing compounds that differ greatly in column retention times. The optimum flow rate for a flash separation is related to the particle size and dimensions of the column. Typical sorbents for normal phase flash chromatography are polar (e.g. silica, NH_2) and elution solvents are non-polar. In reverse phase chromatography, the stationary phase is non-polar (such as C18) and the mobile phase is polar. Compounds are retained by the interaction of their non-polar functional groups with the non-polar groups on the packing surface. Therefore, the most polar compounds elute first followed by other compounds in decreasing order of polarity. To achieve a desired separation, one must select a sorbent that effectively retains the compounds of interest under solvent conditions that are appropriate for the sample's solubility. Sample loading onto a flash column can be done with wet loading (the liquid sample is loaded directly and allowed to percolate into the sorbent bed) or dry loading (when samples are pre-absorbed to a small amount of sorbent which is then loaded onto the column).

12.1 Introduction

Mikhail Semyonovich Tsvet of Russia invented the first chromatographic technique in 1901 during his research on chlorophyll. He used a liquid adsorption column containing calcium carbonate to separate plant pigments. The method was described on 30 December 1901 at the XIth Congress of Naturalists and Doctors in St. Petersburg. The first printed description was published in 1903 in the *Proceedings of the Warsaw Society of Naturalists,* section of biology. He first used the term chromatography in print in 1906 in his two papers about chlorophyll in the German botanical journal, *Berichte der Deutschen Botanischen Gesellschaft.*

In 1952, Archer John Portor Martin and Richard Laurence Millington Synge were awarded the Nobel Prize in Chemistry for their invention of partition chromatography. Since then, the technique has advanced rapidly. Researchers have found that the principles underlying Tsvet's chromatography can be applied in many ways, giving rise to the different varieties of chromatography and allowing increasing similar molecules to be resolved.

12.2 Flash Chromatography

Flash chromatography, also known as medium pressure chromatography, is a rapid form of preparative column chromatography that uses optimized, prepacked columns through which a solvent is pumped at a high flow rate. Initially developed in 1978 by W. C. Stills of Columbia University, New York, USA, flash chromatography is now a method of purification and separation using normal phases. Use of reverse phase packing materials is opening up the technique to a wider range of preparative separations. Currently, it is considered to be a simple and economical approach to preparative liquid chromatography (LC).

Flash chromatography differs from conventional techniques in two ways. First, slightly smaller silica gel particles (250-400 mesh) are used. Second, due to the limited flow of solvents caused by the small gel particles, pressurized gas (10-15 psi) is used to drive the solvent through the column of stationary phase. The net result is rapid ("over in a flash") and high resolution chromatography.

12.2.1 Theory of Flash Chromatography

Chromatography is a separation method that exploits the differences in partitioning behavior between a mobile phase and a stationary phase to separate the components in a mixture. Compounds of a mixture may interact with the stationary phase based on charge, relative solubility or adsorption. Retention is a measure of the speed at which a substance moves in a chromatographic system. In continuous development systems like high performance LC (HPLC) and gas chromatography (GC), where the compounds are eluted with the eluents, retention is usually measured as the retention time (Rt or t_R), i.e. the time between injection and detection. In uninterrupted development systems like thin layer chromatography (TLC), retention is measured as the retention factor (R_f), i.e. the run length of the compound divided by the run length of the eluent front:

$$R_f = \frac{\text{Distance travelled by the analyte}}{\text{Distance travelled by the solvent front}}$$

12.2.2 Converting TLC to Flash Chromatography

TLC separations can be used to help determine effective solvent compositions for flash chromatography. R_f is a common TLC unit and ΔR_f is the distance between the compounds:

$$\Delta R_f = R_{f1} - R_{f2}$$

The ideal solvent system for TLC is one that moves the compound of interest in the mixture to an R_f of 0.15-0.35 and that separates

this component from the others nearest to it by a ΔR_f value of at least 0.15. In contrast to TLC, flash chromatography separations are governed by column volumes.

A column volume (CV) is defined as the volume of solvent required to fill all the adsorbent pores and interstitial spaces between adsorbent particles in a given column. The volume required to elute a compound of interest from a column is expressed in terms of the number of CV. The volume that separates the elution of two substances from the same volume is called column volume difference (ΔCV). The ideal flash chromatography solvent system is one that elutes the desired compound of interest in 3-6 CV and that separates this component from others nearest to it by a ΔCV greater than 1.

The relationship between numbers of CV to R_f for a given compound is $1/ R_f$; therefore, for two compounds $\Delta CV = 1/ R_{f1} - 1/ R_{f2}$. For a particular set of separation conditions, a weakly retained, fast-eluting component with an R_f=0.9 can be eluted in just over 1 CV, whereas a strongly retained, slow-eluting component with an R_f =0.1 requires 10 CV for complete elution (Table 1).

Table 1: Relationship between R_f and CV

R_f	CV
0.90	1.10
0.70	1.40
0.50	2.00
0.30	3.33
0.10	10.0

Due to factors such as change in the TLC solvent flow rate with respect to time and interference from adhesives used to bind TLC sorbents, solvent conditions that provide an acceptable TLC separation will not necessarily work effectively for flash chromatography without modification.

Although some empirical experimentation may be required, the steps below help streamline the process of converting a TLC solvent system into a flash chromatography mobile phase:

1. Use matching sorbent chemistries on the TLC plate and in the flash chromatography column. Stationary phase sorbent chemistries (including silica) can differ from one manufacturer to another. It is important to match these sorbent chemistries if the solvent systems are expected to provide equivalent results.

2. Optimize the TLC solvent mixture so that the compound of interest has an $R_f \approx 0.15\text{-}0.35$ and $\Delta R_f > 0.20$. These conditions will provide the most reliable starting point for a successful flash chromatography separation.

a) *Adjust the solvent selectivity to provide an $\Delta R_f > 0.20$.* Solvent selectivity is defined as the ability to affect the retention of one compound in the mixture relative to the others, therefore affecting ΔR_f and number of CV. Experimenting with different solvent combinations to obtain the desired TLC separation usually reveals appropriate conditions for effective flash chromatography separation. Different solvent mixtures such as hexane:ethyl acetate (1:1) and hexane:dichloromethane (1:2) may provide different solvent selectivities while providing similar solvent strengths. Different solvent mixtures can even reverse the elution order of some of the components in the sample. It is interesting to note that ΔR_f and ΔCV may vary greatly relative to one another for a given separation. ΔCV predicts column capacity, i.e. the amount of material that can be effectively separated in a single column loading (Table 2). Greater the ΔCV, the better the effective capacity of the column.

b) *Adjust the solvent strength to obtain an R_f between 0.15 and 0.35 (CV, 3-6).* Solvent strength refers to the solvent's simultaneous effect on the retention of all compounds in the mixture; therefore, solvent strength affects R_f and CV. Once the optimum separation has been established by modifying solvent selectivity, it may be useful to move some or all of the compounds off the flash column as quickly as possible by increasing solvent strength. Often, slight changes in solvent strength can make large differences in retention. In some cases, a lower-strength mobile phase provides improved separations. It is important to remember that the sample loading solvent should have equal or lower elution strength than the starting strength of the mobile phase. Additional adjustments to the selectivity and strength of the flash solvent system may be necessary to optimize the separation and to achieve a CV≈3-6 and a ΔCV>1. This can often be achieved by using a less polar solvent system or by decreasing the proportion of polar modifier.

Table 2: Approximate capacity of a 20 g/70 ml ISOLUTE Flash Si column (Biotage)

ΔCV	Sample load, g
6	1.0
2	0.5
1	0.25

12.3 Isocratic versus Gradient Chromatography

Two types of solvent systems are used in flash chromatography: isocratic and gradient. The most common is an isocratic (meaning "same solvent strength") system where a single-strength mobile phase mixture brings about the desired separation.

If the mixture is complex and contains compounds that differ greatly in column retention times, chemists may use a gradient solvent system that changes solvent composition during the course of elution. For example, in a normal phase system equipped with a silica column, a non-polar solvent such as hexane is applied to elute non-polar compounds. Then, a more polar solvent such as ethyl acetate is added to the hexane to elute the more polar compounds. The percentage of the polar solvent in the mixture is increased until all components of the mixture have eluted.

In a step-gradient system, the various solvent concentrations are typically changed in large increments (or steps). Alternatively, a linear gradient can be employed whereby a continuous linear change in the concentrations of the solvent (and thus mobile phase strength) is achieved. Chemists can often achieve effective separations more rapidly by using gradient solvent systems. Chemists must select miscible solvents for use in gradient solvent systems. A common solvent system for flash separations using polar sorbents such as silica is hexane and ethyl acetate, where ethyl acetate is the more polar solvent.

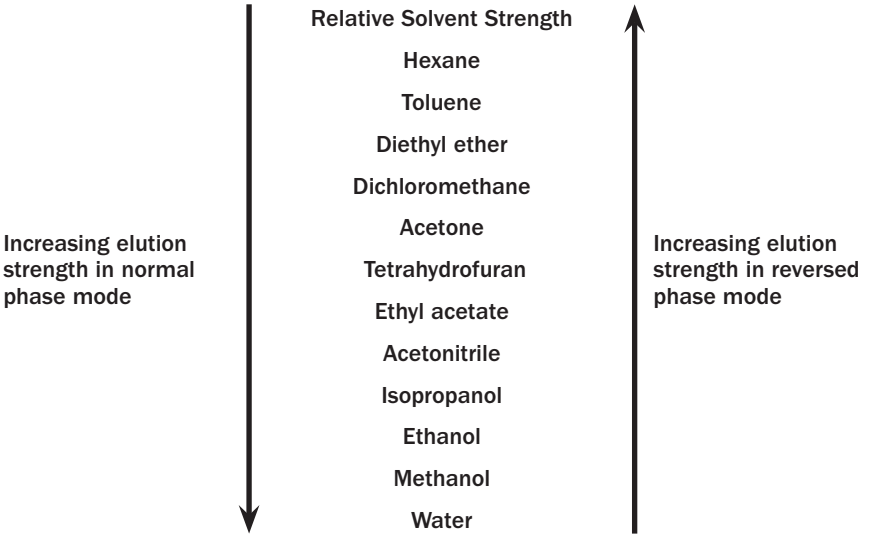

Figure 1: Step-gradient system

12.4 Adsorbent Selection and Mode of Separation

Typical sorbents for normal phase flash chromatography are polar (e.g. silica, NH_2) and elution solvents are non-polar (e.g. hexane, heptane, dichloromethane, sometimes modified with small amounts of more polar solvents such as isopropanol). The sample is usually applied in a weak (very non-polar) solvent, and separation occurs when the elution solvent is applied. In normal phase flash chromatography, the most non-polar sample component elutes first, followed by successively more polar compounds.

12.4.1 Isolute Flash Columns

Isolute flash columns (Biotage) are polypropylene columns prepacked with Isolute flash sorbents. These columns are appropriate for use in both off-line and on-line flash chromatography. In off-line flash chromatography, chemists apply the sample and elution solvent volumes to the top of an Isolute column fixed in a FlashVac Sample Processing Station. In off-line flash chromatography, chemists perform isocratic or step gradient separations. In on-line flash chromatography, an Isolute column is mounted on a system that connects the column to an external liquid pump system to produce a continuous flow of solvent through the column. Depending on the capability of the pump, the solvent composition can be isocratic (a single solvent or solvent mixture) or a gradient with an increasing proportion of stronger solvent (either in a step gradient or a linear gradient).

12.4.2 Method Development Using Isolute Flash Columns

12.4.2.1 Column Equilibration

Prior to sample loading, the column should be prepared for the separation by equilibration (prewetting) with a suitable solvent:

- *Off-line*. Apply the equilibration solvent to the top of the column and allow it to flow through the column under gravity.
- *On-line.* Equilibrate the flash column for on-line mode separation in the off-line mode using a vacuum manifold such as the FlashVac system or by mounting the column on the on-line apparatus and pumping a suitable volume of equilibration solvent through the column.

12.4.2.2 Typical Equilibration Solvents

Suitable solvents are non-polar, e.g. hexane or pentane. For best results, prewet the silica and NH_2 columns prior to use. A suitable volume for column equilibration is approximately two bed volumes (Table 3).

Table 3: Typical column solvent volumes for column equilibration

Column size, g	Approximate column volume, ml	Typical solvent volume, ml
2	2.5	5
5	6.5	13
10	12.5	25
20	25	50
25	31.3	62.5
50	62.5	125
70	88	176
100	125	250
150	188	376

Normal phase flash columns can be used without prewetting, but some column-to-column variation may be experienced.

12.5 Sample Application

There are two popular approaches to flash chromatography sample loading: wet loading and dry loading.

12.5.1 Wet Loading

Load the liquid sample directly onto the top frit of the column, and allow it to percolate through the top of the sorbent bed. For best results, load the sample onto a prewetted column in a non-polar solvent.

12.5.1.1 Practical Tips for Wet Loading

- Dissolve the sample in as non-polar as solvent as possible.
- If compounds are not easily soluble in a non-polar solvent, either dissolve them in a small volume of polar solvent and then dilute with a non-polar solvent to reduce the elution strength, or consider dry loading of the sample (discussed in next section).
- Position column on a FlashVac vacuum manifold equipped with PTFE stopcock needles.
- Apply the sample evenly over the entire area of the top frit. To do so, seal the column by closing the stopcock.
- Alternatively, load samples onto the column in on-line mode using a Flash Master system equipped with a 3-way injection valve.

12.5.2 Dry Loading

Dry loading is the method of choice for loading reaction mixtures consisting of polar solvents onto silica or other normal phase columns. Pre-absorb the reaction mixture onto a small amount of bulk material of the chosen sorbent. Evaporate off the majority of the solvent leaving the compounds bound to the surface of the sorbent.

Add this blend to the top of the prepacked (and prewetted) flash column; allow settling and add a top frit to secure the blend in place. The top frit can be placed using a suitably sized frit inserter.

A popular alternative sorbent for dry loading using the flash sorbent is a diatomaceous earth such as Isolute HM-N. This can be used in the same way as the flash sorbent, but has several advantages including more efficient desorption of the compounds into the mobile phase.

12.5.2.1 Practical Tips for Dry Loading

- Dissolve the sample initially in a suitable solvent, ensuring complete dissolution if possible. Use the smallest volume possible.
- Add the bulk material of choice. The ideal proportion of sample to bulk material ranges from 1:1 to 1:3 by volume.
- Evaporate off the residual solvent using a rotary evaporator to ensure even adsorption of the sample on the bulk material.
- Pack the dry blend on top of the flash column (above the top frit) and add another frit. Push down the new surface to prevent movement of the new blend. When loading with Isolute HM-N, ensure that the material is not crushed at this stage.
- When dry loading using bulk silica, ensure that it is identical to the material in the flash column. If this is not possible, use a lower surface area material, ensuring that the surface pH and moisture content are as similar as possible to the column packing. All Isolute sorbents are available as bulk material.

Table 4: Capacity guidelines

Reaction scale, g	Column size, g
0.1	1-2
0.25	2-5
0.50	5-10
1.0	10-20
2.5	25-20
5.0	50-70
10.0	70-100

As a general guideline, the amount of sample loaded onto the normal phase flash column should be 5%-10% of the column size. Factors affecting the capacity of the column include compounds, sample matrix, concentration of reaction products and elution solvent used.

