HPLC

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HPLC

A Practical User's Guide

SECOND EDITION

Marvin C. McMaster

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PREFACE

High-pressure liquid-solid chromatography (HPLC) is rapidly becoming the method of choice for separations and analysis in many fields. Almost anything that can be dissolved can be separated on some type of HPLC column. However, with this versatility comes the necessity to think about the separation desired and the best way to achieve it. HPLC is not now and probably never will be a turn-key, push-button type of operation. Many dedicated system-in-a-box packages are sold for specific separations, but all of these still offer wide possibilities for separation. Changing the column and the flow rate lets you change the separation and the amount of sample you can inject. This is not the worst thing in the world, for it does create great opportunity for the chromatographer and a great deal of job security for the instrument operator.

Fortunately, controlling separations is not nearly as complicated as much of the literature may make it seem. My aim is to cut through much of the detail and theory to make this a usable technique for you. The separation models I present are those that have proven useful to me in predicting separations. I make no claim for their accuracy, except that they work. There are many excellent texts on the market, in the technical literature, and on the Internet, continuously updated and revised, that present the history and the current theory of chromatography separations.

This book was written to fill a need, hopefully, your need. It was designed to help the beginning as well as the experienced chromatographer in using an HPLC system as a tool. Twenty-five years in HPLC, first as a user, then in field sales and application support for HPLC manufacturers, and finally working as a teacher and consultant has shown me that the average user wants an instrument that will solve problems, not create new ones.

I will be sharing with you my experience gained through using my own instrument, through troubleshooting customer's separations, and from field demos; the tricks of the trade. I hope they will help you do better, more rapid separations and methods development. Many of the suggestions are based on tips and ideas from friends and customers. I apologize for not giving them credit, but the list is long and my memory is short. It has been said that plagiarism is stealing ideas from one person and research is borrowing from many. This book has been heavily researched and I would like to thank the many

who have helped with that research. I hope I have returned more than I borrowed.

I have divided this guide into three parts. The first part should give you enough information to get your system up and running. When you have finished reading it, put the book down and shoot some samples. You know enough now to use the instruments without hurting them or yourself. When you have your feet wet (not literally I hope), come back and we will take another run at the material in the book.

Part II shows you how to make the best use of the common columns and how to keep them up and running. (Chapter 6 on column healing should pay for the book in itself.) It discusses the various pieces of HPLC equipment, how they go together to form systems, and how to systematically troubleshoot system problems. We will take a look at the newest innovations and improvements in column technology and how to put these to work in your research. New detectors are emerging to make possible analysis of compounds and quantities that previously were not detectable.

Finally, in Part III, we will talk about putting the system to work on real-world applications. We will look at systematic methods development, both manual and automated, and the logic behind many of the separations that others have made. We will discuss how to interface the HPLC system to computers and robotic workstations. I will also give you my best guesses as to the direction in which HPLC columns, systems, detectors, and liquid chromatography/mass spectrometer (LC/MS) systems will be going.

It is important to give credit where it is due. Christopher Alan McMaster created many of the illustrations in this text before he died of the ravages of muscular dystrophy six years ago. I supplied hand-drawn sketches of the illustrations I used on boards in my classes. Chris turned them into art on his Macintosh. His collaborative efforts are greatly missed.

A brief note is required about the way I teach. First, I have learned that repetition is a powerful tool, not a sign of incipient senility as many people have hinted. Second, I have found in lecturing that few people can stand more than 45 minutes of technical material at one sitting. However, I have also learned that carefully applied humor can sometimes act as a mental change of pace. Properly applied, it allows us to continue with the work at hand. So, occasionally, I will tiptoe around the lab bench. I do not apologize for it, but I thought you ought to know.

The instrument itself is the most effective teacher. Think logically about the system and the chemistry and physics occurring inside the column. You will be surprised how well you will be able to predict and control your separation.

Remember! HPLC is a versatile, powerful, but basically simple separation tool. It is a time machine that can speed your research and, thereby, allow you to do many things not possible with slower techniques. It is both an analytical and a preparative machine. When I finish, I hope you will have the confidence to run your instrument, make your own mistakes, and be able to find your own solutions.

Your HPLC success depends on three things:

1. The suitability of the equipment you buy,
2. Your ability to keep it up and running (or find someone to service it), and
3. The support you receive, starting out in new directions or in solving problems that come up.

Marvin C. McMaster
Florissant, MO

I

HPLC PRIMER

1

ADVANTAGES AND DISADVANTAGES OF HPLC

The first things we need to understand are how an HPLC system works, its best applications and advantages over other ways of separating compounds, and other techniques that might compliment or even replace it. Is there a faster, easier, cheaper, or more sensitive method of achieving your results? The answer is yes, no, maybe. It really depends on what you are trying to achieve.

HPLC's virtue lies in its versatility! I have used it to separate compounds of molecular weights from 54 to 450,000 Daltons. Amounts of material to be detected can vary from picograms and nanograms (analytical scale) to micrograms and milligrams (semi-preparative scale) to multigrams (preparative scale). There are no requirements for volatile compounds or derivatives. Aqueous samples can be run directly after a simple filtration. Compounds with a wide polarity range can be analyzed in a single run. Thermally labile compounds can be run. I had one customer whose company made explosives for primers. Her first job of the day was to explode samples of the previous day run with a rifle. Her second job was to carry out an HPLC analysis of that day's run.

An HPLC offers a combination of speed, reproducibility, and sensitivity. Typical runs take from 10 to 30 min, but long gradients might consume 1 to 2 hrs. I have seen 15- to 30-sec stat runs on 3-μm columns in hospital laboratories. Retention times on the same column, run to run, should reproduce by 1%. Two columns of the same type from the same manufacturer should give 5% or better retention time reproduction on the same standard set.

While the HPLC can be used in a variety of research and production operations, there are a few places where it really shines. Because it can run

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underivatized mixtures, it is a great tool for separating and analyzing crude mixtures with minimum sample preparation. I began my HPLC career analyzing herbicide production runs as a method of trouble-shooting product yield problems. HPLC was routinely used in the quality control lab to fingerprint batches of final product using a similar analysis. I have helped my customers run tissue extracts, agricultural run-off waters, urine, and blood samples with minimum clean up. These samples obviously are not very good for columns whose performance degrades rapidly under these conditions. Columns can usually be restored with vigorous washing, but an ounce of prevention is generally more effective than a pound of cure and also much more time effective.

Standards purification is another role in which the HPLC excels. It is fairly easy to purify microgram to milligram quantities of standard compounds using the typical laboratory system.

Finally, used correctly, HPLC is a great tool for rapid reaction monitoring either in glassware or in large production kettles. I started my analytical career with a HPLC system cast-off by the Analytical Department and a 15-min training course by another plant monitoring chemist. He gave me an existing HPLC procedure for my compound and turned me loose. The next day I was getting research information. I could see starting material disappear, intermediates form, and both final product and by-products appear. It was like having a window on my reaction flask through which I could observe the chemistry of the ongoing synthesis. Later, I used the same technique to monitor a production run in a 6000-gallon reactor. The sampling technique was different, but the HPLC analysis was identical.

Versatility, however, brings with it challenge. An HPLC is easily assembled and easily run, but to achieve optimum separation, the operator needs to understand the system, its columns, and the chemistry of the compounds being separated. This will require a little work and a little thought, but the skills required do offer a certain job security.

I don't want to leave you with the impression that I feel that HPLC is the perfect analytical system. The basic system is rather expensive compared with some analytical tools; columns are expensive with a relatively short operating life, solvents are expensive and disposal of used solvent is becoming a real headache. Other techniques offer more sensitivity of detection or improved separation for certain types of compounds (i.e., volatiles by GLC, large charged molecules by electrophoresis). Nothing else that I know of, however, offers the laboratory the wide range of separating modes, the combination of qualitative and quantitative separation, and the basic versatility of the HPLC system.