12.6 Elution

12.6.1 Step Gradient Elution

Step gradients provide controlled elution with discrete changes in eluent strength. Each step is optimized to elute only those components that are soluble in that eluent. This technique can be applied to both off-line and on-line flash chromatography.

1. Use TLC to determine a suitable solvent strength to elute the components at discrete intervals, choosing different solvent mixtures that elute each component separately with an R_f of 0.2-0.5.
2. Calculate the volume of solvent required to elute each component using $CV=1/R_f$. Values of CV for different column sizes are listed in Table 3.
3. Apply between 2 and 5 CV of solvent for each step, starting with the solvent with weakest strength.
4. Collect the eluent from each step in a separate vessel.

12.6.2 Linear Gradient Elution

Liner gradients are a quick way of separating complex mixture, reducing the complexity of the subsequent purification of the fractions collected. This technique is suitable for on-line flash chromatography.

1. Use TLC to find both the weakest and strongest elution solvent. The weak solvent (solvent A) should give retention of the majority of components ($R_f<0.1$). The strong solvent (solvent B) should allow elution of all of the components of interest ($R_f>0.5$).
2. Run a gradient starting with 100% solvent A and ending with 100% solvent B.
3. Collect the eluent at regular intervals.

Automated flash chromatography instrumentation such as the FlashMaster II and Solo systems can monitor the signal from a UV detector, and collect only the fractions of eluent carrying the compounds of interest.

12.6.3 Method Development Using Gradient Elution

Method development can be performed without TLC. Gradient elution analysis offers a useful approach to method development, particularly for non-silica-based flash chromatography (e.g. reverse phase) where suitable TLC plates are not available.

1. Load the sample onto a prewetted (equilibrated) flash column in as weak a solvent as possible (or use dry loading); for example, for normal phase work use hexane for loading.
2. Elute the column with aliquots (2 CV each) of successively increasing solvent strength. A typical scheme for mixing the two solvents is given in Table 5.
3. Collect each fraction and analyze for the presence of the components of interest.
4. Using these data, identify the solvent mixture that elutes the components of interest separately, and set up a step or continuous gradient as described.

Table 5: Typical scheme of mixing solvents for method development. Solvent A is a weak solvent (e.g. hexane) while solvent B is a strong solvent (polar modifier, e.g. isopropanol)

Aliquot number	Solvent A, %	Solvent B, %
1	100	0
2	99	1
3	98	2
4	97	3
5	06	4
6	95	5
7	94	6
8	93	7
9	92	8
10	91	9

12.6.4 Practical Tips for Gradient Elution

1. The use of a gradient does not improve the selectivity of a separation if isocratic elution using the same solvent system does not effectively separate the sample components. However, a gradient can be used to decrease the time required to achieve a separation.

2. The gradient starting conditions must not cause chromatography separation. Start the gradient with a weak solvent that matches the sample loading conditions.
3. The flow rate at the beginning of a gradient can be high but, for best results, it should be reduced to the optimum flow rate at the separation area.
4. To speed up a gradient separation, use either a higher flow rate (most suited to samples with many components, high sample load) or a steeper gradient (most suited to samples with few components, low sample load).

12.6.5 Optimizing Flow Rate

The optimum flow rate for a flash separation is related to the particle size and dimensions of the column. Theoretical optimum flow rate for flash columns of different dimensions can be predicted. However, in practice, increasing the flow rate has not had a significant effect on separation and offers important productivity advantages (Table 6). Other factors, such as mobile phase composition and back pressure, also affect the range of effective flow rates. For recommended flow rates, see Table 6.

Table 6: Recommended flow rates

Column diameter (configuration)	Flow rate range ml/min
16 mm (D)	5-25
20 mm (E)	5-25
27 mm (F)	10-30
37 mm (J)	20-50
40 mm (V, W, X)	20-50

12.7 Fraction Collection

12.7.1 Off-line Flash Chromatography

When performing flash chromatography on a vacuum manifold such as the FlashVac system, successive fractions can be collected as follows:

- Load collection rack with vials of a suitable volume in each position.
- Place a single flash column in position 1 of the collection rack and apply the first solvent aliquot.
- Collect the aliquot in the vial in position 1 of the collection rack.
- Move the column to position 2 and apply the second solvent aliquot.

- Collect the aliquot in the vial in position 2 of the collection rack.
- Continue until all the components of interest have been collected.

Alternatively, multiple columns can be processed by replacing collection vials at each elution step. A typical volume for each fraction is two CV.

12.7.2 On-line Flash Chromatography

Using an automated system equipped with a fraction collector, fractions can be collected in a variety of ways, for example:

- Fixed volume fraction collection.
- Individual peak fraction collection under microprocessor control with UV detector input.

12.8 Low Pressure Liquid Chromatography

In low pressure column chromatography, a column of particulate material such as silica or alumina has a solvent passed through it at atmospheric or low pressure. There are different kinds of low pressure chromatographic techniques:

i) Gel filtration chromatography (separation on the basis of size)
ii) Ion exchange chromatography (separation of the basis of charge)
iii) Affinity chromatography (separation on the basis of specific binding sites on the proteins)

12.8.1 Gel Filtration Chromatography

Proteins of different sizes are separated on a column in which the stationary phase consists of polymerized agarose or acrylamide beads with pores of particular sizes. A small protein in the mobile phase (aqueous buffered solution) can enter the pores in the beads while a large protein cannot due to size restriction. The result is that a smaller fraction of the overall volume of the column is available to the large protein than to the small protein, which thus spends a longer time on the column and is eluted by the mobile solvent after the large protein.

12.8.2 Ion Exchange Chromatography

The material used for this type of chromatography consists of an agarose, acryl amide or cellulose resin or bead which is derivatized to contain covalently linked positively or negatively charged groups. Proteins

in the mobile phase bind through electrostatic interactions to the charged groups on the column.

In a mixture of proteins, positively charged proteins bind to a resin containing negatively charged groups like carboxymethyl ($-OCH_2COO^-$) or sulfopropyl ($-OCH_2CH_2CH_2SO_3^-$), while the negatively charged proteins pass through the column. The positively charged proteins are eluted from the column with a mobile phase containing either a gradient of increasing salt concentration or a single higher salt concentration. The most positively charged proteins are eluted last, at the highest salt concentration.

Likewise, negatively charged proteins bind to a resin containing positively charged groups, like diethylaminoethyl ($-CH_2CH_2NHEt_2^+$) or a quaternary ethyl amino group. These proteins are separated in an analogous way.

12.8.3 Affinity Chromatography

In this technique, the chromatography resin is derivatized with a group that binds to a specific site on a protein of interest. It may be a group that binds to the active site of an enzyme (such as benzamidine-agarose used for the purification of trypsin) or an antibody that recognizes a specific amino acid sequence on a protein.

This method exploits the specific binding of antibody to antigen held on a solid matrix. Antigen is bound covalently to small, chemically reactive beads which are loaded in the column and the antiserum is allowed to pass over the beads. The specific antibodies bind while all other proteins in the serum including antibodies to other substances are washed away.

Affinity chromatography can also be used to purify antigens from complex mixtures by using beads coated with the specific antibody.

12.9 Conclusions

Flash chromatographic systems have been developed for the separation and purification of organic molecules from natural sources and from reaction mixtures. Method development for separations can be optimized with proper selection of adsorbents, solvent systems and flow rate of solvents used for the separation. Scale-up of flash chromatographic methods can easily be achieved with minimum optimization. Therefore, they are considered to be efficient and cost-effective methods to purify compounds in little time.

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13 Counter-current Chromatography

S. K. Srivastava

Abstract

Counter-current Chromatography (CCC) is based on liquid-liquid partitioning and is an excellent alternative to circumvent the problems associated with solid phase adsorbents and to preserve the chemical integrity of mixtures subjected to fractionation. The uniqueness of this technique is that it does not use a stationary phase and separation is achieved between two immiscible liquid phases. One phase is used as a stationary phase while the other one is used as mobile phase. The separation takes place on the basis of partition of a sample between the two phases. In contrast to other chromatographic techniques, since there is no irreversible adsorption of sample on the stationary phase, recovery is up to 100%. Centrifugal Partition Chromatography (CPC) and High Speed Counter-current Chromatography (HSCCC) are the two variants of CCC being used today. Separation of compounds with a wide range of polarities is possible with the use of aqueous and non-aqueous solvent systems. Separation of crude plant extracts, semipurified fractions and synthetic mixtures can be carried out, with sample loads ranging from milligrams to grams.

13.1 Introduction

Natural products have been a key source for the discovery of new drugs. Isolation of the active components from a natural product has always been associated with complex separation problems due to the enormous chemical complexity of the extracts, but recent advances in separation sciences have facilitated the isolation of these active components from natural products.

Crude extracts of natural products which show desirable biological activity are subjected to activity-guided fractionation until an active component is isolated and identified. This exploratory process of fractionation typically involves suboptimal chromatographic conditions; hence, in order to avoid destruction of potentially labile components, utmost care must be taken throughout the entire process of isolation.

At present, most of the chromatographic separations of natural products are being carried out on solid supports. However, SiO_2, Al_2O_3 and reverse phase adsorbents are not chemically inert. Separation of a natural product on alumina or silica gel sometimes results in recovery of only 70%–90%. Sometimes severe losses of valuable materials result because of irreversible adsorption on a solid support. In addition, isolation of artifacts has also been reported due to chemical reactions of the substrates with solid phase adsorbents.

Counter-current Chromatography (CCC) is a unique form of liquid partition chromatography which utilizes a separation column free of solid support matrix. Because of this support-free system, the method provides an important advantage over other chromatographic methods by eliminating various complications such as adsorptive loss and deactivation of samples and contamination. During the 1970s, the method was steadily improved by accelerating the separation speed and efficiency. In the early 1980s, an epoch-making advance was achieved by the advent of high-speed CCC (HSCCC) which can yield highly efficient separation in a short period of time. Because of its high performance, the recent research and development of the CCC technology have been almost entirely focused on HSCCC, high performance centrifugal partition chromatography (HPCPC), and fast centrifugal partition chromatography (FCPC).

Recent developments in CCC instruments, explaining the use of CCC for better separation and their applications in the separation of bioactive natural products from plants, are discussed in this paper.

13.2 Principles and Development of Counter-current Chromatography

Anyone conversant with the technique of liquid-liquid extraction (using a separatory funnel) can readily understand the principles of Counter-current Chromatography (CCC), where separation is based on the partition of solutes between two immiscible liquid phases. In CCC, one of the two phases is retained in the column and is called the stationary phase. The second phase, which is called the mobile phase, percolates through the stationary one.

13.2.1 Liquid-liquid Extraction

Liquid-liquid extraction is a simple means of separating large quantities of materials, using a minimum of solvent. After dissolving the sample in a two-phase solvent system (in a separatory funnel), the steps in performing liquid-liquid extractions are as follows:

- Shake vigorously to thoroughly mix the two phases.
- Allow the mixture to settle into two phases.
- Separate the phases from each other.

These steps are crucial to achieve the separation of sample components.

The chief disadvantage of liquid-liquid extraction is that it provides only one plate of separation in the original sense.

Accordingly, either this single-step separation must be designed to suit one's needs, or multiple liquid-liquid extractions must be used to increase the separation.

CCC has its origin in the work of Archer John Porter Martin and Richard Laurence Millington Synge (Martin and Synge, 1941; Synge, 1946) carried out in Britain during World War II. For their pioneering work, Martin and Synge shared the 1952 Nobel Prize in Chemistry. Soon after their work appeared, Lyman Creighton Craig and Otto Post developed an apparatus that essentially consisted of a series of separatory funnels ("tubes") (Craig and Post, 1949). The sample was "automatically" transferred through the Craig-post apparatus (Figure 1). Over 1000 mixing and separation steps could be accomplished in one day.

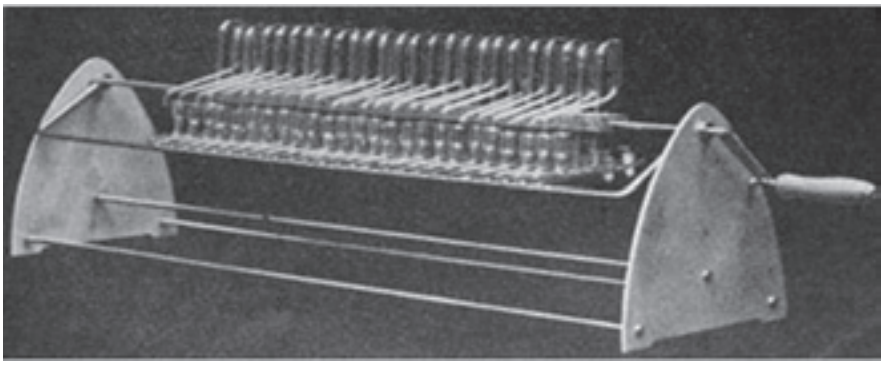

Figure 1: Craig-post apparatus

Individual components were separated based on their partitioning behavior. Craig and Post continuously improved their apparatus and were commercially quite successful. Over 1000 publications on "counter-current distribution" appeared during the period 1950-1970 citing the use of the Craig-post apparatus.

13.2.2 Partition Coefficient

For a given substance A, the partition coefficient KA is defined the concentration of A in the upper phase divided by that in the lower phase (Figure 2).

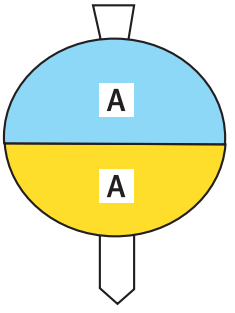

Figure 2: KA= [A] upper phase/[A] lower phase

KA is a constant at any given temperature, and it is unaffected by the presence of other substances or by the concentration of the solute. Usually it is expressed as the amount of solute in the stationary phase divided by that of the mobile phase as in conventional liquid chromatography. For a better CCC separation, the K values of the target compounds should be in the range of $0.5 \leq K \leq 1.0$. A smaller K value elutes the solute closer to the solvent front with lower resolution while a larger K value tends to give better resolution but broader, more dilute peaks due to a longer elution time. In CCC, one can choose either the upper or lower phase as the stationary phase, but before deciding which phase is to be used as the stationary phase, one should temporarily express the partition coefficient as $K_{U/L} = C_U/C_L$, where C_U is the solute concentration in the upper phase and C_L is that of the lower phase. If $K_{U/L} = 2$, the lower phase should be used as the stationary phase, which gives $K = 0.5$. It is important that this preliminary $K_{U/L}$ be clearly distinguished from K using the subscripts to avoid confusion. The measurement of $K_{U/L}$ values may be performed by the shake-flask method.

Add a small amount (a few milligrams) of each target compound (one at a time) to the two mutually equilibrated solvent phases (1–2 ml each) in a stoppered test tube (13 mm × 100 mm). Thoroughly mix to equilibrate the contents. After settling, pipette and deliver an equal volume of the upper and the lower phases (100–200 µl) each into a separate test tube, dilute each with an equal volume (2 ml) of a suitable solvent such as methanol, and measure the absorbance with a spectrophotometer at a suitable wavelength to obtain the $K_{U/L}$ value. If the pure sample is not available, one can subject each phase to HPLC analysis to compare the peak heights (or areas under the peaks). If the sample does not absorb in the UV or visible wavelengths, the $K_{U/L}$ value may be determined by other ways such as thin layer chromatography (color reaction) or evaporative light scattering detection (ELSD). Thin layer chromatography combined with densitometry is effectively used for simultaneous determination of $K_{U/L}$ of multiple components from a sample mixture.

13.2.3 Droplet Counter-current Chromatography

In the early 1970s, Ito and colleagues at the US National Institutes of Health introduced Droplet Counter-current Chromatography (DCCC). In DCCC, as with the Craig-post apparatus, the stationary phase is held in place only with unit gravity.

The apparatus consists of a set of vertical straight tubes serially connected with narrow transfer tubing (Figure 3). The original CCC apparatus was equipped with 300 glass tubes, each 60 cm × 1.8 mm i.d.

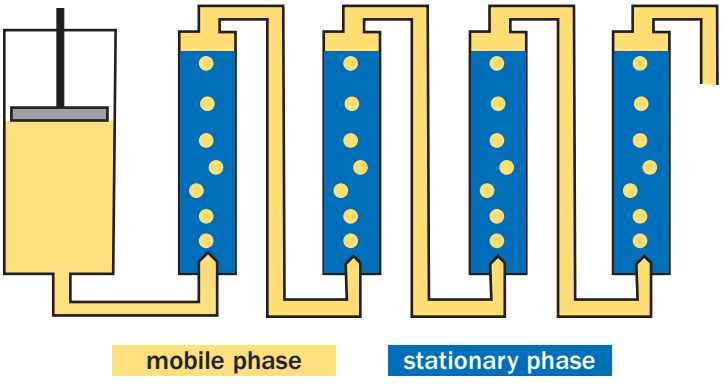

Figure 3: Droplet counter-current chromatography

The total capacity is about 600 ml including the volume in the transfer tubing (about 15% of total capacity). The operation of DCCC is initiated by filling the entire column with the stationary phase of an equilibrated two-phase solvent system followed by injection of sample solution. Then, the other phase is introduced into the first unit in such a way that the mobile phase travels through the column of the stationary phase by the effect of gravity, i.e. the mobile phase is introduced from the bottom if it is the lighter phase, and from the top if it is heavier. Consequently, the solutes are separated according to their partition coefficients. As with all types of chromatography, compounds more soluble in the mobile phase move more quickly, while those more soluble in the stationary phase lag behind. Under optimum flow rate, the mobile phase forms multiple droplets in the stationary phase to divide the column space into numerous partition units; this process is repeated in each partition unit.

DCCC necessitates a proper choice of solvent systems for producing a droplet flow of the mobile phase in the column. The most popularly used solvent system is composed of chloroform, methanol and water at various volume ratios, although a sizable separation usually takes a few days.

13.2.4 Applications of Droplet Counter-current Chromatography

This simple DCCC instrument has been used to perform efficient preparative separations such as:

- Separation of non-polar compounds.
- Isolation of virginiamycin-M1 and parthenolide.
- Isolation of vitamin B12.
- Pharmacognostical studies of *Tabernaemontana* species: ion-pair DCCC of indole alkaloids from suspension cultures.
- Chiral resolution of a carboxylic acid.
- Separation of natural polar substances by reverse phase HPLC, centrifugal thin layer chromatography and DCCC.
- Efficient isolation of ecdysteroids from the silkworm, *Bombyx mori*.
- Analytical DCCC isolation of 20-hydroxyecdysone from *Vitex thyrsiflora* (Verbenaceae).
- Increasing the speed of DCCC separations.
- Complete resolution of isoleucine.
- DCCC of anthocyanins.
- Efficient isolation of phytoecdysones from *Ajuga* plants by high-performance liquid chromatography and DCCC.
- DCCC with non-aqueous solvent systems.
- Use of DCCC in log *P* determinations.
- Purification of *Stevia rebaudiana* sweet constituents (potential sweetening agent of plant origin).
- Water-free solvent system for DCCC and its suitability for the separation of non-polar substances.
- Isolation of phorbol, 4α-phorbol and croton oil.
- Purification of antibiotics such as gramicidins, tyrocidines and tetracyclines.

13.2.5 Limitations of DCCC

- Extremely low flow rates (sometimes solute retention is measured in days).
- Only biphasic solvents systems that form stable droplets can be used.
- Poor mixing of phases, which results in relatively low efficiency.

13.2.6 Modern Counter-current Chromatography

Modern CCC has split into two basic directions. The first, which is called High-speed Counter-current Chromatography (HSCCC), uses an apparatus with a variable gravity field produced by a double axis gyratory motion (Figure 4). The second, termed Centrifugal Partition Chromatography

(CPC), employs a constant gravity field produced by a single axis rotation, together with rotatory seals for supply of solvent. Separation takes place in cartridges or disks. CPC with cartridges or disks is a hydrostatic equilibrium system. If the coil is filled with the stationary phase of a biphasic solvent system and then the other phase is pumped through the coil at a suitable speed, a point is reached at which no further displacement of the stationary phase occurs and the apparatus contains approximately 50% of each of the two phases. Steady pumping-in of mobile phase results in elution of mobile phase alone. This basic system uses only 50% of the efficient column space for actual mixing of the two phases.

A more effective way of using the column space is to rotate the coil around its central axis while eluting the mobile phase. A hydrodynamic equilibrium is rapidly established between the two phases and almost 100% of the column space can be used for their mixing. CCC with rotating coil instruments is an example of this latter mechanism.

- The planetary motion is produced by engaging a planetary gear mounted on the column holder axis to an identical stationary sun gear rigidly fixed to the centrifuge framework.
- This 1:1 gear coupling produces a particular type of planetary motion of the column holder, i.e. the holder rotates about its own axis while revolving around the centrifuge axis at the same angular velocity (synchronous) in the same direction.
- This planetary motion provides two major functions for performing CCC separation. The first is a rotary-seal-free elution system so that the mobile phase is continuously eluted through the rotating separation column.
- The second and more important function is that it produces a unique hydrodynamic motion of two solvent phases within the rotating multilayer coiled column mainly due to the Archimedean screw effect.

13.3 HSCCC Instrument and Mechanism

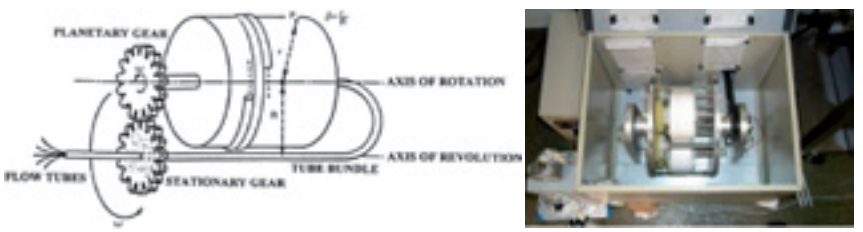

Figure 4: High speed counter-current chromatography instrument

When two immiscible solvent phases are introduced in an end-closed coiled column, the rotation separates the two phases completely

along the length of the tube where the lighter phase occupies one end (called the head) and the heavier phase occupies the other end (called the tail). Although the cause of this bilateral hydrodynamic phase distribution of two immiscible solvents is still unknown, it can be efficiently used for performing CCC. In Figure 5A the coil at the top shows bilateral hydrodynamic distribution of the two phases in the coil where the white phase (head phase) occupies the head half and the black phase (tail phase) the tail half.

This condition clearly indicates that the white phase introduced at the tail end will move toward the head and similarly the black phase introduced at the head will move toward the tail. This hydrodynamic trend is effectively used for performing CCC (Figure 5B). The coil is first entirely filled with the white phase followed by pumping the black phase from the head end (Figure 5B, top). Similarly, the coil is filled with the black phase followed by pumping the white phase from the tail (Figure 5B, bottom). In either case, the mobile phase quickly moves through the coil, leaving a large volume of the other phase stationary in the coil.

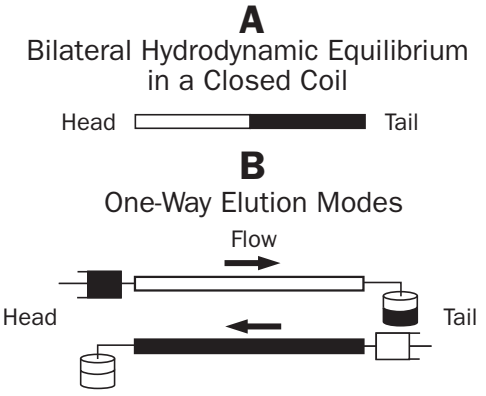

Figure 5: Mechanism of HSCCC. (A) Bilateral hydrodynamic distribution of two phases in the coiled column. (B) Elution mode of both lighter and heavier phases through the rotating coiled column.

- The motion and distribution of the two phases in the rotating coil were observed under stroboscopic illumination, and are illustrated in Figure 6A.
- A spiral column undergoes type-J planetary motion. The area in the spiral column is divided into two zones: the mixing zone occupying about one-quarter of the area near the center of revolution and the settling zone in the rest of the area.
- In Figure 6B, the spiral column is stretched and arranged according to the positions I–IV to visualize the motion of the mixing zones along the tubing.
- Each mixing zone travels through the spiral column at a rate of one round per revolution.

- The solute in the spiral column is subjected to the repetitive partition process of mixing and settling at an enormously high rate of over 13 times per second (at 800 rpm).
- HSCC is highly efficient.

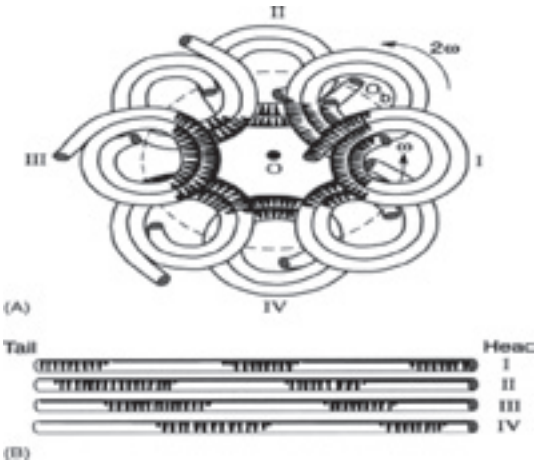

Figure 6: (A) Motion and distribution of two phases in the rotating coil under stroboscopic illumination. (B) Column in stretched position to visualize the motion of the mixing zones along the tubing.

There are numerous potential variants of this instrument design. The most significant of these is represented by the third instrument in the Pharma-tech product line: TCC-1000-toroidal CCC (Figure 7). In some respects it is like CPC, but retains the advantage of not needing rotary seals. In addition, it employs a capillary tube, instead of the larger-diameter tubes employed in the helices of the other CCC models. This capillary passage makes the mixing of two phases very thorough, despite lack of any shaking or other mixing forces.

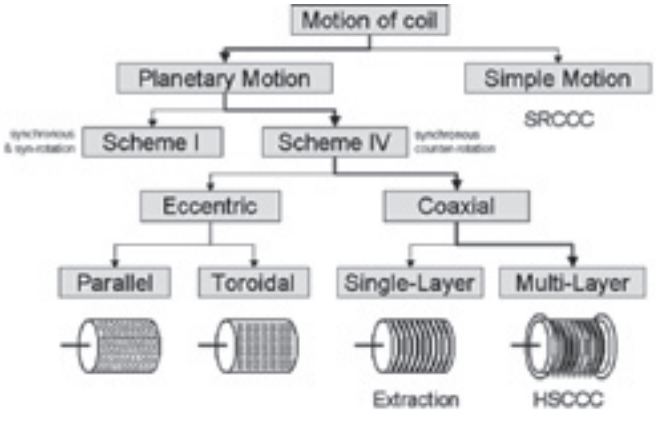

Figure 7: The TCC-1000-toroidal CCC

13.4 CPC Instrument and Setup

Figure 8: Centrifugal partition chromatography instrument

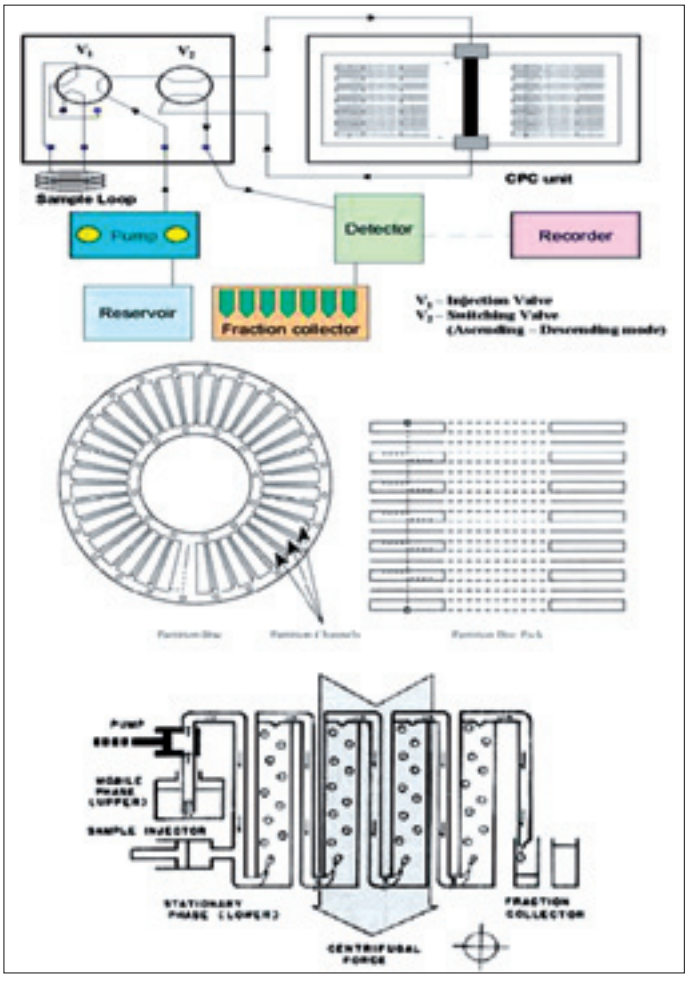

Figure 9: Centrifugal partition chromatography setup

13.4.1 Various Kinds of CPC Instruments

CPC instruments are available in various sizes depending upon

the rotor volume. As per the sample size and the purpose, one can choose on the basis of the following:

Rotor volume	Scale	Sample amount
100 ml	Analytical screening	Milligrams
200 ml	Analytical preparative	<5 g
1000 ml	Pilot scale	<40 g
5000 ml	Production scale	100–200 g
10000 ml	Production scale	200–400 g
Other volumes	On request	Up to one kilogram

13.5 How to Achieve Good Separation of Various Kinds of Natural Products Using HSCCC and CPC

In order to achieve the best separation of a desired natural product, one must systematically follow the guidelines presented in this section on choice of a solvent system.

13.5.1 Search for a Suitable Solvent System

In Table 1, sets of two-phase solvent systems are arranged from top to bottom in decreasing order of hydrophobicity in the organic phase.