1.1 HOW IT WORKS

The HPLC separation is achieved by injecting the sample dissolved in solvent into a stream of solvent being pumped into a column packed with a solid sep-

arating material. The interaction is a liquid-solid separation. It occurs when a mixture of compounds dissolved in a solvent can either stay in the solvent or adhere to the packing material in the column. The choice is not a simple one since compounds have an affinity for both the solvent and the packing.

On a reverse-phase column, separation occurs because each compound has different partition rates between the solvent and the packing material. Left alone, each compound would reach its own equilibrium concentration in the solvent and on the solid support. However, we upset conditions by pumping fresh solvent down the column. The result is that components with the highest affinity for the column packing stick the longest and wash out last. This differential washout or elution of compounds is the basis for the HPLC separation. The separated, or partially separated, discs of each component dissolved in solvent move down the column, slowly moving farther apart, and elute in turn from the column into the detector flow cell. These separated compounds appear in the detector as peaks that rise and fall when the detector signal is sent to a recorder or computer. This peak data can be used either to quantitate, with standard calibration, the amounts of each material present or to control the collection of purified material in a fraction collector.

1.1.1 A Separation Model of the Column

Since the real work in an HPLC system occurs in the column, it has been called the heart of the system. The typical column is a heavy-walled stainless steel tube (25-cm long with a 3–5 mm i.d.) equipped with large column compression fittings at either end (Fig. 1.1).

Immediately adjacent to the end of the column, held in place by the column fittings, is a porous, stainless steel disc filter called a frit. The frit serves two purposes. It keeps injection sample particulate matter above a certain size from entering the packed column bed. At the outlet end of the column it also serves as a bed support to keep the column material from being pumped into the tubing connecting out to the detector flow cell. Each column end fitting is drilled out to accept a zero dead volume compression fitting, which allows the column to be connected to tubing coming from the injector and going out to the detector.

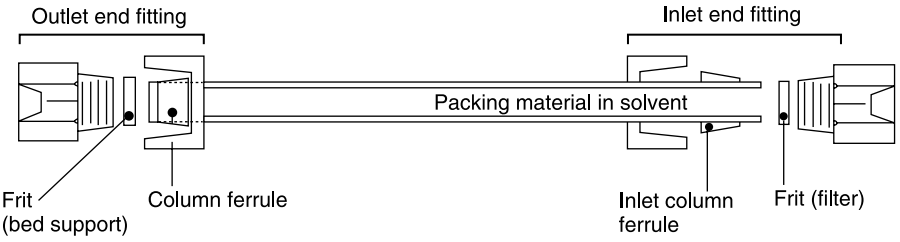

Figure 1.1 HPLC column design.

The most common HPLC separation mode is based on separating by differences in compound polarity. A good model for this partition, familiar to most first-year chemistry students, is the separation that takes place in a separatory funnel using immiscible liquids such as water and hexane. The water (very polar) has an affinity for polar compounds. The lighter hexane (very nonpolar) separates from the water and rises to the top in the separating funnel as a distinct upper layer. If you now add a purple dye made up of two components, a polar red compound and a nonpolar blue compound, and stopper and shake up the contents of the funnel, a separation will be achieved (Fig. 1.2).

The polar solvent attracts the more polar red compound, forming a red lower layer. The blue nonpolar dye is excluded from the polar phase and dissolves in the relatively nonpolar upper hexane layer. To finish the separation, we simply remove the stopper, open the separatory funnel's stopcock, and draw off the aqueous layer containing the red dye, and evaporate the solvent. The blue dye can be recovered in turn by drawing off the hexane layer.

The problem with working with separatory funnels is that the separation is generally not complete. Each component has an equilibration concentration in each layer. If we were to draw off the bottom layer and dry it to recover the red dye, we would find it still contaminated with the other component, the blue dye. Repeated washings with fresh lower layer would eventually leave only insignificant amounts of contaminating red dye in the top layer, but would also remove part of the desired blue compound. Obviously, we need a better technique to achieve a complete separation.

The HPLC column operates in a similar fashion. The principle of "like attracting like" still holds. In this case, our nonpolar layer happens to be a moist, very fine, bonded-phase solid packing material tightly packed in the column. Polar solvent pumped through the column, our "mobile phase," serves as the second immiscible phase. If we dissolve our purple dye in the mobile phase, then inject the solution into the flow from the pump to the column, our two compounds will again partition between the two phases. The more non-

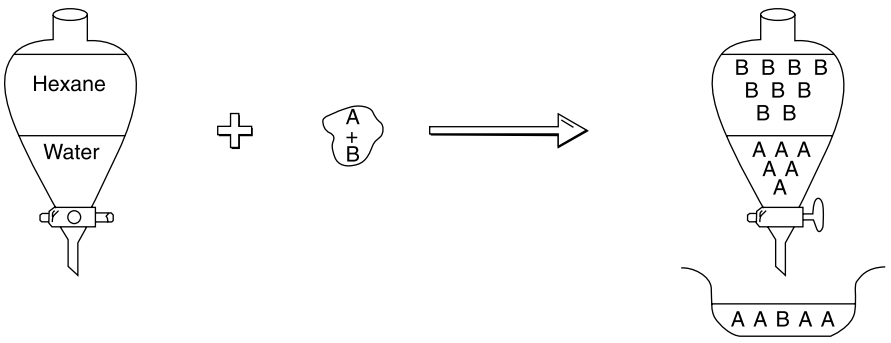

Figure 1.2 Separation model 1 (separatory funnel).

polar blue dye will have a stronger partition affinity for the stationary phase. The more polar red dye favors the mobile phase, moves more rapidly down the column than the blue dye, and emerges first from the column into the detector. If we could see into the column we would see a purple disc move down the column, gradually separating into a fast moving red disc followed by a slower moving blue disc (Fig. 1.3).

1.1.2 Basic Hardware: A Quick, First Look

The simplest HPLC system is made up of a high-pressure solvent pump, an injector, a column, a detector, and a data recorder (Fig. 1.4).

Note: The high pressures referred to are of the order of 2000–6000 psi. Since we are working with liquids instead of gases, high pressures do not pose an explosion hazard. Leaks occur on overpressurizing; the worse problems to be expected are drips, streams, and puddles.

Solvent (mobile phase) from a solvent reservoir is pulled up the solvent inlet line into the pump head through a one-way check valve. Pressurized in

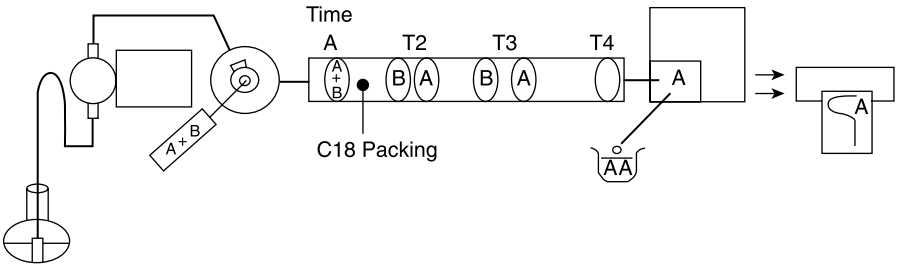

Figure 1.3 Separation model 2 (HPLC column).

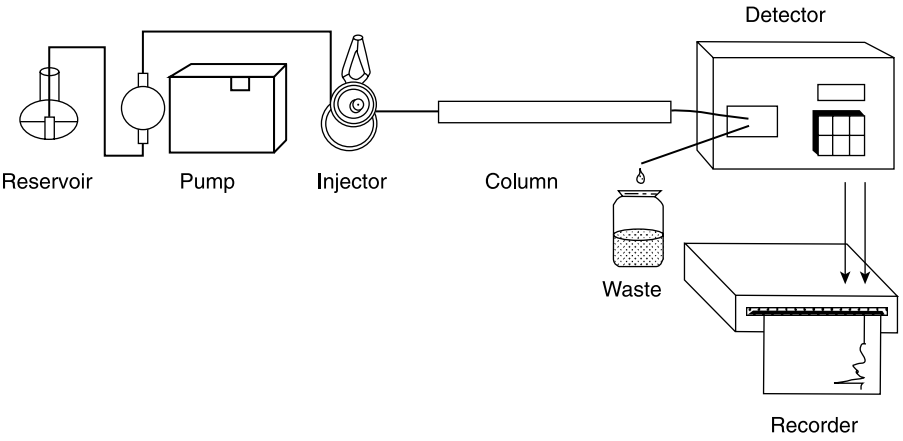

Figure 1.4 An isocratic HPLC system.

the pump head, the mobile phase is driven by the pump against the column back-pressure through a second check valve into the line leading to the sample injector. The pressurized mobile phase passes through the injector and into the column, where it equilibrates with the stationary phase and then exits to the detector flow cell and out to the waste collector.

The sample, dissolved in mobile phase or a similar solvent, is first loaded into the sample loop and then injected by turning a handle swinging the sample loop into the pressurized mobile phase stream. Fresh solvent pumped through the injector sample loop washes the sample onto the column head and down the column.

The separated bands in the effluent from the column pass through the column exit line into the detector flow cell. The detector reads concentration changes as changes in signal voltage. This change in voltage with time passed out to the recorder or computer over the signal cable and is traced on paper as a chromatogram, allowing fractions to be detected as rising and falling peaks.

There are always two outputs from a detector, one electrical and one liquid. The electrical signal is sent to the recorder for display and quantitation (analytical mode). The liquid flow from the detector flow cell consists of concentration bands in the mobile phase. The liquid output from nondestructive detectors can be collected and concentrated to recover the separated materials (preparative mode).

It is very important to remember that HPLC is both an analytical and a preparative tool. Often the preparative capabilities of the HPLC are overlooked. While normal analytical injections contain picogram to nanogram quantities, HPLCs have been used to separate as much as 1 lb in a single injection (obviously by a candidate for the *Guinness Book of World Records*). Typical preparative runs inject 1–3 g to purify standard samples.

To be effective, the detector must be capable of responding to concentration changes in all of the compounds of interest, with sensitivity sufficient to measure the component present in the smallest concentration. There are a variety of HPLC detectors. Not all detectors will see every component separated by the column. The most commonly used detector is the variable ultraviolet (UV) absorption detector, which seems to have the best combination of compound detectability and sensitivity. Generally, the more sensitive the detector, the more specific it is and the more compounds it will miss. Detectors can be used in series to gain more information while maintaining sensitivity for detection of minor components.

1.1.3 Use of Solvent Gradients

Solvent gradients are used to modify the separations achieved in the column. We could change the separation by changing the polarity of either the column or the mobile phase. Generally, it is easier, faster, and cheaper to change the character of the solvent.

The key to changing the separation is to change the difference in polarity between the column packing and the mobile phase. Making the solvent polarity more like the column polarity lets compounds elute more rapidly. Increasing the difference in polarities between column and mobile phase makes compounds stick tighter and come off later. The effects are more dramatic with compounds that have polarities similar to the column.

On a nonpolar column running in acetonitrile, we could switch to a more polar mobile phase, such as methanol, to make compounds retain longer and have more time to separate. We can achieve much the same effect by adding a known percentage of water, which is very polar, to our starting acetonitrile mobile phase (step gradient). We could also start with a mobile phase containing a large percentage of water to make nonpolar compounds stick tightly to the top of the column and then gradually increase the amount of acetonitrile to wash them off (solvent gradient). By changing either the initial amount of acetonitrile, the final amount of acetonitrile, or the rate of change of acetonitrile addition, we can modify the separation achieved. Separation of very complex mixtures can be carried out using solvent gradients. There are, however, penalties to be paid in using gradients. More costly equipment is required, solvent changes need to be done slowly enough to be reproducible, and the column must be re-equilibrated before making the next injection. Isocratic separations made with constant solvent compositions can generally be run in 5–15 min. True analytical gradients require run times of around 1 hr with about a 15-min re-equilibration. But some separations can only be made with a gradient. We will discuss gradient development in a later section.

1.1.4 Ranges of Compounds

Almost any compound that can be retained by a column can be separated by a column. HPLC separations have been achieved based on differences in polarity, size, shape, charge, specific affinity for a site, stereo, and optical isomerism. Columns exist to separate mixtures of small organic acid present in the Krebs cycle to mixtures of macromolecules such as antibody proteins and DNA restriction fragments. Fatty acids can be separated based on the number of carbons atoms in the chains or a combination of carbon number and degree of unsaturation. Electrochemical detectors exist that detect separations at the picogram range for rat brain catecholamines. Liquid crystal compounds are routinely purified commercially at 50 g per injection. The typical injection, however, is of 20 μL of solvent containing 10–50 ng of sample. Typical runs are made at 1–2 mL/min and take 5–15 min (isocratic) or 1 hr (gradient).

1.2 OTHER WAYS TO MAKE MY SEPARATION

Obvious there are many other analytical tools in the laboratory that could be used to make a specific separation. Other techniques may offer higher

sensitivity, less expensive equipment, different modes of separation, or faster and dirty tools for cleaning a sample before injection into the HPLC. Often, a difficult separation can only be achieved by combining these tools in a sequential analysis or purification. I'll try to summarize what I know about these tools, their strengths and drawbacks.

1.2.1 FPLC—Fast Protein Liquid Chromatography

FPLC is a close cousin of the HPLC optimized to run biological macromolecules on pressure-fragile agarose or polymeric monobead-based columns. It uses the same basic system components, but with inert fluid surfaces (i.e., Teflon, titanium, and glass), and is designed to operate at no more than 700 psi. Inert surfaces are necessary since many of the resolving buffers contain high concentrations of halide salts that attack and corrode stainless steel surfaces. Glass columns are available packed with a variety of microporous, high-resolution packings: size, partition, ion exchange, and affinity modes. A two-pump solvent gradient controller, injector valve, filter variable detector, and a fraction collector complete the usual system. The primary separation modes are strong anion exchange or size separation rather than reverse-phase partition as in HPLC.

FPLC advantages include excellent performance and lifetimes for the monobead columns, inert construction against the very high salt concentrations often used in protein chromatography, capability to run all columns types traditionally selected by protein chemist, availability of smart automated injection and solvent selection valves, and very simple system programming. Disadvantages include lack of capability to run high-pressure reverse phase columns, lack of a variable detector designed for the system, and lack of a true autosampler. HPLC components have been adapted to solve the first two problems, but have proved to be poor compromises. The automated valves can partially compensate for the lack of an autosampler.

1.2.2 LC—Traditional Liquid Chromatography

LC is the predecessor of HPLC. It uses slurry packed glass column filled with large diameter (35–60 μm) porous solid material. Materials to be separated are dissolved in solvent and applied directly to the column head. The mobile phase is placed in a reservoir above the column and gravity fed to the column to elute the sample bands. Occasionally, a stirred double-chamber reservoir is used to generate linear solvent gradients and a peristaltic pump is used to feed solvent to the column head. Packing materials generally made of silica gel, alumina, and agarose are available to allow separation by partition, adsorption, ion exchange, size, and affinity modes.

A useful LC modification is the quick clean-up column. The simplest of this is a capillary pipette plugged with glass wool and partially filled with packing material. The dry packed column is wetted with solvent, sample is applied, and

the barrel is filled with eluting solvent. Sample fractions are collected by hand in test tubes. A further modification of this is the sample filtration and extraction columns (SFE). These consist of large pore packing (30–40 μm) trapped between filters in a tube or a syringe barrel. They are used with either a syringe to push sample and solvent through the cartridge or a vacuum apparatus to pull solvent and sample through the packed bed into a test tube for collection. Once the sample is on the bed, it can be washed and then eluted in a step-by-step manner with increasingly stronger solvent. These are surprising powerful tools for quick evaluation of the effectiveness of a packing material, sample clean-ups, and broad separations of classes of materials. They are available in almost any type of packing available for HPLC separations: partition, ion exchange, adsorption, and size.