Table 1: Solvent systems arranged in decreasing order of hydrophobicity in the organic phase

	n-Hexane	EtOAc	MeOH	n-BuOH	Water	
	10	0	5	0	5	HYDROPHOBIC ↑
	9	1	5	0	5	
	8	2	5	0	5	
	7	3	5	0	5	
	6	4	5	0	5	
	5	5	5	0	5	
	4	5	4	0	5	
START →	3	5	3	0	5	
HERE	2	5	2	0	5	
	1	5	1	0	5	
	0	5	0	0	5	
	0	4	0	1	5	POLAR ↓
	0	3	0	2	5	
	0	2	0	3	5	
	0	1	0	4	5	
	0	0	0	5	5	

When the polarity of the target compounds is unknown, the search may start with the two-phase solvent system composed of hexane–ethyl acetate–methanol–water at a volume ratio of 3:5:3:5, which has a moderate degree of polarity. If the partition coefficient is slightly off from the proper range, it can be adjusted by modifying the volume ratio. For example, if $K_{U/L}$ is slightly over 2, the volume ratio may be modified toward more hydrophobic such as 3.2:5:3.2:5, and if $K_{U/L}$ is slightly less than 0.5, the volume ratio may be modified in the opposite direction to 2.8:5:2.8:5.

If the target compound is mostly distributed in the upper organic phase, the search is directed upward along the arrow. If it is mostly distributed in the lower aqueous phase, the search is directed downward along the arrow. If the sample is an extract of plant material, the search may start at any point according to the polarity of the solvent used for the extraction.

If the sample is an ethyl acetate extract (relatively hydrophobic solvent), the search may start at hexane–ethyl acetate–methanol–water (1:1:1:1), whereas if the sample is a methanol extract (polar solvent), the search may start at 1-butanol–water. The search should be continued until a suitable range of $K_{U/L}$ values for all of the compounds of interest is obtained.

13.5.1.1 Solvent Systems for the Separation of a Large Variety of Natural Products

Table 2 presents different kinds of solvent systems that have been used for the separation of a large variety of natural products, which may be of immense importance for the researcher to find a suitable solvent system for the desired separation of natural products.

Table 2: Solvent systems used for the separation of natural products

Sample	Solvent system		Mobile phase
	Solvents	Ratio	
Aryl ketones			
Phenylethanone derivatives	*n*-Heptane–EtOAc–MeOH–H_2O	4:2:2:1	Lower
Styrylpyrones			
Kava lactones	*n*-Hexane–acetone–MeOH–H_2O	4:1:3:1	Upper
Benzofurans			
Dibenzofurans	*n*-Heptane–CH_2Cl_2–CH_3CN	10:3:7	Lower, upper

Sample	Solvent system		Mobile phase
	Solvents	Ratio	
Phenolic glycosides			
Gastrodin	EtOAc–1-BuOH–H_2O	3:2:5	Lower
Phenylpropanoids			
Honokiol, magnolol	*n*-Hexane–EtOAc–MeOH–H_2O	5:2:5:2	Lower
Phenylpropanoid glycosides			
Verbascoside	EtOAc–CH_3CN–H_2O	13:13:24	Lower
Acteoside	EtOAc–H_2O	1:1	Lower
Hydrolyzable tannins			
Ellagic acid and corilagin	1-BuOH–HOAc–H_2O	4:1:5	Lower
Catechins			
Epigallocatechin	*n*-Hexane–EtOAc–H_2O	1:9:10	Lower
Tea catechins	*n*-Hexane–EtOAc–MeOH–H_2O	3:10:3:10	Lower
Proanthocyanidins			
Tea proanthocyanidins	*n*-Hexane–EtOAc–MeOH–H_2O	1:5:1:5	Lower
Anthocyanins	MTBE–1-BuOH–CH_3CN–H_2O	2:2:1:5	Lower
Bilberry anthocyanins	MTBE–1-BuOH–CH_3CN–H_2O–TFA	1:4:1:5:0.01	Lower
Flavonoids			
Flavones and chalcones	$CHCl_3$–CH_2Cl_2–MeOH–H_2O	2:2:3:2	Lower
	CH_2Cl_2–MeOH–CH_3CN–H_2O	40:11:25:20	Lower
	EtOAc–1-PrOH–H_2O	140:8:80	Upper
Flavonoid glycosides	*n*-Hexane–EtOAc–MeOH–H_2O	1:8:1:8	Lower
	n-Hexane–EtOAc–MeOH–H_2O	2:12:3:15	Lower
	$CHCl_3$–MeOH–H_2O	8:7:4	Lower
C-Glycosyl flavones	$CHCl_3$–MeOH–H_2O	6:25:29	Lower
	EtOAc–1-BuOH–MeOH–H_2O	35:10:11:44	Lower
Icariin	*n*-Hexane–1-BuOH–MeOH–H_2O	1:4:2:6	Lower
Isoflavone glycosides	EtOAc–1-BuOH–EtOH–H_2O	30:10:6:50	Lower
	EtOAc–EtOH–HOAc–H_2O	16:4:1:20	Lower
Flavonol glycosides	1-BuOH–H_2O	1:1	Lower
Flavonolignans			

Sample	Solvent system		Mobile phase
	Solvents	Ratio	
Silymarin	*n*-Hexane–EtOAc–MeOH–H_2O	1:4:3:4	Lower
Lignans			
Arctiin	EtOAc–1-BuOH–EtOH–H_2O	10:1:2:10	Lower
Lignan diglucoside	MTBE–1-BuOH–CH_3CN–H_2O	1:3:1:5	Upper
Coumarins			
Psoralen and isopsoralen	*n*-Hexane–EtOAc–MeOH–H_2O	10:7:10:8	Lower
Inflacoumarin A	*n*-Hexane–$CHCl_3$–MeOH–H_2O	5:6:3:2	Lower
Chalcones			
Licochalcone A	*n*-Hexane–$CHCl_3$–MeOH–H_2O	3:12:6:4	Lower
Quinones			
Tanshinones	*n*-Hexane–EtOH–H_2O	(a) 20:11:9 (b) 10:7:3	Lower
Shikonin	*n*-Hexane–EtOAc–EtOH–H_2O	16:14:14:5	Lower
Diterpene quinone	*n*-Hexane–CCl_4–MeOH–H_2O	1:3:3:2	Lower
Monoterpenes			
Monoterpenes and monoterpene glycosides	$CHCl_3$–MeOH–H_2O	7:13:8	Upper
Iridoid glycosides	$CHCl_3$–MeOH–1-PrOH–H_2O	5:6:1:4	Lower
Sesquiterpenes			
Artemisinine, artemisitene arteannuin B	*n*-Hexane–EtOAc–EtOH–H_2O	6:4:5:4	Lower
Diterpenes			
10-Deacetylbaccatin III	*n*-Hexane–EtOAc–EtOH–H_2O	2:5:2:5	Lower
	n-hexane–$CHCl_3$–MeOH–H_2O	5:25:34:20	Lower
Trachylobanes and isopimaranes	*n*-Heptane–CH_3CN–CH_2Cl_2	10:7:3	Lower
Triterpenes			
Celastrol	*n*-Hexane–EtOAc–CCl_4–MeOH–H_2O	1:1:8:6:1	Lower
Saponins			
Ginsenosides	$CHCl_3$–MeOH–2-BuOH–H_2O	5:6:1:4	Lower
	EtOAc–1-BuOH–H_2O	1:1:2	Upper

Sample	Solvent system		Mobile phase
	Solvents	Ratio	
	n-Hexane–1-BuOH–H_2O	3:4:7	Lower
Phytolacca saponins	$CHCl_3$–MeOH–2-PrOH–H_2O	5:6:1:4	Lower
Glycyrrhizin	EtOAc–MeOH–H_2O	5:2:5	Lower
Carotenoids			
Zeaxanthin	*n*-Hexane–EtOAc–EtOH–H_2O	8:2:7:3	Lower
Lycopene	*n*-Hexane–CH_3CN–CH_2Cl_2	20:13:7	Upper
Alkaloids			
Aporphine alkaloids	CH_2Cl_2–MeOH–5% HOAc	5:5:3	Lower
Naphthylisoquinoline alkaloids	$CHCl_3$–EtOAc–MeOH–0.1 M HCl	5:3:5:3	Lower
Diterpene alkaloids	*n*-Hexane–CH_2Cl_2–MeOH–H_2O	15:15:24:8	Lower
	C_6H_6–$CHCl_3$–MeOH–H_2O	5:5:7:2	Lower
Tetramethylpyrazines			
Chuanxiongzine	*n*-Hexane–EtOAc–EtOH–H_2O	5:5:3:7	Lower
Glucosinolates	PrOH–CH_3CN–$(NH_4)_2SO_4$–H_2O	10:5:12:10	Upper
Cyclodepsipeptides	*n*-Heptane–EtOAc–MeOH–H_2O	2:8:2:8	Lower

13.5.1.2 Retention of the Stationary Phase

- Successful separation in HSCCC-CPC largely depends on the amount of the stationary phase retained in the column. In general, higher the retention of stationary phase, better the peak resolution.
- The amount of stationary phase retained in the column is highly correlated with the settling time of the two phases in a test tube.
- Measure the settling time of the two-phase solvent system to be used for the separation.
- The procedure is as follows: the two phases are first equilibrated in a separatory funnel. Deliver 2 ml of each phase, a total volume of 4 ml, into a test tube or graduated cylinder, which is then capped. Gently invert the container for several times and then immediately place it in an upright position to measure the time required for the two phases to form clear layers with a distinct interface.
- If the settling time is less than 20 seconds, the solvent system will provide satisfactory retention of the stationary phase, usually over 50% of the total column capacity, in a proper range of flow rates.

13.5.1.3 Preparation of Sample Solution

- The sample for HSCCC-CPC may be dissolved directly in the stationary phase or in a mixture of the two phases.
- The recommended sample volume is less than 5% of the total column capacity.
- Introduction of a larger sample volume into the column will reduce peak resolution of the analytes, especially for those having small *K* values.
- Ideally the analyte is injected in a small volume of the stationary phase to preserve the sharpness of the early elution peak.

13.5.1.4 Separation Column

- We must understand the head–tail orientation of the separation column.
- A lower (heavier) mobile phase should be introduced through the head toward the tail, and an upper (lighter) mobile phase in the opposite direction.
- This is extremely important because the elution of either phase in the wrong direction results in an almost complete loss of the stationary phase from the column.

13.5.1.5 Choice of the Mobile Phase

- In HSCCC-CPC, either phase can be used as the mobile phase provided that the *K* value of the analyte is in a proper range.
- If one has a choice, the lower phase may be used as the mobile phase, because the system provides more stable retention of the stationary phase and one can avoid trapping air bubbles in the flow cell of the detector by introducing the effluent from the lower end of the cell.
- On the other hand, when the upper organic mobile phase (excluding the chloroform system) is used as the mobile phase, it will facilitate the evaporation of solvent from the collected fractions.

13.5.1.6 Flow Rate of the Mobile Phase

- The flow rate of the mobile phase determines the separation time, the amount of stationary phase retained in the column, and therefore the *peak resolution*.
- A lower flow rate usually gives higher retention level of the stationary phase and improves the peak resolution, although it requires a longer separation time.
- The typical flow rates for the commercial multilayer coil are as follows:
  i) 5–6 ml/min for a preparative column with 2.6 mm i.d. PTFE tubing (600–800 rpm) (up to 1 g sample load);

ii) 2–3 ml/min for a semi preparative column with 1.6 mm i.d. PTFE tubing (800–1000 rpm) (up to 500 mg sample load);
iii) 1 ml/min for an analytical column with 0.85–1.0 mm i.d. PTFE tubing (1000–1200 rpm) (up to 50 mg sample load).

- These flow rates should be modified according to the settling time of the two-phase solvent system as well as other factors. When the settling time is around 20 s and the *K* value of the analyte is small, a lower flow rate is recommended.

13.5.1.7 Revolution Speed

The optimum revolution speed (revolution and planetary rotation speeds are always same) for the commercial HSCCC-CPC instrument for preparative separation ranges between 600 and 1400 rpm.

- Use of a lower speed will reduce the volume of the stationary phase retained in the column, leading to lower peak resolution.
- On the other hand, the higher speeds may produce excessive sample band broadening by violent pulsation of the column because of elevated pressure.

13.5.1.8 Filling the Column with the Stationary Phase

- In each separation, the column is first entirely filled with the stationary phase.
- Before introducing the stationary phase, the column may be flushed with a column volume of a solvent miscible with the two phases used in the previous run (e.g. ethanol or methanol) to wash out materials remaining in the column.
- This will also ensure a stable, clean baseline before the solvent front emerges.
- Avoid trapping the air in the column, especially in a preparative column. This can be easily tested as:
  i) If no air is present in the column, the flow from the column outlet is ceased shortly after stopping the pumping.
  ii) If the solvent keeps flowing from the outlet for more than several seconds, the air trapped in the column should be eliminated by resuming the pumping of the stationary phase under low speed column rotation (100–200 rpm) in a tail to head elution mode to accelerate air movement toward the outlet of the column.

13.5.1.9 Sample Loading

There are two ways to load samples; both are satisfactory for HSCCC-CPC separations:

- In the first method, the column is entirely filled with stationary phase and this is immediately followed by sample injection.

- The mobile phase is then eluted through the column while the column is rotated at the optimum rate.
- In the second method, after the column is filled with stationary phase, the mobile phase is eluted through the column at a desired rate until the solvent front emerges and hydrodynamic equilibrium is established throughout the column as evidenced by diminished carryover of the stationary phase.
- The sample is then injected into the column through the sample port.

Each method has advantages. The second method gives a clear tracing of the elution curve because of the minimum carryover of the stationary phase from the column.

The first method produces a distinct solvent front and saves separation time by eliminating the waiting period to reach hydrodynamic equilibrium.

One can conveniently use an injection valve with a sample loop as in HPLC.

13.5.1.10 On-line Monitoring of Effluent

- The effluent from the outlet of the HSCCC-CPC columns may be continuously monitored by a UV–VIS detector as in conventional liquid chromatography.
- An important difference between these two methods is that HSCCC-CPC uses the liquid stationary phase which, if carried over from the column, tends to disturb the tracing of the elution curve.
- Avoid trapping the stationary phase in the vertical flow cell by eluting the lower mobile phase upward from the bottom; do the reverse if the upper is used as the mobile phase.
- When the upper mobile phase is eluted from the top of the flow cell downward, it is important to prevent the formation of air bubbles which may become trapped in the flow cell and disturb the tracing of the elution curve.
- Bubble formation can be largely avoided by degassing the two phases in the separatory funnel before use, and also by connecting fine PTFE tubing (typically 30 cm × 0.4–0.5 mm i.d.) to the outlet of the monitor so that the pressure within the flow cell is substantially increased.

13.5.1.11 Measurement of Stationary Phase Retention

- When the separation is completed, rotation is stopped and the column contents are collected into a graduated cylinder by connecting the column inlet to a nitrogen cylinder (ca. 50 psi; 1 psi = 6894.76 Pa).