The basic advantages of LC technique are low equipment cost and the variety of separation techniques available. Very large and very small columns can be used, they can be run in a cold room, and cartridge columns are reusable with careful handling and periodic washing. Disadvantages included relatively low resolving power, overnight runs, and walking pneumonia from going in and out of cold rooms.

1.2.3 GLC—Gas Liquid Chromatography

GLC uses a column packed with a solid support coated with a viscous liquid. The volatile sample is injected through a septum into an inert gas stream that evaporated the sample and carries it onto the column. Separation is achieved by differential partition of the sample components between the liquid coating and the continuously replaced gas stream. Eventually, each compound flushes off the column and into the detector in reverse order to their affinity for the column. The column is placed in a programmable oven and separation can be modified using temperature gradients.

Advantages of the technique include moderate equipment prices, capillary columns for high-resolution, rapid separations, and high-sensitivity detectors and the possibility of direct injection into a mass spectrometer because of the absence of solvents. Disadvantages include the need for volatile samples or derivatives, limited range of column separating modes and eluting variables, the requirement for pressurized carrier gases of high purity, and the inability to run macromolecules.

1.2.4 SFC—Supercritical Fluid Chromatography

SFC is a relatively new technique using a silica-packed column in which the mobile phase is a gas, typically carbon dioxide, which has been converted to a "supercritical" fluid under controlled pressure and temperature. Sample is injected as in a GLC system, carried by the working fluid onto the packed column where separation occurs by either adsorption or partition. The separated components then wash into a high-pressure UV detector flow cell. At

the outlet of the detector, pressure is released and the fluid returns to the gaseous state leaving purified sample as a solid. Doping of carrier gas with small amounts of volatile polar solvents such as methanol can be used to change the polarity of the supercritical fluid and modify the separation.

Advantages of SFC include many of the characteristics of an HPLC separation: high resolving power and fast run times, but with much easier sample recovery. The technique is primarily used as a very gentle method for purifying fragile or heat-labile substances such as flavors, oils and perfume fragrances. Disadvantages include high equipment cost, the necessity of working with pressurized gases, poor current range of column operating modes and available working fluids, and the difficulty of producing supercritical fluid polarity gradients.

1.2.5 TLC—Thin Layer Chromatography

TLC separations are carried out on glass, plastic, or aluminum plates coated with thin layers of solid adsorbant held to the plate with an inert binder. Plates are coated with a thick slurry of the solid and binder in a volatile solvent, then allowed to dry before using. Multiple samples and standards are each dissolved in volatile solvent and applied as spots across the solid surface and allowed to evaporate. Separation is achieved by standing the plate in a shallow trough of developing solvent and allowing solvent to be pulled up the plate surface by capillary action. Once solvent has risen a specific distance, the plates are dried and individual compounds are detected by UV visualization or by spraying with a variety of reactive chemicals. Identification is made by calculating relative migration distances and/or by specific reaction with visualizing reagents. TLC can be used in a preparative mode by streaking the sample across the plate at the application height, nondestructive visualization, and scraping the target band(s) from the plate and extracting them with solvent. Short (3–4 in) TLC strips are an excellent quick and dirty tool for checking reaction mixtures, chromatography fractions, and surveying LC and HPLC solvent/packing material combinations. Two-dimensional TLC, in which each direction is developed with a different solvent, has proven useful for separating complex mixtures of compounds.

Advantages of TLC include very inexpensive equipment and reagents, fairly rapid separations, a wide variety of separating media and visualizing chemicals, and use of solvents and mobile phase modifiers, such as ammonia, not applicable to column separations. Disadvantages include poor resolving power and difficulty in quantitative recovery of separated compounds from the media and binder.

1.2.6 EP—Electrophoresis

EP takes advantage of the migration of charged molecules in solution toward electrodes of the opposite polarity. Electrophoresis separating gels are cast in

tube or slab form by either polymerizing polyacrylamide support material or casting agarose of controlled pore size in the presence of a buffer to carry an electrical current. Sample is applied to the gel surface, buffer reservoirs and positive and negative electrodes are connected to opposite end of the gel, and electrical current is applied across the gel surface. Because electrical resistance in the media generates heat, the gel surface is usually refrigerated to prevent damage to thermally labile compounds. Compounds migrate within the gel in relation to the relative charge on the molecule and, in size-controlled support matrices, according to their size, charge, and shape. Two-dimensional GEP, in which separation is made in one direction with buffer and in the second direction with denaturing buffers, has proved a powerful tool for protein and polypeptide separations in proteomics laboratories.

Advantages of electrophoresis include relatively low-priced equipment, solvents, and media, and very high resolving power for charged molecules, especially biological macromolecules. Disadvantages of EP include working with high-voltage power supplies and electrodes in recovering separated components from a polymeric matrix contaminated with buffer, relatively long separation times in many cases, and the effect of heat on labile compounds.

1.2.7 CZE—Capillary Zone Electrophoresis

CZE is a relatively new technique involving separations in a coated capillary column filled with buffer under the influence of an electrical field. Samples are drawn into and down the column using electrical charge potential. Migration is controlled by the molecule's charge and interaction with the wall coating. Separated components are detected through a fine, drawn-out, transparent area of the column using a variable UV detector or a fluorometer. Still under development, CZE offers great potential as improvements are made in injection techniques and in column coatings to add modified partition, size, ion exchange, and affinity capability. Mass spectrometer interfaces are used to provide a definitive compound identification.

Advantages of CZE include very high resolving power, fairly short run times, and lack of large quantities of solvent to be disposed. Disadvantages include the fact that this is primarily an analytical tool with little capacity for preparative sample recovery and that, again, there is the necessity of working with relatively high-voltage transformers and electrodes. Resolving variables are limited to column coating, applied voltage, buffer character, strength, and pH.

2

SELECTING AN HPLC SYSTEM

Over the years, I have encountered a common customer problem when it came to buying HPLC systems. My customers wanted to buy exactly what they needed to get the job done at the very best price. They wanted to be prepared for future needs and problems, but they did not want to buy equipment they did not need or that would not work.

Trustworthy advise on buying a system is often difficult to obtain. The commissioned salesman for the HPLC company obviously could not be considered completely objective. The customer referral from the salesman might be a little better, but companies seldom hand out a list of customers who have encountered problems. Your local in-house HPLC guru would certainly be more objective, but his information might not be current and his application might be completely different from what you are trying to do. A consultant would be more expensive, probably suffer from the same problems as the guru, and is hard to find without connections to one company or another.

This section, I hope, is the answer! I currently have no connection to an HPLC company, although I have worked for four of the major players in the past. I have taught HPLC extension courses for 16 years and have consulted on a variety of other manufacturers' systems for at least that long. I will try to give you an objective look at the various types of problems that HPLC can solve and my best recommendation for the equipment you'll need to solve each one and, at least, a ballpark price (2006 vintage) for each system.

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2.1 CHARACTERISTIC SYSTEMS

Like buying computer software, the first step is to decide exactly what you will be using the HPLC for today and possibly in the future. I'm not talking about specific separations at this point; those decisions will be used to control column selection, which we will discuss in a moment. What I'm really looking for is an overall philosophy of use.

2.1.1 Finding a Fit: Detectors and Data Processing

Before we start, let me offer some general comments. In the past, fixed or filter variable wavelength UV detectors have been sold with inexpensive systems. Variable detectors were expensive and replacement lamps were expensive and very short lived. This is no longer true and I would not consider buying an HPLC system without a good single-channel variable UV detector. By the same token, photo diode array UV detectors have been oversold. They may have specific applications in method development laboratories, but in their current form they provide useful array information only in a post-run batch mode, not real time. Real time they are only used as very expensive variable detectors. The computer necessary to extract useful information from the three-dimensional output simply increases their cost. An inexpensive diode array detector that could display a real-time summation chromatogram, similar to a MS total ion chromatogram, with peaks annotated with retention times and maximum absorption wavelength, would probably be worth purchasing, but probably exists only in chemical science fiction.