- After nitrogen appears at the outlet, the column is slowly rotated (100 rpm) in the tail to head elution mode so that solvent remaining inside the column is pumped out by an Archimedean screw force assisted by the nitrogen flow. Measure the retained stationary phase in a graduated cylinder.
- Measurement of the retained stationary phase is useful in efforts to improve the separation: when the peak resolution is unsatisfactory, a measure of stationary phase retention will serve as a guide for the next trial.
- If it is less than 30%, the separation may be improved by increasing retention, by applying a lower flow rate of the mobile phase, increasing the revolution speed, or modifying the solvent system to shorten the settling time.
- If instead the stationary phase retention is over 50%, efforts should be directed to search for a new two-phase solvent system, which provides an improved separation factor (α) between the analytes.

13.5.2 Applications of HSCCC-CPC Technologies in Natural Products Isolation

HSCCC-CPC technologies have applications in the following industries: nutraceuticals, fine chemicals, pharmaceuticals, biomedical, biotechnology, fats and oils, and fermentation.

Compounds that can be isolated in high purity by HSCCC-CPC technologies include: saponins, alkaloids, chlorophylls, tannins, carotenoids, phospholipids, fat-soluble vitamins, mono- and oligosaccharides, anthocyanins, lignans, phenolic compounds, synthetic compounds, other active compounds present in medicinal and aromatic plants (e.g. herbs and spices) and much more. The numerous applications of CCC have resulted in a growth in the annual number of publications in which this separation technology has been cited (Figure 10).

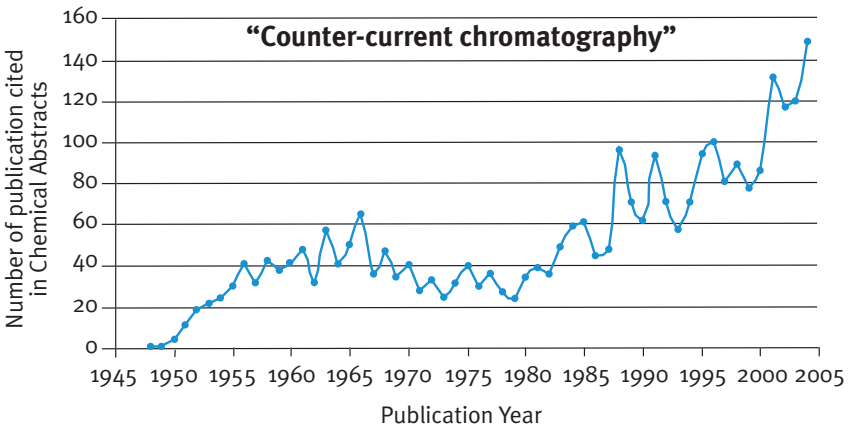

Figure 10: Increasing number of publications on separation by CCC

Examples of some important natural products that have been separated using HSCCC-CPC in the recent past are summarized herein.

13.5.2.1 Purification of Coenzyme Q_{10} from Fermentation Extract: HSCCC versus Silica Gel Column Chromatography

HSCCC was applied to the purification of coenzyme Q_{10} (CoQ_{10}) for the first time. CoQ_{10} was obtained from a fermentation broth extract. A non-aqueous two-phase solvent system composed of heptane–acetonitrile–dichloromethane (12:7:3.5, v/v/v) was selected by analytical HSCCC and used for purification of CoQ_{10} from 500 mg crude extract. The separation yielded 130 mg CoQ_{10} at an HPLC purity of over 99%. The results showed the advantages of HSCCC over an alternative of silica gel chromatography followed by recrystallization. These advantages regard purity, recovery and yield (Table 3).

Table 3: Purification of coenzyme Q_{10} from fermentation extract: HSCCC vs. silica gel column chromatography with subsequent crystallization

	Crude extract	CoQ_{10} purified by silica gel chromatography	CoQ_{10} purified by HSCCC
HPLC purity, %	89.2	96.0	99.2
Absolute purity, %	29.4	93.3	97.8
Recovery, %*	–	74.3	88.0
Yield, %*	–	23.4	26.4

* *Recovery, amount of CoQ_{10} in purified product/amount of CoQ_{10} in crude extract. Yield,amount of purified product/amount of crude extract (Journal of Chromatography A 1127 (1-2), 15 September 2006, 92-96)*

13.5.2.2 Preparative Separation of Gambogic Acid and its C-2 Epimer by HPCCC

For the preparative separation of epimers, gambogic acid and epigambogic acid, from *Garcinia hanburyi,* a two-phase solvent system composed of *n*-hexane–methanol–water (5:4:1, v/v/v) was used. From 50 mg mixture, 28.2 mg gambogic acid and 18.4 mg epigambogic acid were separated. The purities of both were above 97% as determined by HPLC. The chemical structures were then identified by their ^{1}H NMR and ^{13}C NMR spectra.

13.5.2.3 Separation and Purification of 10-deacetylbaccatin III by HSCCC

At present, the most promising approach is the semisynthesis of paclitaxel or its analogs from 10-deacetylbaccatin III, a compound available in a relatively high quantity from the foliage of several yew species. HSCCC was used for the separation and purification of 10-deacetylbaccatin III (Figure 11). A crude needle extract (500 mg/5 ml) from Chinese yew (*Taxus chinensis*) was

first separated with a two-phase solvent system composed of *n*-hexane–ethyl acetate–ethanol–water (2:5:2:5, v/v). The partially purified fraction was again purified with a different solvent system composed of *n*-hexane–chloroform–methanol–water (5:25:34:20, v/v). HPLC analysis of the final fraction showed that the purity of 10-deacetylbaccatin (20 mg) was over 98%.

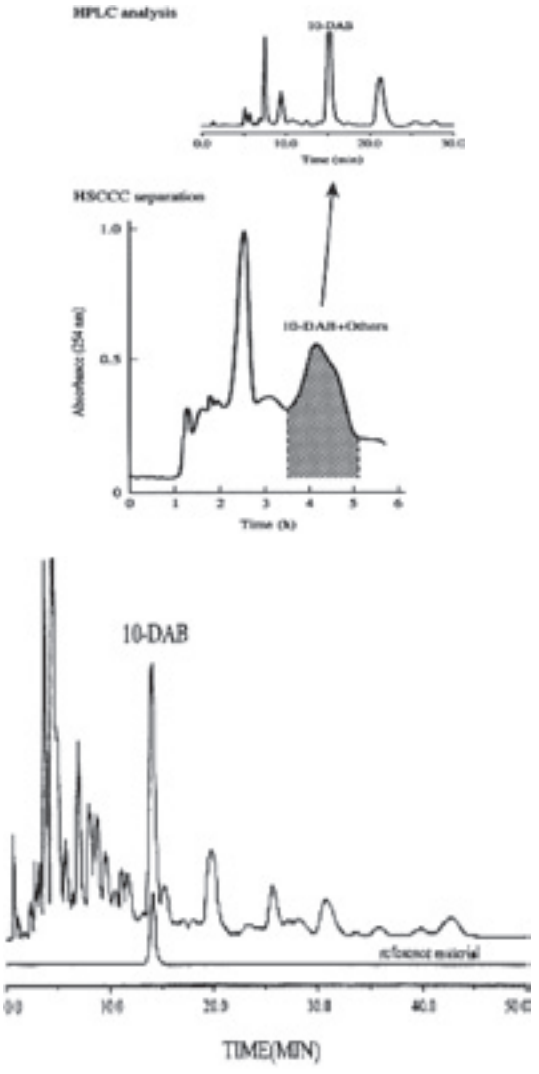

Figure 11: Separation and purification of 10-deacetylbaccatin III (DAB) by HSCCC

13.5.2.4 Large-scale Separation of Resveratrol and Anthraglycoside A and B from *Polygonum cuspidatum* by HSCCC

HSCCC was successfully applied to the large-scale (5 g) separation of resveratrol, anthraglycoside A and anthraglycoside B from a crude extract of *Polygonum cuspidatum* Sieb. et Zucc (Figure 12). A two-phase solvent system composed of chloroform, methanol and water (4:3:2, v/v) was used. The separation yielded 200 mg to 1 g of these three compounds, each at over 98% purity as determined by HPLC. Resveratrol is important as it has a cancer chemopreventive activity.

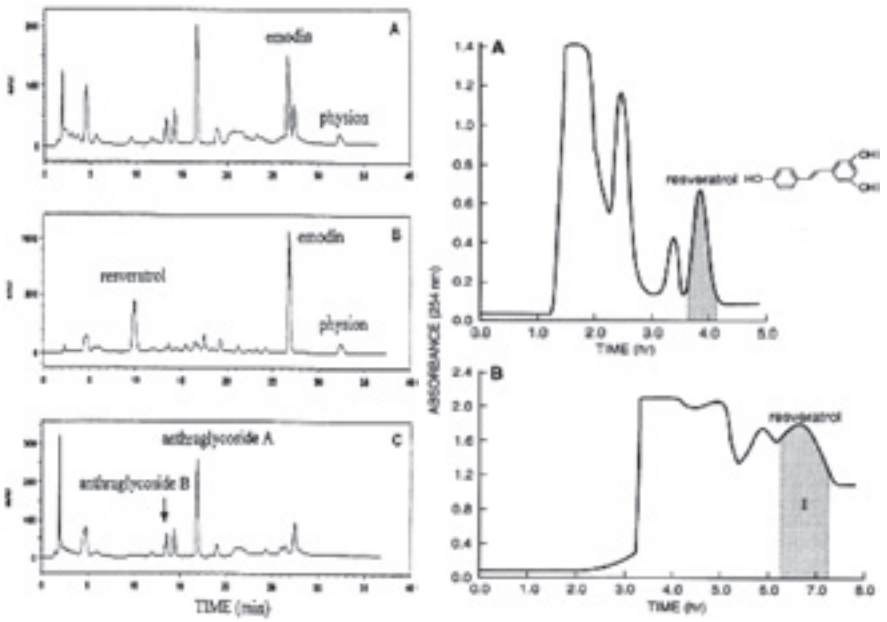

Figure 12: Separation of resveratrol, anthraglycoside A and anthraglycoside B from a crude extract of Polygonum cuspidatum Sieb. et Zucc

13.5.2.5 Separation of Andrographolide and Neoandrographolide from the Leaves of *Andrographis paniculata* using HSCCC

The bioactive diterpenes andrographolide and neoandrographolide from the leaves of *Andrographis paniculata* NEES (Acanthaceae) were successfully separated by CCC (Figure 13). A single 280-min separation yielded 189 mg of 99.9% andrographolide and 9.5 mg of 98.5% neoandrographolide. Water–methanol–ethyl acetate–*n*-hexane (2.5:2.5:4:1) solvent system was used.

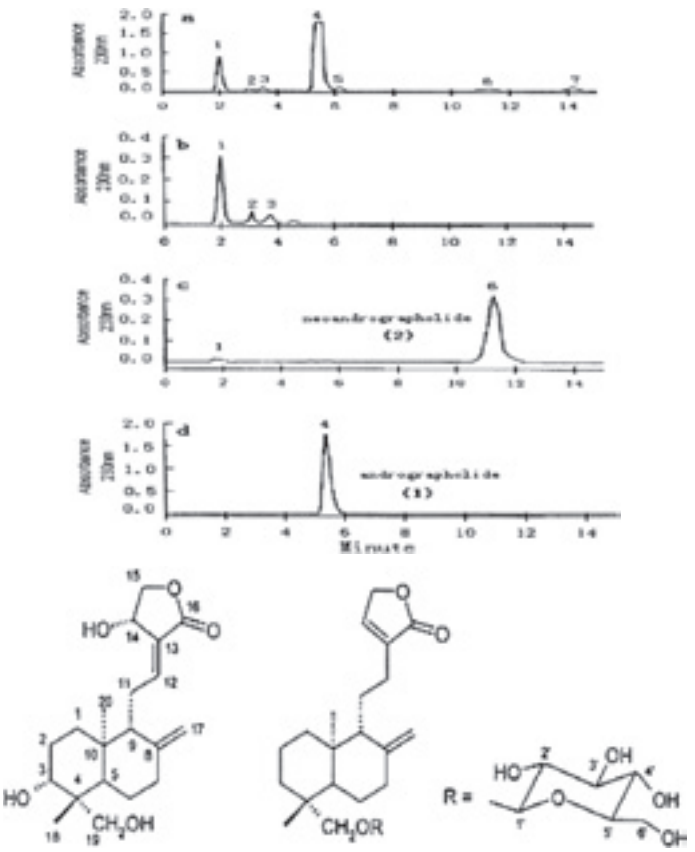

Figure 13: Separation of Andrographolide and Neoandrographolide from the leaves of Andrographis paniculata using HSCCC

13.5.2.6 Separation of WAP-8294A Components, a Novel Anti-methicillin-resistant *Staphylococcus aureus* Antibiotic, using HSCCC

The WAP-8294A complex was isolated from the fermentation broth of *Lysobacter* sp. WAP-8294 (Figure 14). The major component, WAP-8294A2, shows strong activity against gram-positive bacteria including methicillin-resistant *Staphylococcus aureus* (MRSA) and vancomycin-resistant *Enterococci* in vitro, and also exhibited a potent activity against MRSA in vivo.

The previous separations were unsatisfactory. Hence HSCCC was applied. *n*-Butanol–ethyl acetate–aqueous 0.005 *M* trifluoroacetic acid (1.25:3.75:5, v/v/v) was used as biphasic solvent. A sample size of 25 mg yielded pure fractions of three components (1–6 mg). The method will contribute to the clinical development of WAP-8294A2 as an anti-MRSA agent.

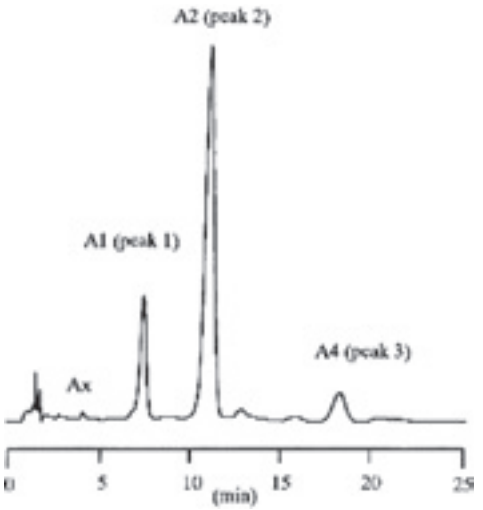

Figure 14: Separation of WAP-8294A components using HSCCC

13.5.2.7 Other Examples of Separation of Phytoconstituents by CCC

Apart from the previously mentioned examples, isolation and purification of polymethoxylated flavones from tangerine peel, catechin constituents from five tea cultivars, rupestonic acid from the Chinese medicinal plant *Artemisia rupestris* L., lycopene from tomato paste, spiramycin, gallic acid from *Cornus officinalis*, lutein from the microalga *Chlorella vulgaris*, naphthopyranone glycosides, salvianolic acid B from the Chinese medicinal plant *Salvia miltiorrhiza*, dammarane saponins from *Panax notoginseng*, isoflavan and pterocarpan glycosides from *Astragalus membranaceus* Bge. var. *mongholicus* (Bge.), glycyrrhizin from the root of liquorice and active principles from the roots of *Sophora flavescens* have been carried out successfully using HSCCC.