The other piece of mandatory equipment that has changed recently is the data acquisition computer. Previously, every inexpensive HPLC had to have a strip chart recorder. The price differential between a computer-generated annotated chromatogram and a strip chart has dropped to the point that it doesn't make sense not to have that capability in the lab. You may only integrate 1 run out of 10, but when you need it, the capability will be there. Try and avoid a computer system using a thermal or inkjet printer. The paper does not store well for a permanent record. Often, it will be necessary to photocopy the "keeper" chromatograms for further reference and archival storage.

2.1.2 System Models: Gradient Versus Isocratic

There are four basic system types. *Type I* are basic isocratic systems used for simple, routine analysis in a QA/QC environment; often for fingerprinting mixtures or final product for impurity/yield checking. *Type II* systems are flexible research gradient systems used for methods development, complex gradients, and dial-mix isocratics for routine analysis and standards preparation. They fit the most common need for an HPLC system. *Type III* systems are fully automated, dedicated systems used for cost-per-test, round-the-clock analysis of a variety of gradient and isocratic samples typical of clinical and environmental analysis laboratories. *Type IV* systems are fully automated gra-

dients with state-of-the-art detectors used for methods development and research gradients.

2.1.3 Vendor Selection

If you're looking for the name of "the" company to buy an HPLC from, I'm afraid I'm going to have to disappoint you. First, that answer is a moving target. Today one company might be the right choice; tomorrow they might have manufacturing and design problems. For one type of system, such as a microbore gradient HPLC or an ultra-fast system to interface to a mass spectrometer, one company may be superior to the competition. For one application, such as a biological purification, another company may stand out. Second, HPLC equipment has improved so much that you are fairly safe no matter which hardware you select. Control and data processing software design has become critically important in the last few years in getting the most out of your HPLC. Service and support have always been the differentiating factors that really separate and define the best companies.

2.1.4 Brand Names and Clones

A single supplier working on an O.E.M. basis has always produced single components that are used in different private-label systems sold by a variety of manufacturers. It is still important to buy all of your components from the same supplier to prevent a major outbreak of finger pointing in case of problems. Buying bits and pieces from many companies in a search for the "best price" can produce many headaches when the pieces do not play well together. If everything comes from one company, they are the ones who are responsible to help solve the problem. Just make sure they have a current reputation of being in the business of providing for customer needs. Buying expensive systems does not guarantee good customer support. Buying from the lowest bidder or buying the cheapest system possible almost always ensures that *you* are the customer support system. Low margin companies do not have large budgets to plow into support facilities. By the same token, large companies often have so much overhead that little is left for support.

A company's support reputation may change with time and owners. Support is expensive and only the best companies believe in it over the long haul. Find out what a company's reputation for customer support is today from current users.

Your best support will probably come from your local sales and service representatives. If they are good they can help you interface with the company and make sure problems get solved. Remember that service representatives solve electrical and mechanical, not chemical and column problems. If your only tool is a hammer, every problem looks like a nail.

You must be able to distinguish between these two types of problems. With luck, the sales representative will have the proper background and training to be of some assistance in separating these problems. If that training consists of selling used cars, it may not be of much assistance when your column pressure

reaches 4,000 psi and your peaks have merged into a single mass. Find out how much help your sales representative has been to you colleague in the lab across the hall.

2.1.5 Hardware–Service–Support

With many laboratory instruments, equipment specifications alone control the decision of which instrument you should buy. However, HPLC systems are so flexible, can run so many types of columns, and have enough control variables, that hardware decisions alone are insufficient in helping you decide which system you need to solve your application problems. I finally designed a diagram to aid in explaining how to buy an HPLC system (Fig. 2.1).

If you are buying a water bath for the laboratory, you need only consider the temperature range and whether it is UL rated. All you do is turn it on and set the temperature. Price and hardware considerations are enough to make your decision. If it is critical to your work that the water bath always work, you either buy a backup unit or you buy from a company that will provide excellent and prompt on-site service. At this point, the second leg of the success triangle comes into play. In an HPLC system, hardware, service, and support are all critical to guarantee your HPLC success. If you buy from a company that provides only hardware, you must provide the service and support. If the company has good hardware and a responsive serviceperson, but no support, then you must provide the support. This might mean reading a book and attending courses to become "the HPLC expert." It might mean hiring an HPLC consultant. It might mean getting only a portion of the capability of your system.

The HPLC should be a tool to help you solve your research problems, not a new research problem of its own. Think how much your time is worth. (If you do not know, ask you boss, who knows well!) Selecting a company that can provide excellent hardware, responsive and knowledgeable service, and

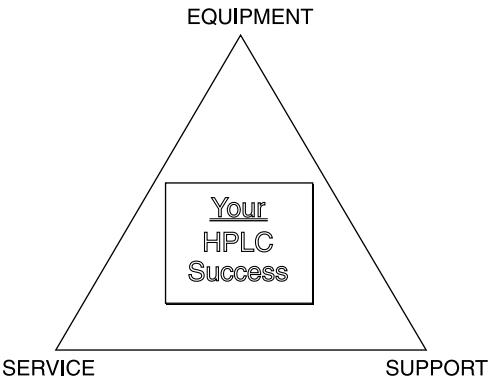

Figure 2.1 Hardware–service–support.

application support after the sale can be one of the most economical decisions you will ever make, no matter what the initial cost of the system turns out to be.

2.2 SYSTEM COST ESTIMATES

HPLC companies tend to sell Type II systems when a Type I will do, a Type III system when a Type II would be sufficient for the job. I've tried to estimate a range of prices for which I last sold these systems (1993). Precise system prices are difficult to obtain from the manufacturers unless you are on GSA pricing or a bid system. Inflation will drive these prices up; the very real competition in this field tends to hold prices down. Let's look at each type of system in turn.

2.2.1 Type I System—QC Isocratic (Cost: $10–15,000)

This system is made up of a reservoir, pump, injector, detector, and an integrator. The Rheodyne manual injector has pretty much become the standard in the industry. It gives good, reproducible injections, but the fittings used on it are specific for this injector and are different from any other fitting in the system and very difficult to connect or disconnect because of tight quarters in the back of the injector. I recommend a variable UV detector as your workhorse monitor, then add other monitors as the need arises (i.e., electrochemical detector for catacholamines, fluorometer for PNAs). The integrator lets you record or integrate. If you dislike working with thermal paper, you can photocopy for long-term storage or look around for a plain-paper integrator. Stay with modular systems. Systems in a box are cheaper because of a common power supply, but not nearly as flexible in case of problems with a single component, with upgrading as required by a new application, or as available equipment changes.

2.2.2 Type II System—Research Gradient (Cost: $20–25,000)

The Type II system comes in two flavors. They vary by the type of gradient pumping system they contain: low-pressure mixing or high-pressure mixing. The rest of the system is the same: injector, variable detector, and computer-based data acquisition and control. Autosamplers would allow 24-hr operation, but most university research laboratories find graduate students to be less expensive.

A few years ago I would have always recommended the high-pressure mixing system, even though it was more expensive; performance merited the difference in price. Today, it depends on the applications you anticipate running. If you plan on running 45-min gradients to separate 23 different components, some of them as minor amounts such as with PTH amino acids, then

I recommend a dynamically stirred, two-pump, high-pressure mixing system. If, on the other hand, you'll mainly be doing scouting gradients, dial-a-mix isocratics, and the occasional uncomplicated gradient, the low-pressure mixing system would be excellent and save you about $4,000. This system has the advantage of giving you three- or four-solvent capability, which would be of advantage in scouting and automated wash-out, but it requires continuous, inert gas solvent degassing. I generally find low-pressure mixing gradient reproducibility performance to be about 95% that of the high-pressure mixing system. Gradients from 0 to 5% and 95 to 100% B may be worse than 95% and should be checked before buying (see Chapter 9).

You can replace an integrator-based data acquisition system with a computer-based system, but let the buyer beware. I am not impressed with most of the control/data acquisition add-on systems I've seen. The system made by Axxion runs on most systems, is competitively priced, and is reasonably friendly. For maximum control and processing benefit, the computer and software have to be carefully matched to the HPLC hardware. If I was going to buy anything, I'd get data acquisition/processing only. My operating rule is to "*try it before you buy it*" and think again. I've been using personal computers for 25 years; I'm a fan, but I'm still not convinced that most people can upgrade to a useful component system. Manufacturers carefully match computer and HPLC hardware with optimized software and, even then, many control/processing systems leave much to be desired. If you do buy a computer to acquire data, keep your integrator or strip chart recorder. You will thank me.

2.2.3 Type III System—Automated Clinical (Cost: $25–35,000)

The most common job for these systems is the fast-running isocratic separation. They could be built up from the QC isocratic, but dial-a-mix isocratic is faster and more convenient since they switch easily from job to job. These systems come in the same two flavors as the research gradient, low- and high-pressure mixing, but replace the manual injector with an autosampler, allowing 24-hr operation. For thermally labile samples that need to be held for a period of time before being injected, there are autosampler chillers available.

The components in these systems tie together, start with a single start command, and may be capable of checking on other components to make sure of their status. The controllers usually will allow different method selection for different injection samples. The more expensive autosamplers allow variable injection volumes and bar code vial identification for each vial. Since these laboratories must retain chromatograms and reports for regulatory compliance and good laboratory practice, they are moving more toward computer control/data acquisition. At the moment, this will add an additional $5,000 to the cost above for software and hardware. This assumes that the computer system replaces the controller and integrator at purchase.

2.2.4 Type IV System—Automated Methods (Cost: $30–50,000)

Another fully automated gradient system, this system is most commonly found in industrial methods development laboratories. They usually have an autosampler, a multi-solvent gradient, at least a dual-channel, variable UV detector and computer-based control, and data processing system for reports. They may add a fraction collector to be used in standards preparation. Some laboratories will replace the variable detector with a diode array detector/computer combination that can run the cost of this system to $60,000. Of course, you could have two Type II systems for the same price. Other detectors, such as a caronal charged aerosol detector or a mass spectrometer and interface module, will dramatically increase the system price. In 2004, I talked to a laboratory director who had just purchased an automated gradient HPLC system with a linear ion trap mass spectrometer that cost $220,000! It depends on what you are trying to achieve and how heavily budgeted your department is at the moment.

2.3 COLUMNS

The decision about which HPLC column to choose is really controlled by the separation you are trying to make and how much material you are trying to separate and/or recover. I did a rather informal survey of the literature and my customers 15 years ago to see which columns they used. I found 80% of all separations were done on some type of reverse-phase column (80% of those were done on C_{18}), 10% were size separation runs (most of these on polymers and proteins), 8% were ion-exchange separations, and 2% were normal-phase separation on silica and other unmodified media, such as zirconium and alumina. The percentage of size- and ion-exchange separations has increased recently because of the importance of protein purification in proteomics laboratories and the growing use in industry of ion exchange on pressure-resistant polymeric and zirconium supports.

2.3.1 Sizes: Analytical and Preparative

Columns vary in physical size depending on the job to be accomplished and the packing material used. There are four basic column sizes: microbore (1–2 mm i.d.), analytical (4–4.5 mm i.d.), semipreparative (10–25 mm i.d.), and preparative (1–5 in i.d.). Column lengths will range from a 3-cm ultrahigh resolution, 1–3-μm packed microbore column to a 160-cm semipreparative column with 5 μm packing. The typical analytical column is a 4.2-mm i.d. $\times$ 25-cm C_{18} column packed with 5 μm media.

Size separation columns need to be long and thin to provide a sufficiently long separating path. Preparative ion exchange and affinity columns should be short and broad to provide a large separating surface.

2.3.2 Separating Modes: Selecting Only What You Need

Column decisions should be made in a specific order based on what you are trying to achieve. First, decide whether you are trying to recover purified material or simply analyzing for compounds and amounts of each present (see Fig. 5.4).

If you are going to make a preparative run, how much material will you inject? Deciding this allows you to decide on an analytical (microgram amounts) column, a semipreparative (milligram) column, or a preparative (grams) column depending on the amounts to be separated (see Table 11.1).

Once the column size is decided, the next column decision is based on the types of differences that will be needed to separate the molecules. The separating factors might be size, the charge on the molecules, their polarities, or a specific affinity for a functional group on the column.

For size differences, select a size-exclusion or gel-permeation column. A further decision needs to be made based on the solubilities of the compounds. Size separation columns are supposed to make a pure mechanical separation dependent only on the diameters of the molecules in the mixture. Compounds come off the column in order of size, large molecules first. Solvent serves only to dissolve the molecules to they can enter the column pores and be separated based on their resident times. Size columns come packed with either silica-based, polymer-based, or gel-based packing in solvents specific for samples dissolved in either aqueous or organic solvents. Do not switch solvents or solvent types on gel-packed columns; differential swelling can change the separating range of the column, cause column voiding, or even crush the packing.

For charge differences, select either an anion-exchange or cation-exchange column, either gel-based or bonded-phase silica or chelated zirconium. Anion-exchange columns retain and separate anions or negatively charged ions. Cation-exchange columns retain and separate positively charged cations. Silica-based ion exchange columns are pressure resistant, but are limited to pH 2.5–7.5 and degrade in the presence of high salt concentrations, which limits cleaning charged contaminants off the column or separation of strongly bound compounds. Zirconium-based ion exchange columns are resistant to pressure, high temperature, and pH from 1-11, but they have Lewis acid functionality that must be blocked to prevent non-ion exchange interacts that will interfere with the separation. Column packing with bonded chelators has been produced to overcome this problem. The functional group on either positively or negatively charged columns can have permanent charges (strong ion exchangers, either quaternary amine or sulfonic acid) or inducible charges (weak ion exchangers, with carboxylic acid or secondary/tertiary amine). The latter types can be cleaned by column charge neutralization through mobile-phase pH modification. Ion exchangers do not retain or separate neutral compounds or molecules with the same charge as the column packing.

For polarity differences, select a partition column. Look at solubilities in aqueous and organic solvents again. Compounds soluble only in organic sol-

vents should be run on normal-phase (polar) columns. Compounds with structural or stereo isomeric differences should be separated on normal-phase columns. Most compounds soluble in aqueous organic solvents should be run on reverse-phase columns. Although C_{18} columns are commonly used, intermediate phase columns, such as the phenyl, C_8, cyano, and diol columns, offer specificity for double bonds and functional groups. Additives to the mobile phase can modify polarity-based separations, such a strong solvent changes, pH modification, and ion pairing agents.

This selection of separating modes is an oversimplification, but it serves as a good first approximation and will be expanded on in later sections of this book. There is rarely such a thing as a pure size column or column packing that separates solely by partition. Many size columns control pore size by adding bonded phases that can exhibit a partition effect. The underlying silica support can have a cation-exchange effect on a partition separation. A bonded phase column's pore size can introduce size exclusion effects. Most separations are a combination of partition, size, and ion-exchange effects, generally with one separating mode dominating and others modifying the interactions. This can be a problem when trying to introduce simple, clean changes in a separation, but it can be used to advantage if you are aware that it might be present.