13.6 Advantages of CCC

- Quick (high throughput in preparative separation).
- Inexpensive (only solvent costs, which are 5 times less than for other LC techniques).
- Gentle and versatile, for separation of varied compounds, with less chance of decomposition.
- Able to resolve from milligrams to tens of grams on the same instrument.
- Able to switch between normal and reverse phase at will.
- A CCC machine, which is a chromatographic column with a liquid stationary phase, can be used as a liquid–liquid reactor for chemical reactions involving a liquid catalyst.

- No irreversible adsorption to a solid support (100% recovery of sample).
- Increased capacity for the same volume of stationary phase; a CPC column gives a higher capacity than the HPLC one.
- Quantity of sample depends on two factors: solubility of the sample and properties of the solvent system.
- No sample loss as a simple rinsing of the instrument allows a full recovery of the noneluted fractions.
- Easy maintenance, no costly solid phase to change.
- No degradation or denaturation of compounds and no interaction with silica.
- No polarity restriction; all biphasic mixtures can be used.
- Dual mode (off-line and on-line) exchange of stationary and mobile phase (CPC).

13.6.1 Advantages of HSCCC-CPC Technologies over HPLC

HSCCC-CPC	HPLC
No column	Expensive columns
High recovery	Irreversible adsorption
High throughput	Poor loadability
Retention of fragile compounds (molecular integrity)	Loss of biological activity (denaturation)
Volume ratio of stationary/ mobile very high (better resolution)	Ratio is low

13.7 Manufacturers of CCC Instruments

13.7.1 Manufacturers of HSCCC Machines

- AECS (http://www.ccc4labprep.com/)
- Conway Centri Chrom (http://www.centrichrom.com/)
- Dynamic Extractions (http://www.dynamicextractions.com/)
- Pharma-tech Research Corporation (http://www.pharma-tech.com/)

13.7.2 Manufacturers of CPC Machines

- EverSeiko Corporation (http://www.everseiko.co.jp)
- Kromaton Technologies (http://www.kromaton.com/)
- Partus Technologies (http://www.partus-technologies.com)

13.8 Selected Reviews on CCC

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13.9 Conclusions

Counter-current Chromatography is an excellent alternative to circumvent the problems associated with solid-phase adsorbents and to preserve the chemical integrity of mixtures subjected to fractionation. It provides efficient resolution of samples by a mechanism which relies solely on partition. Separation of compounds with a wide range of polarities can be achieved with the use of aqueous and non-aqueous solvent systems. CCC is very flexible: solvent gradients are possible; flow rates can be varied during a chromatographic run; lower and upper phases can be interchanged as mobile phases during a separation, provided that the flow direction is also changed accordingly; and instruments can be stopped during chromatography and re-started hours later without affecting separation efficiency. A wide range of pH is tolerated in CCC, with implications in the separation of acidic and basic samples, notably in the technique of pH-zone-refining. Separation of crude plant extracts, semipurified fractions or synthetic mixtures can be carried out, with samples of any quantity ranging from 100 mg to 1500 g (for the largest model). With these advantages, CCC is gaining popularity as a separation method for natural products, and especially in the bioassay-guided fractionation of natural products.

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14 Quality Control of Medicinal and Aromatic Plants and their Extracted Products by HPLC and High Performance Thin Layer Chromatography

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Abstract

The interest in medicinal plants and their products has increased manifold in recent years. The increasing public demand for natural medicines has resulted in increased commercial activity and production of these medicines. There is also increasing concern for ensuring quality and safety of plant medicines. Plant drugs, unlike active pharmaceutical ingredients, possess some inherent limitations which deter the process of laying standards for these drugs. This aspect has received considerable attention from different quarters including policy planners, scientists and manufacturers. This paper describes briefly the roles of high performance thin layer chromatography and high performance liquid chromatography in quality assurance of plant products. Some practical aspects of these techniques are also discussed.

14.1 Introduction

The use of medicinal plants products has increased several fold during the last decades. Individual countries are also giving increasing emphasis to promote their use under the direction of the World Health Organization (WHO). Besides this, one finds enormous interest in natural products from the public, which is attributable to several factors. These medicines are affordable, safer and better tolerated by the biological system. This has led to an increased consumption and cross-country movement of raw materials of medicinal plants.

In some parts of the world, e.g. several places in Africa and Asia, traditional medicines are the only affordable option. On the other hand, the same medicines are the option of choice in developed nations like Japan and the United States and in the European States. Despite being the more common medical option in Africa, use of traditional medicines has not matured to the expected level. But, some countries in Asia, especially India and China, have developed them to a level that has benefited all countries of the world.

Europe did not inherit a well-developed traditional system of medicine but it has put in place the strongest evidence-based and scientifically supported system of plant medicines. North America is the most prolific and flamboyant market for plant products, but government regulations are such that except for select plant drugs most are used as dietary supplements or

nutraceuticals. South America, which has given the world some of the all-time great plant medicines like quinine and pilocarpine, follows the medical system of North America. Australia has a strong impact from Chinese medicines on the continent which is otherwise dominated by the modern system of medicine.

14.2 Quality Control of Medicinal Plants and their Products

The quality control of consumer products has become more challenging and demanding. The quality considerations of drugs are the most stringent among all consumer products. The purity of active pharmaceutical ingredients has been stretched to an all-time high with more and more restrictions on the level of the impurities. The situation is opposingly different in the case of plant-derived medicine, where we are still striving to define specifications to ensure consistency and safety. Therefore, the standards of plant drugs are more relaxed and are in the process of development. The inherent problems of plant drugs are obvious; unlike single chemical entities of modern drugs, they are combinations of infinite chemical molecules, known and unknown; the knowledge of the active components is incomplete; the natural variations in content and quantity of the chemical constituents are large and exercising a precise control is impractical; and the complete chemical profiling of plant drugs is beyond scope. Therefore, laying standards for such drugs is not an easy task and a comprehensive system of standards cannot be laid down for such drugs. As our knowledge of plant drugs will advance, the standards for them will become more meaningful and complete.

The quality issue of plant drugs was irrelevant in ancient times when these medicines were dispensed by the medical men for their patients. However, the issue has taken front seat with the commercialization of plant drugs. The matter has been further complicated by the vested interest of manufacturers who are out to exploit the loopholes in the standards and laws governing the production and distribution of plant drugs.

Several national and international agencies have prioritized the issue of assuring the quality of plant drugs. The effort of the World Health Organization is outstanding: over 20 years ago it first published *Quality control methods for medicinal plant materials*, which has been regularly updated and followed by a series of monographs on globally important medicinal plants.

The quality of a plant product cannot be assured without assuring the quality of the raw material. Also required to ensure quality products are in-process control, quality control of the finished product, good manufacturing practice (GMP) controls and process validation. In this regard, it is imperative to define specifications of raw materials to minimize variations in the quality of finished products and to achieve consistency. The specifica-

tions of plant materials include macro- and microscopic descriptions, tests of identity, and analytical and physicochemical determinations. The expected results of these tests and measurements are presented as numerical limits or as a range or discretely observable result.

While fixing the limits of specifications, naturally met variations in plant drugs need to be accommodated. A plant material conforming to the prescribed specifications should be considered acceptable for intended use. Several factors contribute to variation in the content and composition of raw materials. These factors can broadly be grouped in four categories of climatic, nutritional, collection and post-harvest factors. Climatic factors include prevailing temperature, rainfall, humidity, daylight and altitude of the growing region. The nutritional factors are those which affect the health of a growing plant and are reflected in the production of biomass and its composition; several soil factors such as availability of micro- and macronutrients, pH and cation exchange capacity are important for optimal growth of plants. Collection factors control the content of active components by giving due attention to the age, season, collection time and part of the plant collected. Post-harvest factors are important as the collected material is still live and carries out metabolic processes and respires; the enzymatic processes continue after collection until they are deactivated by drying or other suitable treatment; the crushing and cutting of material leads to de-compartmentalization of reactive chemical constituents of plants which were naturally located in intact cells; and the collected material faces direct impact of oxidation by air and light besides physical loss of some components.

The World Health Organization, in its volume *Quality control methods for medicinal plant materials,* has listed several parameters which are valuable in assuring quality of plant drugs. These include identification, visual inspection, sensory characters, macro- and microscopic characteristics, moisture content, foreign matter, fingerprint by thin layer chromatography (TLC), ash values, extractive values, volatile matter, microbial load, heavy metals and pesticide residues, radioactive contaminants and, according to the nature of the drug, one or more determinations for bitter value, tanning test, foaming, hemolytic and swelling indices. The European Medicines Agency's "Guideline on quality of herbal medicinal products" and "Guideline on specifications" more precisely describe the quality-related issues of medicinal plants. These documents define and differentiate herbal substances (equivalent to herbal drugs), herbal preparations (equivalent to herbal drug preparations) and herbal medicinal products (equivalent to traditional herbal medicinal products). The guidelines emphasize the quantification of active or analytical markers and describe procedures to ensure quality of raw material, semifinished and finished products.

14.3 Biological and Chemical Standardization of Drugs

All test procedures ultimately aim to determine the intrinsic potency of a drug, which is attributable to the chemical constituents present. Evaluating the biological potency of a drug provides a direct assessment of its quality. But, the complexity of the procedures and methods forbids implying this assessment. Moreover, it is not the practical option when one handles large numbers of samples. The other option, besides biological testing, is chemical testing, which uses assay procedures to determine the quantity of chemical compounds, preferably the active ones, to assess the quality of a product. This is complementary to recording other specifications like macro- and microscopic characteristics.

14.3.1 Chemical Standardization and Markers

Chemical standardization requires first to identify and select a chemical constituent of a drug and then to elaborate the assay procedure for quantification of the chosen compound. Selection of the constituent, called marker, is a difficult task based on several considerations: the chemical profile of the drug, biological activity of the chemical constituents, successful development of the assay procedure, ease of procuring or isolating the marker, and stability of the marker.

The European Union Guidelines define markers as “Chemically defined constituents or groups of constituents of a herbal substance, a herbal preparation or a herbal medicinal product which are of interest for control purposes independent of whether they have any therapeutic activity.” The WHO defines markers as “constituents of a medicinal plant material which are chemically defined and of interest for control purposes.”

Markers serve to calculate the quantity of herbal substances or herbal preparations in the herbal medicinal product if the marker has been quantitatively determined in the herbal substance or herbal preparations. The European Medicines Agency differentiates an active marker from an analytical marker: an active marker contributes to the therapeutic activity of the drug, whereas an analytical marker serves only the analytical purposes.

The choice of a marker, in first place, should be for a chemical constituent of the drug responsible for the activity. More than one marker can be employed for control purposes, separately or in combination. A second choice of marker is for a defined chemical constituent that is uniquely associated with the drug. This is true when active constituents of the drug are not known or active constituents are unobtainable or unstable for assay purposes. As a last choice, a marker can be selected among more commonly or ubiquitously present phytoconstituents. In rare cases, a chemical compound

not associated with the plant or its activity can also be used as a marker. Such chemical compounds allow the estimation of an active constituent of the plant, which itself is unstable and unfit for the purpose of analysis. For example, dantron is recommended by the European Pharmacopoeia for the estimation of valerenic and acetoxyvalerenic acids in valerian root. Valerenic and acetoxyvalerenic acids, which are active constituents of drugs, are highly unstable and difficult to isolate and to use as markers. The test response measured for these acids in the drug is interpreted from the standard plot of dantron to calculate the concentration of valerenic and acetoxyvalerenic acids in the test solutions. It has been experimentally shown that the standard curves of dantron and these two acids, in the range of estimation, are linear and parallel.

Identification of a marker requires knowledge of the chemical and active constituents of the plant drug. Markers are usually generated in-house and a limited number of markers are commercially available from different sources. Sigma Chemicals, Chromadex, Regional Research Laboratory (Jammu) and Natural Remedies (Bangalore) are some commercial sources of markers.

14.3.2 Analytical Techniques for Quantifying a Marker

After a marker has been identified, it needs to be quantified or assayed in the test material for the purpose of quality control. Any of the major analytical techniques, including high performance liquid chromatography (HPLC), high performance thin layer chromatography (HPTLC), gas chromatography (GC), radioimmunoassay, ultraviolet or infrared spectrometry, and mass spectrometry, can be used to determine the quantities of marker in the test samples. These techniques possess some advantages and disadvantages. Some are more commonly used while the others have limited applications. The assay method requires the procedure and technique to be simple, quick, specific, economical and robust. The ideal method can be used in different laboratories across the world without compromising on the accuracy and precision, which is possible if the procedure has a minimum number of critical variables. Whenever possible, the assay procedure should use simple, inexpensive equipment which is affordable in most places. The assay methods are developed through experimentation and from the existing knowledge of the drug and of techniques.

14.3.3 Validation of Analytical Procedures

Development of the assay method is followed by its validation. The validation procedure considers issues of specificity, linearity, range, accuracy, precision, detection limit, quantitation limit, robustness and system suitability. The International Conference on Harmonisation (ICH) has issued guidelines on validating analytical procedures, which are widely accepted and commonly used. Besides validation of a procedure, the instruments

used are also validated from time to time. Practically all manufacturers of modern equipment supply detailed instructions and methods of equipment validation. The validation record is maintained and, in case a deviation is observed, a service engineer is called.

The ICH guidelines on validation are available from the internet for comprehensive understanding of the subject. A brief explanation to the validation procedure is provided here.

14.3.3.1 Specificity

Specificity indicates the extent to which an assay procedure specifically measures the analyte of interest. It is not always possible to demonstrate that an analytical procedure is specific for a particular analyte (complete discrimination). It may require two or more assay procedures to demonstrate the necessary level of discrimination. Spiking the sample with the analyte or related compounds and observing the effect on the estimations demonstrates assay specificity. If spiking with compounds related to the analyte produces no effect on the result, the procedure is considered specific. Specificity is particularly valuable when analyzing an analyte among several similar compounds present in the sample.

The issue of specificity with respect to herbal materials has lesser relevance as in several instances; we tend to determine the content of total active compounds rather than one active constituent, e.g. total sennosides in senna and not a particular sennoside. In such cases, a procedure that is able to assay all sennosides together is preferred than a specific procedure which discriminates and estimates only one sennoside.

14.3.3.2 Linearity

The linearity of an analytical procedure is its ability (within a given range) to obtain test results which are directly proportional to the concentration (amount) of the analyte in the sample. A linear relationship (between amount of analyte and response) should be demonstrated across the range used in the assay procedure. A minimum of 5 concentrations is recommended. A regression line, using appropriate statistical methods, should be drawn to calculate correlation coefficient, y-intercept and slope. A plot of the data should be included in the test report. Another important feature, which estimates the degree of linearity, is calculation of the deviation of the actual data points from the regression line. In some cases, mathematical transformation of data, prior to the regression analysis, may be required.

14.3.3.3 Range

The range is the interval between the lower and upper analyte concentrations for which it has been demonstrated that the analytical pro-

cedure has a suitable level of accuracy, precision and linearity. It is normally derived from linearity studies.

14.3.3.4 Accuracy

The accuracy of an analytical procedure is the closeness of agreement between the conventional true value (or an accepted reference value) and the value calculated. It tells how closely the analyte amount is determined to its true amount present in the test sample. Accuracy should be specified across the range of analytical procedure and inferred from 9 measurements (triplicates of three concentrations in the range).