2.3.3 Tips on Column Use

Here are a few tips on column usage that will make your life easier:

1. Keep the pH of bonded-phase silica column between 2.0 and 8.0 (better is pH 2.5–7.5). Solvents with a pH below 2.0 remove bonded phases; all silica columns dissolve rapidly above pH 8.0 unless protected with a saturation column.
2. Always wash a column with at least six column volumes (approximately 20 mL for a 4 mm × 25 cm analytical column) of a new solvent or a bridging solvent between two immiscible solvents.
3. Do not switch from organic solvents to buffer solution or vice versa. Always do an intermediate wash with water. Buffer precipitation is a major cause of system pressure problems. You may be able to go from less than 25% buffer to organic and get away with it, but you are forming a very bad habit and that will get you into trouble later on. I usually keep a bottle of my mobile phase minus buffer on the shelf for column washout at the end of the day. This also can be used for buffer washout, but a water bridge is still the best.
4. Do not shock the column bed by rapid pressure changes, by changes to immiscible solvents, by column reversing, or by dropping or striking the column or the floor or the desktop.
5. Pressure increases are caused by compound accumulation, by column plugging with insoluble materials, or by solvent viscosity changes. It is

poor practice to run silica-based columns above 4,000 psi (see Chapter 10 on troubleshooting for cleaning). Keep organic polymer columns and large-pore silica size columns below 1,000 psi or lower if indicated in the instructions supplied with the column. Set your pump overpressure setting, if it has one, to protect your column. Solvents mixtures such as water/methanol, water/isopropanol, and DMSO/water undergo large viscosity changes during gradient runs and washouts. Adjust your flow rates and overpressure setting to accommodate these increases so the systems does not shut down or overpressure columns.

6. Use deoxygenated solvents for running or storing amine or weak anion-exchange columns (see "Packing Degradation," in Chapter 6, for a deoxygenating apparatus).
7. Wash out buffer, ion pairing reagents, and any mixture that forms solids on evaporation before shutting down or storing columns. Store capped columns in at least 25% organic solvent (preferably 100% MeOH or acetonitrile) to prevent bacterial growth.

3

RUNNING YOUR CHROMATOGRAPH

This chapter is designed to help you get your HPLC up and running. We will walk through making tubing fittings, putting the hardware together, preparing solvents and samples, initialization of the column, making an injection, and getting information from the chromatogram produced. Let us begin with the hardware and work our way toward acquiring information.

3.1 SET-UP AND START-UP

When your chromatograph arrives, someone will have to put it together. If you bought it as a system, a service representative from the company may do this for you. No matter who will put it together, you should immediately unpack it and check for missing components and for shipping damage.

If you only bought components or if you are inheriting a system from someone else, you will have to put it together yourself. More than likely, you will need, at a minimum, the system manual, a 10-foot coil each of 0.010-in (10 thousandths) and 0.020-in (20 thousandths) tubing, compression fittings appropriate to your system, cables to connect detectors to recorder/integrators and pumps to the controller, and tools. Our model will be a simple, isocratic system: a single pump, a flush valve, an injector, a C_{18} analytical column, a fixed-wavelength UV detector, and a recorder (see Fig. 1.4). The first thing we need to do is to get the system plumbed up or connected with small internal diameter tubing. For now, check the columns to make sure they were shipped or were left with the ends capped. We will put them aside until later.

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3.1.1 Hardware Plumbing 101: Tubing and Fittings

We will need 1/8-in stainless steel HPLC tubing with 0.020-in i.d. going from the outlet check valve of the pump to the flush valve and on to the injector inlet. Three types of tubing are used in making HPLC fittings, 0.04-in, 0.02-in, and 0.01-in i.d.; the latter two types are easily confused. If you look at the ends of all three types, 0.04-in looks like a sewer pipe, more hole than tube. Look at the tubing end on; if you can see a very small hole and think that it is 0.01-in it probably is 0.02-in. If you look at the end of the tubing and at first think its a solid rod and then look again and can just barely see the hole, that's 0.01-in. From the injector to the column and from the column on to the detector we will use 4-in pieces of this 0.010-in tubing.

It is critically important to understand this last point. There are two tubing volumes that can dramatically affect the appearance of your separation; the one coming from the injector to the column and from the column to the detector flow cell. It is important to keep this volume as small as possible. The smaller the column diameter and the smaller the packing material diameter, the more effect these tubing volumes will have on the separation's appearance (peak sharpness).

A case in point is a trouble-shooting experience that I had. We were visiting a customer who had just replaced a column in the system. The brand new column was giving short, broad, overlapping peaks. It looked much worse than the discarded column, but retention times looked approximately correct. Since the customer was replacing a competitive column with one that we sold, I was very concerned. I asked her if she had connected it to the old tubing coming from the injector and she replied that the old one did not fit. She had used a piece of tubing out of the drawer that already had a fitting on it that would fit. This is always dangerous since fittings need to be prepared where they will be used or they may not fit properly. They can open dead volumes that serve as mixing spaces. I had her remove the column and looked at the tubing. Not only was tubing protruding from the fitting very short, the tubing was 0.04-in i.d. This is like trying to do separations in a sewer pipe. We replaced it with 0.01-in tubing, made new fittings, and reconnected the column. The next run gave needle-sharp, baseline-resolved peaks!

To make fittings, you need to be able to cleanly cut stainless steel tubing. Do not cut tubing with wire cutters; that is an act of vandalism. Tubing is cut like glass. It is scored around its circumference with a file or a micro-tubing cutter. The best apparatus for this is called a Terry Tool and is available from many chromatography suppliers. If adjusted for the internal diameter of the tubing, it almost always gives cuts without burrs. If you do not have such a tool, score around the diameter with a file. Grasp the tube on both sides of the score with blunt-nosed pliers and gently flexed the piece to be discarded until the tubing separates. Scoring usually causes the tubing to flare at the cut. A flat file is used to smooth around the circumference. Then, the face of the cut is filed at alternating 90° angles until the hole appears as a dot directly in the

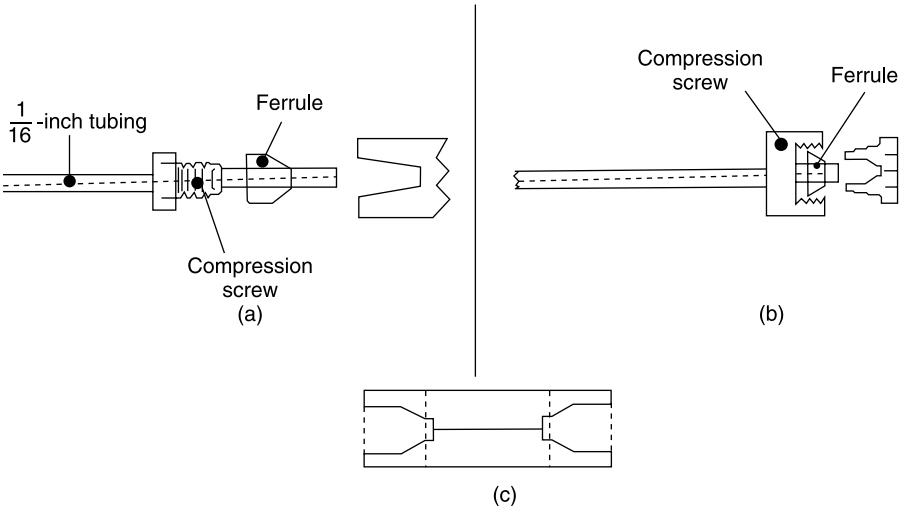

Figure 3.1 Compression fittings. (a) Male fitting; (b) female fitting; (c) zero dead volume union.

center of a perfect circle. The ferrule should then slide easily onto the tubing. Make sure not to leave filings in the hole. Connect the other end to the pumping system and use solvent pressure from the pump to wash them out.

The tubing is connected to the pump's outlet check valve by a compression fitting. The fitting is made up of two parts: a screw with a hex head and a conical shaped ferrule (Fig. 3.1a). The top of the outlet valve housing has been drilled and threaded to accept the fitting.