Accuracy can be demonstrated by application of the proposed procedure to an analyte of known purity or by comparing the results of the proposed analytical procedure with those of a second well-characterized procedure, whose accuracy is already known. Application of the procedure to test samples after spiking at three different levels of 50%, 100% and 150% of expected analyte concentration helps to determine the accuracy of the procedure.

A sample containing 1.0 mg analyte may, in different analyses, be found to contain 1.2, 0.9 and 0.8 mg. The assay procedure determining 0.9 mg is more accurate than the two other procedures.

14.3.3.5 Precision

The precision of an analytical method is closeness of results for a series of measurements of multiple samples from the same homogeneous material. The precision may suffer upon varying the experimental conditions, which are therefore assumed to be kept constant. System precision demonstrates error in recording the response and can be determined by repeatedly analyzing a sample within a short period of time.

It is possible that the results are precise but not accurate or vice versa. Triplicate measurements of 1.0 mg true quantity as 0.6, 1.0 and 1.4 result in an average value of 1.0 mg which is accurate but the three measurements themselves are not precise. Similarly, triplicate measurements of the same quantity as 0.6, 0.7 and 0.6 are precise but not accurate. Triplicate measurements of 1.0, 0.9 and 1.0 are precise as well as accurate.

Precision is expressed at three levels of short, medium and long intervals, which are respectively referred to as repeatability, intermediate precision and reproducibility.

14.3.3.5.1 Repeatability

Repeatability is precision under the same operating conditions over a short interval of time (intra-day precision). It is demonstrated by a minimum of 9 determinations covering the specified range for the procedure (e.g. 3 concentrations/3 replicates each) or a minimum of 6 determinations at 100% of the test concentration.

14.3.3.5.2 Intermediate Precision

Intermediate precision expresses intra-laboratory variations: different days, different analysts, different equipment, etc.

14.3.3.5.3 Reproducibility

Reproducibility is precision at the inter-laboratory level. It is especially important if the analytical procedure is to be used in different laboratories, for instance, a pharmacopoeial procedure.

14.3.3.6 Detection Limit

Detection limit (DL) is the lowest amount of analyte in a sample which can be detected, but not necessarily quantitated as an exact value. It is determined by analyzing samples containing known concentrations of analyte and by establishing the minimum level at which the analyte can be reliably detected. Other methods of determining DL are based on the signal-to-noise ratio and on the standard deviation of the response and the slope. A signal-to-noise ratio of 3:1 or 2:1 is a fair estimate of DL. Using the standard deviation of the response (σ) and slope of the calibration curve (S), $DL=3.3\sigma/S$.

14.3.3.7 Quantitation Limit

Quantitation limit (QL) is the lowest concentration of the analyte that can be determined with acceptable precision and accuracy under the stated experimental conditions. It is generally determined by the analysis of samples with known concentrations of analyte and by establishing the minimum level at which the analyte can be quantified with acceptable accuracy and precision. QL can also be defined from the signal-to-noise ratio (a typical signal-to-noise ratio is 10:1), or from the standard deviation of the response (σ) and the slope (S) of the calibration curve ($QL=10\sigma/S$).

14.3.3.8 Robustness

Robustness of an analytical method ensures that it performs well and has few variables affecting its performance. For example, a robust HPLC analytical procedure does not show variation when columns of differ-

ent lots or from different manufacturers are used, when there is a slight variation in pH or composition of the mobile phase, or when temperature and flow rate vary. Steps of extraction, purification or enrichment of an analyte in herbal material should be simple and time effective. Solutions used in the analysis should be stable over a reasonable period of time. Lastly, the procedure should be feasible in most laboratories.

If measurements are prone to variations with small changes in the test conditions then such conditions should be suitably controlled or a precautionary statement should be included in the procedure.

14.4 Thin Layer Chromatography in Quality Control of Plant Products

Thin layer chromatography (TLC), also called planar chromatography, is a widely accepted and extensively used separation technique that is over 65 years old. The technique is simple, cost effective, versatile, and useable in all laboratories around the globe. It can be easily adapted to any given situation of qualitative, quantitative or preparative separation. Despite the great variety and complete automation of the technique, it still lags behind other chromatographic techniques when it comes to its use as analytical technique. However, there is no substitute for this technique for situations requiring qualitative analyses of plant extracts. TLC has nearly become indispensable for the standardization of plant materials, be it the fingerprint profiling or analysis of a marker. The advantages of the technique over other analytical techniques are many when handling plant materials. The samples can be applied without undertaking tedious, time-consuming processes of sample preparation. The loss in sensitivity is far compensated by the gain on several fronts, including ease of assays, multiple sample analyses and low cost per sample.

The two prominent uses of TLC in the standardization of plant materials include fingerprint profiling for the assessment of chemical constituents of a drug and quantitative analysis of markers in plant drugs. A typical TLC procedure involves sample preparation, selection of the chromatographic layer and the mobile phase, sample application, development and drying of the plate, derivatization (if required) and chromatogram evaluation.

14.4.1 Sample Preparation

Methods of sample preparation for fingerprinting and estimation of marker differ significantly. Whereas in fingerprinting, only proportionate quantities of components must be extracted, in assaying the marker complete and exhaustive extraction has to be ensured. Correspondingly, particle size of the crude drug, the solute-solvent ratio, extraction period and

number of extractions assume greater significance in marker estimations. Normally 1-2 g of moderately fine powder (unless specified) of plant material is extracted with 25-50 ml solvent at room temperature, in a Soxhlet apparatus or under reflux on a water-bath. The extraction is repeated a number of times to ensure complete and exhaustive extraction of the marker from the drug matrix. The extract is filtered and solvent is removed from the combined filtrate. The residue is dissolved in the solvent, filtered again, and the volume is adjusted. The concentration of the marker is determined in the solution.

On the other hand, in comparing fingerprint profiles, the procedure requires a shorter extraction scheme. A powdered specimen of pharmacopoeial quality may be required as the reference material for comparison of the fingerprint profiles. The test and sample solutions are prepared under identical conditions of extraction and concentration. Usually 0.1-1.0 g material is extracted with 1-10 ml solvent for 5-30 min, by shaking at room temperature or heating to boiling. The extract is filtered, concentrated and used. Sometimes the solvent is completely evaporated and the residue is dissolved in a small volume of solvent (typically less than 1 ml) and filtered to separate the insoluble particles. The solution of a marker, of preferably known strength, is required if marker presence is to be ascertained. Using known strength of marker additionally provides semiquantitative information.

In certain cases, the extracts require further purification using extraction of the residue with another solvent at different pH or using distillation, sublimation or other appropriate method.

14.4.2 Selection of Chromatographic Layer

A wide variety of options is available for the adsorbent layer. Laboratory-made plates have given way to precoated plates marketed by several manufacturers. The precoated plates are machine-made of glass, aluminium or plastic base coated with different adsorbents. The different adsorbents include normal phase silica gel (most commonly used), reverse phase silica gel (RP_2, RP_8, RP_{18}, cyano, diol and amino plates), aluminium oxide, cellulose, kieselguhr, hybrid (capable of being used as normal and reverse phase) and derivatized adsorbent layers. They come in different sizes, from small strips to continuous rolls (20 x 20 cm^2 is most common).

The nature of the compounds defines the choice of adsorbent layer; a stronger adsorbent (aluminium oxide) is used for weakly adsorbed compounds and a weak adsorbent (cellulose) is used for strongly adsorbed compounds. Normal phase silica gel is more suited for non-polar components and reverse phase silica gel is more suited for polar constituents, which are eluted first on reverse phase TLC. The silica gel plates containing fluorescent dye (F_{254}) of aluminium base are most widely used; about 80% of the analyses are done using these plates as they are optimally efficient and cost-effective.

14.4.3 TLC versus HPTLC Layers

High performance TLC (HPTLC) plates use thin layers of adsorbent (100 µm instead of 200-250 µm) and smaller particles (5-6 µm versus 10-12 µm) of more homogeneous size (4-8 µm versus 5-20 µm). Moreover, they give better resolution (5- to 10-fold more) over shorter runs (3-6 cm versus 8-15 cm), reduce separation time (3-20 min versus 20-200 min), accommodate more samples per plate (more than double), use smaller sample volumes (0.1-0.5 µl versus 1-5 µl) with improved detection limits (100-500 pg), and significantly improve the precision, accuracy and sensitivity. HPTLC plates are substantially more expensive (4- to 6-times more) than normal plates but are an efficient alternative when high sensitivity, accuracy and precision are required in situations demanding high performance. More improvements in adsorbent layers include use of spherical particles of narrow size distribution (reducing resolution time and size of spots while improving the detection limit) and ultrathin layers (10 µm) that improve the resolution and sensitivity and drastically reduce the development time.

14.4.4 Selection of the Mobile Phase

Infinite combinations and a wide choice of solvents are available for TLC developments. Unlike HPLC, where choice is limited, TLC provides no or few restrictions. A mobile phase with 1-3 components is preferred over a multicomponent mobile phase. The polarity of the compounds of interest is the key to selection of a mobile phase. Personal experience applied to existing knowledge and a trial and error method is used to select the composition of the mobile phase. The mobile phase is freshly prepared for each run and the constituting solvents are mixed outside before transferring to the developing chambers. It is advised to allow the developing chamber to saturate unless otherwise specified. Saturation of the chamber is quickened by lining half or more of the total area of the inside walls with filter paper and pouring the mobile phase over it. Closing the chamber and allowing it to stand at room temperature saturates the chambers. It is possible to use another solvent alongside the mobile phase for chamber saturation in twin troughs, e.g. ammonia placed in one trough and mobile phase in another.

The TLC results are sensitive to temperature and humidity variations. All operations during which the plate is exposed to the air should be carried out at a relative humidity of 50%-60% under controlled temperature of 20°-30° C.

14.4.5 Application of Sample

Three typical options of delivering the sample solution onto the plate are manual, semi-automatic and automatic application. Manual application is done using a capillary, which can have a specific volume of 1, 2 or 5 µl for quantitative purposes. The solution is applied by the technique of touch

and deliver. The precision and accuracy, as known to the author from personal experience, is fairly high after a short experience. The semi-automatic application uses devices such as Linomat 5 from Camag and Applicator AS 30 from Desaga, which use a syringe that has to be manually cleaned and filled. The remaining part of the application is automated through computer commands. The solution is applied as a spot or band of predetermined size at predetermined points by touch and delivery or spray-on technique. The needle touches the surface of the adsorbent layer and delivers, whereas in spray-on technique the predetermined volumes are sprayed onto the plate. In the fully automated application, all steps are controlled through a computer including washing of the delivery line.

The typical concentration of the applied samples ranges from 0.1 to 1 mg/ml for qualitative analysis but is usually much lower for quantitative purposes, which further depends on the molar absorption of the marker. The typical volume for spot application is 1-5 µl, and 10 µl for band application. These volumes are drastically reduced in HPTLC plates or ultrathin TLC plates. Bands are known to give better resolution and results than spots, as a narrow band is better suited to the optics of the TLC scanner.

14.4.6 Developing the Chromatogram

Development of plates is carried out in chambers which are special purpose jars or simple containers good enough to hold the solvent in an air-tight environment. There is no doubt that special purpose chambers produce better chromatograms. Twin-trough chambers allow use of another mobile phase in the chamber for the purpose of saturation, besides consuming smaller quantities of solvent. The cost of the chamber, which seems high in the beginning, is recovered by way of savings on the quantity of expensive solvents. Presaturation of the chambers decreases R_f values and corrects side distortions of the solvent front. The plate is placed as nearly vertical as possible in the chamber, ensuring that the points of application are above the surface of the mobile phase and the sides of the plate do not touch the container walls.

The developing chamber should always be kept out of direct sunlight. It should be protected from light during development, if the components being investigated are suspected to be unstable. If sun rays fall directly on the developing chamber, they may be refracted to different degrees through the glass walls, producing areas of high temperature on the plate and resulting in erratic flow of the mobile phase.

The technique of development has been largely improved in horizontal developing chambers and completely automated in automated development chambers or automated multiple development chambers. However, the cost of this equipment (except for the horizontal development chamber) is excessively high.

14.4.7 Drying the Plate

After development, the plate is dried. This is an automatic procedure in automated development chambers, but it has to be accomplished in air at room temperature, in a vacuum desiccator or by heating or blowing hot-air over the surface of the plate. In all instances, the mobile phase should be as completely removed as possible before proceeding to derivatization or scanning the plate.

14.4.8 Derivatization

Derivatization involves treatment of developed chromatograms with suitable spray reagents for locating the position of the constituents for qualitative evaluation and for quantifying ultraviolet-insensitive markers. Two methods are employed for derivatization of plates: spraying with a fine mist of a reagent (a traditional method) and dip-in technique, which of late has become more popular. The spray method does not allow the uniform wetting of the plate, producing areas of high wetting and deficient spray. This affects the precision and accuracy in case of quantitative determinations. The dip-in technique produces more uniform wetting; special equipment is available for this purpose. In most cases of derivatization, heating is required after spraying the plate. Heating the plate uniformly in the open air produces better results than heating in an oven. The fumes from heating are strongly reactive and damage the inner walls of the oven. The plate is heated at about 110° C for about 10 min or until the spots are best seen. Special purpose heating plates are available from the manufacturers of TLC equipment.

14.4.9 Evaluation of the Chromatograms

The TLC plate is observed in daylight, under short-wave and long-wave ultraviolet light, for comparing the chromatograms of standard and test samples or for observing the presence of a marker or compounds of interest in the test chromatogram. The centre of each spot is marked with a needle. The distance from the centre of each spot to the point of application is measured to record the R_f value (the ratio of the distance travelled by a given compound to that travelled by the solvent front) or the R_r value (the ratio of the distances moved by a compound and a stated reference substance). R_f values may vary depending on the temperature, degree of saturation, the activity of the adsorbent layer and the composition of the mobile phase.

Quantitative evaluation is done by scanning the plate in a TLC densitometer or scanner. The densitometer uses two modes of transmittance and reflectance depending upon the available optics. It uses fluorescence mode, ultraviolet absorption or visible light for quantitation of the marker depending upon the option exercised. Ultraviolet and visible light absorption modes come as a standard option on a scanner and the fluores-

cence mode is optional. Data acquisition and analysis is through standard PC-based software. Multi-wavelength scanning, recording and comparing ultraviolet spectra, and generating and acquiring spectra libraries are among several options available on the software provided with the TLC scanner.

The determination of analyte concentration is through a standard plot or single or double point calibrations.

14.4.10 Improving the Efficiency of TLC

Several precautions can be taken to improve the efficiency of TLC analysis. These include carefully selecting the range of concentrations for analysis; using correct instrument parameters like slit dimensions, wavelength selection, scanning speed, base line correction; using HPTLC plates for high sensitivity and resolution; use of appropriate sorbent from a wide range of sorption properties to optimize selectivity; use of automated sample application, development and detection; use of precise in situ recording and quantitation of chromatograms; and avoiding derivatization in assay procedures and, if necessary, using dip-in technique of derivatization.

The following example, of one of the Ayurvedic drugs, illustrates the use of TLC in quality control of plant material. The drug was analyzed for one of the active compounds and the TLC fingerprint profile was used for the purpose of positively identifying the plant material.

To prepare the fingerprint profile, about 5 g plant material was extracted with 50 ml methanol for 30 min at 50° C in a conical flask. The extract was filtered and the filtrate was concentrated to about 5 ml under vacuum. One of the active constituents isolated from this plant (code name DPH-1) was used as a reference. The solution of the reference substance was prepared by dissolving about 5 mg in 1 ml chloroform. About 10 µl of each test and reference solution was manually applied in band form on aluminium base, silica gel 60 F_{254}, 0.2-mm thick TLC plates (Merck). The plate was developed using mobile phase containing 95 volumes toluene and 5 volumes ethyl acetate. The plate after development was dried and visualized under 254 nm ultraviolet light (Figure 1A). The same plate was sprayed with anisaldehyde-sulphuric acid reagent and heated for about 10 min to visualize the spots (Figure 1B). These profiles can be used to confirm the identity of the plant material and to obtain semiquantitative information on the amount of DPH-1 present in the drug.

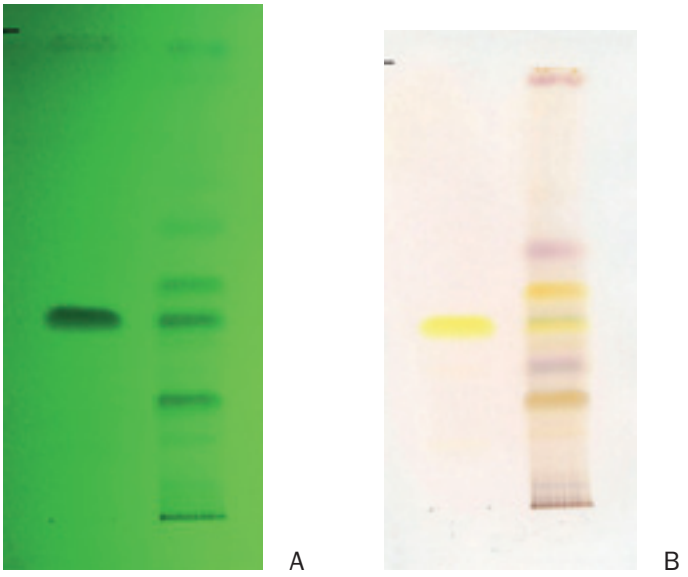

Figure 1: TLC chromatograms as visualized (A) under 254 nm UV and (B) after being sprayed with anisaldehyde-sulphuric acid reagent

Besides developing the fingerprint profile of the drug, the quantity of DPH-1 was also estimated in different samples of the plant material. For the purposes of analysis, 5 g moderately fine powder of drug material was extracted with methanol in a Soxhlet apparatus for 4 h. The extract was filtered and the volume was adjusted with methanol to 50 ml in a volumetric flask. One milliliter of this solution was diluted to 10 ml in a volumetric flask and used for analysis. A standard solution of DPH-1 was prepared by dissolving 4.95 mg DPH-1 in 10 ml methanol and diluting 0.5 ml of this solution to 10 ml in a volumetric flask. Six different concentrations of this solution were applied in triplicate on a precoated TLC plate, which was developed using mobile phase containing 90 volumes toluene and 10 volumes ethyl acetate. The developed and dried plate was scanned at 305 nm in a TLC scanner and the standard plot was constructed (Figure 2). One microliter of test solution was similarly analyzed using the same conditions as used for DPH-1, and the amount of DPH-1 in the test sample was calculated from the response obtained in a TLC scanner. The analyzed drug samples showed large variations in the content of DPH-1, ranging from below 0.3% to over 1.4%. The method was validated according to ICH guidelines.

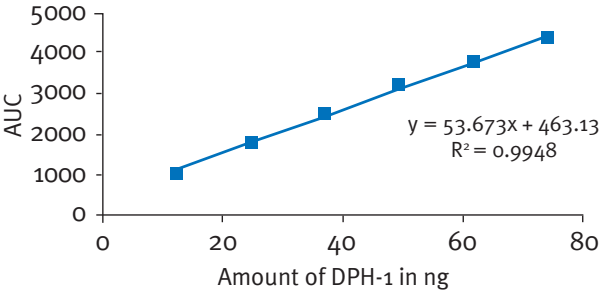

Figure 2: Calibration curve of DPH-1

The TLC-based method of analysis and fingerprint development is quick and reliable, and can be used conveniently in different laboratories. Similarly, it is possible to apply this technique to other plant drugs to develop fingerprint profiles and also to estimate the percentage of marker substances in the crude drugs or in finished products.

14.5 High Performance Liquid Chromatography

In a period of less than 50 years, HPLC has become the most widely used analytical tool in most laboratories of the world. The technique has received great attention for innovations leading to its overall development, regarding both consumables and equipment. HPLC separations are achieved using any of the five basic chromatographic modes: liquid-solid (adsorption), liquid-liquid (partition), bonded-phase (partition), ion exchange, and size exclusion chromatography. The selected mode depends on the nature and properties of the analyte. Bonded-phase chromatography, in which a stationary phase of organosilanes of varying carbon lengths is chemically bonded to silanol groups, is the most commonly used mode of separation. In liquid-liquid chromatography, the solid support (usually silica or kieselguhr) is mechanically coated with a film of high boiling point organic liquid, unlike bonded-phase chromatography where non-polar hydrocarbon chains are chemically bonded to hydroxyls of the silica support. Liquid-liquid chromatography, by virtue of its mechanism, is more susceptible to changes by interaction with mobile phase than bonded-phase chromatography.

A typical HPLC operation includes pumping of mobile phase at moderately high pressure through a narrow-bore column containing adsorbent. The separation of the mixture takes place in the column and separated components are detected by employing a suitable detector. As the mobile phase is being pumped at high pressures, a system is required to inject the mixture into the system without dropping the pressure and disturbing the flow characteristics, i.e. rate and pressure. To accomplish these requirements, an HPLC system requires a pump to push the mobile phase against high pressure, an injector to insert a solution of standard substance or test

mixture, a column to effect separation, a detector to reveal the presence of analyte in the eluate, and a suitable data processing unit.

14.5.1 Pumps

The pump is considered a heart of the HPLC system, as all depends on the composition of the mobile phase and its flow rate accuracy. The pump gives a pulse-free flow of mobile phase; the expected variations in flow rate are less than 1.0%. Online mixing of solvents is preferred to manual mixing. However, compositions containing less than 10% of a particular solvent are better prepared by manual mixing. The composition of the mobile phase is either constant during the analysis (isocratic mode) or it is changed (gradient mode). The type and design of modern pumps allow low pressure mixing of up to four solvents; else, different pumps, one for each solvent, are required for gradient operation and the solvents are mixed at high pressure. A typical analytical procedure uses a flow rate of about 1 ml/min and operating pressure between 1000 and 2000 psi. Higher flow rates generating higher pressure should always be used with justification, as they decrease column life besides requiring frequent servicing of the pump.

The flow accuracy of the pumps is critical for analysis. The constancy of retention time of the last eluted peak is a measure of long-term flow accuracy of the pumps, whereas short-term flow accuracy is checked from the average peak areas of each component and their standard deviations. The mobile phase must be free of dissolved gases to ensure an accurate flow and to minimize noise due to bubbles. Vacuum filtration, sonication and helium gas purging are methods for degassing.

14.5.2 Injector

The injector allows a predetermined volume of test solution to be introduced into the flow channel of the system, without disturbing the flow kinetics. Typically, fixed volume injections are preferred over variable volume injections. When using fixed volume loops, it is advisable to flush higher volumes of the sample through the loop to ensure complete filling of the loop with the sample solution. The mobile phase close to the inner walls of the loop can only be assured to have pushed out after injecting volumes larger than the loop volume, e.g. injecting 20 µl test solution into a 20-µl loop cannot assure accurate injection; if the quantity of the test material is not a problem, flush the loop with over 100 µl test solution. Only the appropriate needle (compatible with the injection port) should be employed for the purpose of making an injection. It is important to select a syringe of appropriate size when giving variable injections; a thumb rule for any analytical technique is not to use the volumetric apparatus if less than 20% of its total volume is being used. Thus, a syringe of 25 µl should not be used to measure or inject volumes less than 5 µl.

14.5.3 Columns

Columns come in varied sizes, structural architecture and chemistry. The chromatographic material is usually packed in stainless steel casing. The material is composed of porous particles which vary in nature (inorganic ceramic, organic polymer, or hybrid), shape (irregular or spherical), size (ranging from 2 to 20 μm; normally around 5 μm) and surface modifications (silanes of different carbon lengths, aminopropyl, diol, cyano, sulphonic acid and ammonium ions). The choice of column is based on the type of analysis. Comprehensive information is available on the websites of the leading manufacturers of HPLC columns, which serves as good guides in choosing columns for analysis. Most analyses are reported on reverse phase columns, usually C_{18}, with increasing emphasis on reducing the column length, diameter and analysis time. Most HPLC separations are successful on columns maintained at ambient temperature, but thermostatted column maintained to ±0.2° C is necessary for reproducible results. This is because all mechanisms of separation are temperature-dependent and any shift in temperature has remarkable bearings on the result.

14.5.4 Detectors

A wide variety of detectors is available to cater to diverse needs of the analysts. Ultraviolet detectors of fixed wavelength, dual wavelength or variable wavelength (photodiode array detector) are most frequently used. Other options are refractive index detector, fluorescent detector, electrochemical detector, evaporative light scattering detector and chemiluminescence detector.

14.5.5 Data Processing

The electrical response from the detector is digitalized and fed to a data processing module, which in present days is invariably a computer, and computations are made using special software. Several software programs are available for data processing, from both manufacturers and third parties. Besides computing the data, they also control the entire operation of the machine.

14.5.6 Factors Affecting HPLC Analysis

Numerous variables affect an HPLC analysis. This topic is beyond the scope of this paper, but some critical variables are discussed briefly. Increased emphasis is now paid to control the temperature of the column within a narrow range to ensure precision of the result. This is desirable, as factors such as solubility, solute diffusion, viscosity of the mobile phase, and column plate number all are affected by temperature. Mobile phase composition is another vital parameter that affects the resolution, retention time and peak area. Pumps contribute the most towards variations of results, as

precise composition of mobile phase and flow rate can only be assured by accurate pumps. Gases dissolved in the mobile phase are a source of flow-rate inaccuracies and errors in detector response.

Retention time variations are often discussed to know the tolerable limits. The retention time is affected by flow rate, column temperature, mobile phase composition and integration. An error in flow rate leads to changes in the retention time to the same extent. Small variations in column temperature have more significant effects on retention time. Ideally, a column is thermostatted to ±0.2° C. However, a high precision of 0.1% in retention time requires the column to be thermostatted to ±0.04° C. Changes in mobile phase composition leave a stronger impact on the retention time. It is estimated that in a typical isocratic elution, a variation of ±1% in mobile phase composition occurs, which introduces an error of 0.4%-0.7% in retention times. The observed variations in composition of the mobile phase are more in the gradient elution. Recording devices also introduce variations in the retention time through faulty recording, but the effect is much smaller (in the range of 0.1% to 0.04%).

Peak area is affected by all the factors that affect retention time. Additionally, the recorder response in marking the start and end of the peak is crucial; this has been seen to be the main source of error in recording peak areas.

Several more factors, like injection volume, connecting tubing, end fittings and detector volume, also have bearings on the final results. Large injection volume and quantity of analyte result in broadening of the peak. Preparing the sample in the mobile phase produces the best result and should be taken as the first choice.

14.5.7 HPLC in Quality Control of Plant Products

HPLC is the most popular technique among all the analytical techniques used today. It is therefore understandable that most happenings are taking place in the modernization of this technique. As discussed in the section on TLC, HPLC can be used for similar purposes. There are two applications of HPLC: one to generate the profile, for which TLC is preferred, and one to estimate the quantity of markers, where HPLC is preferred. The initial steps of sample preparation are similar to those for TLC with the exception that the samples for HPLC are filtered through a filter of 0.45 µm or less. Furthermore, it is assured that the test sample does not contain substances which are permanently retained on the HPLC column, which means in most cases purification procedures are applied to extracts before injecting them onto the column.

After the sample has been prepared, it is injected onto the column and the response is recorded preferably using a variable wavelength ultraviolet detector. As the nature of all the compounds of the extract cannot

be known beforehand, the photodiode array detector is useful, especially when constructing profiles of plant extracts. The fingerprint profile of plant extracts can be used for identification purposes and also for obtaining semi-quantitative information if the sample preparation was not done for quantitative analysis. Similarly, the profile can be generated for the finished product and used to record batch to batch variations. The fingerprint profile can be used to study changes in the composition of the finished product or, in other terms, to indicate the stability of the product.

The most important use of HPLC is in estimation of markers in plant drugs. The steps in HPLC analysis are fundamentally the same as used for any other analytical technique. The response of the test sample is compared to that of a known quantity of the marker to quantify the marker in the test substance. The HPLC method is developed from knowledge of the technique and chemistry of the marker. In chemical analysis, HPLC has no parallel and can be customized to produce the most precise and accurate results. The HPLC analysis is vital in the analysis of a finished product and the expected results are superior to those from TLC, as the separations in HPLC are better. However, run time of HPLC analyses usually varies from 15 to 30 min, which restricts its use if large numbers of samples are to be analyzed.

14.6 TLC versus HPLC

TLC has emerged as a major tool in standardization of plant materials. The advancement and automation of the technique has made it a first choice for plant drugs. Its use has become more popular in developing countries where advancements of HPLC are not cost efficient. TLC offers several advantages over HPLC. Sample and mobile phase preparation do not require elaborated steps of purification, degassing, and filtration, which are essential to protect expensive columns from deterioration. Several samples (up to 18) can be accommodated on a single 20 x 20 cm^2 plate. The test samples and standards are analyzed simultaneously under the same conditions. Several analysts can work simultaneously as each step in analysis is carried out independently using separate equipment. The choice of solvent systems is unlimited, unlike for HPLC where column chemistry disallows the use of extremes of pH in mobile phase. The technique allows enormous flexibility of derivatization with chromogenic spray reagents, making possible the detection of an analyte that is transparent to ultraviolet light. It also allows multiple evaluations of the developed chromatogram, which is not possible in HPLC. There is no leftover from the previous analysis to interfere in the next, as each time a fresh plate is employed. Lastly, it saves tremendously on the time and cost of the analysis.

TLC offers many advantages but also has some disadvantages. It fails to match the sensitivity of HPLC and has not kept with the pace of developments and advancements happening in the area of HPLC. TLC is

an open system and hastens the degradation of compounds sensitive to light and air, which in the case of HPLC pass through an enclosed environment. Detection of the analyte in HPLC occurs in solution, permitting high sensitivity, whereas in TLC the solid phase interaction makes detection less sensitive. Finally, recent advances and efficient flow kinetics of HPLC allow more complex separations than TLC.

14.7 Conclusions

Both TLC and HPLC are vital in the analysis and quality control of plant material and the extracted products. Each of these techniques has its own limitations and advantages. TLC is fast, adaptable and economical, whereas HPLC is more precise and accurate. Based on the preferences and demand of the situation, one can opt to use one or the other for quality assurance of plant products.

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