First, the compression screw then the ferrule are pushed on to the tubing; the narrow end of the ferrule and the threads of the screw point toward the tubing's end. The end of the tubing is pushed snugly into the threaded hole on the check valve. The ferrule is slid down the tube into the hole, followed by the compression screw. Using your fingers, tighten the screw as snug as possible; then use a wrench to tighten it another quarter turn. As the screw goes forward, it forces the ferrule against the sides of the hole and squeezes it down onto the tubing, forming a permanent male compression fitting. The fitting can be removed from the hole, but the ferrule will stay on the tubing. The tubing must be cut to remove the ferrule.

It's important not to overtighten the fitting. It should be just tight enough to prevent leakage under pressure. Try it out. If it leaks, tighten it enough to stop the leak. By leaving compliance in the fitting, you will considerably increase its working lifetime. Many people overtighten fittings. If you work at it, it is even possible to shear the head off the fitting. But please, do not.

There is a second basic type of compression fitting (Fig. 3.1b), the female fitting, which you will see on occasion. Some column ends have a protruding, threaded connector and will require this type of fitting. This fitting is made

from a threaded cap with a hole in the center. It slides over the tubing with its threads pointed toward the tubing end. A ferrule is added exactly as above and the tubing and the ferrule are inserted into the end of a protruding tube with external threads. Tightening the compression cap again squeezes the ferrule into the tapered end of the tube and down onto the tubing forming a permanent fitting. The third type of device for use with compression fittings is the zero dead volume union (Fig. 3.1c). A union allows you to connect two male connection fittings. If these fittings are made in the union, it allows tubing to be connected with negligible loss of sample volume.

You will find that stainless steel fittings will cause you a number of headaches over your working career. An easier solution in many cases is the polymeric "finger-tight" fittings sold by many supplier such as Upchurch and SSI. These fittings slide over the tubing and are tightened like stainless steel fittings, but are not permanently "swagged" onto the tubing and can be reused. They are designed to give a better zero-dead-volume fitting, but they have pressure and solvent limits. They are also more expensive, but only in the short run.

3.1.2 Connecting Components

New pumps are generally shipped with isopropanol or a similar solvent in the pump head, and this will need to be washed out. Always try and determine the history of a pump before starting it up. Systems that have not been run for a while may have dried out. If buffer was left in the pump, it may have dried and crystallized. In any event, running a dry pump can damage seals, plungers, and check-valves.

First we will need to hook up the pump inlet line. This usually consists of a length of large-diameter Teflon tubing with a combination sinker/filter pushed into one end and a compression fitting that will screw into the inlet fitting at the bottom of the pump head on the other end. Drop the sinker into the solvent reservoir and screw the other end into the inlet check valve housing.

The next step is to use compression fittings to hook the pump outlet to the flush valve with a length of 0.02-in i.d. tubing. The flush valve is a small needle valve used to prime the pump that allows us to divert solvent away from the column when rapidly flushing the pump to atmospheric pressure. Open the valve and the line is vented to the atmosphere. This removes the back-pressure from the column, a major obstacle when trying to push solvent into a plumbed system.

From the flush valve we can connect with fittings and 0.02-in tubing onto the injector inlet port. The back of the injector usually has ports for an inlet, an outlet, two ports for the injection loop, and a couple of wash ports. If a sample loop is not in place, connect it, then make a short piece of 0.01-in i.d. tubing with fittings to be used in connecting the column. Use the column end to prepare the compression fitting that will fit into it (Fig. 3.2). At the outlet end of the column, hook up with compression fittings a piece of 0.01-in tubing

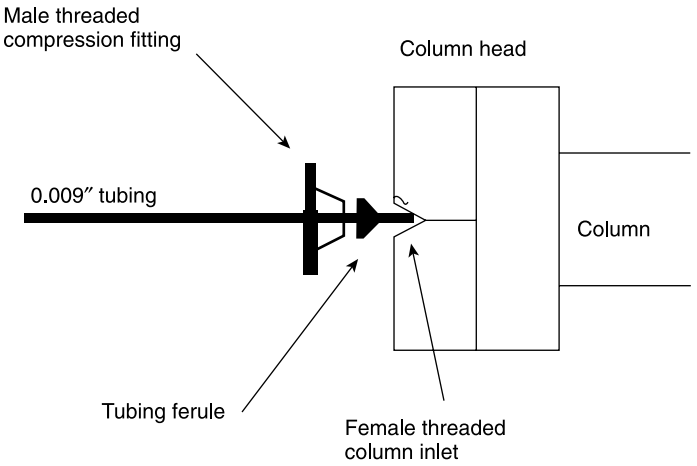

Figure 3.2 Column inlet compression fitting.

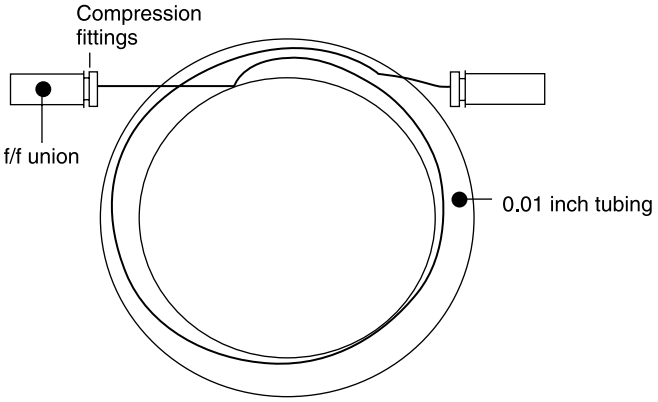

Figure 3.3 Column blank.

that connects to the detector flow cell inlet line. When this is done, remove and recap the column and set it aside.

Next, we are going to create a very useful tool for working with the HPLC system. I call it a "column blank" or column bridge (Fig. 3.3). It bridges over the place in the system where we would normally connect the column. It is very valuable for running, problem diagnosis, and for cleaning a "column less system." It is made up of a 5-ft piece of 0.01-in tubing with a male compression fitting on each end screwed into a zero-dead-volume union (female/female). Our column blank now has two ends simulating the end fittings on the column.

Connect one end of your column blank to the tubing from the injector outlet; the other end is connected to the line leading to the detector flow cell. We have one more fluid line to connect to complete our fluidics. A piece of 0.02-in tubing can be fitted to the detector flow cell outlet port to carry waste to a container. In some systems, this line will be replaced with small-diameter Teflon tubing.

In either case, the line should end in a back-pressure regulator, an adjustable flow resistance device designed to keep about 40–70 psi back-pressure on the flow cell to prevent bubble formation that will interfere with the detector signal. Air present in the solvent is forced into solution during the pressurization in the pump. The column acts as a depressurizer. By the time our flow stream reaches the detector cell, the only pressure in the system is provided by the outlet line. If this is too low, bubbles can form in the flow cell and break loose, resulting in sharp spikes in the baseline. The back-pressure regulator prevents this from happening.

The final connections are electrical. A power cable needs to be connected to each pump. Check the manuals to see whether fuses need to be installed and do so if required. Finally, connect the 0–10 mV analog signal connectors on the back of the detector to the strip chart recorder. Connect red to red, black to black. If a third ground wire is present in the cable, connect it only at one end, either the detector or the recorder end. (*Note*: The ground wire connects to the cable shield, which is wrapped around the other two wires in the cable. If no ground is connected, no shielding of the signal occurs. If both ends of a ground are connected, the shield becomes an antenna; worse than no shield at all.)

Now our system is ready to run. We will need to prepare solvent, flush out each component, then connect, flush out, and equilibrate the column before we are ready to make our first injection of standard.

3.1.3 Solvent Clean-up

Before we tackle the column, let us look at how to prepare solvents for our system. I have found that 90% of all system problems turn out to be column problems. Many of these can be traced to the solvents used, especially water.

Organic solvents for HPLC are generally very good. There are three rules of thumb to remember: always use HPLC grade solvents, buy from a reliable supplier, and filter your solvents and check them periodically with your HPLC. Most manufacturers do both GLC and HPLC quality control on their solvents; some do a better job than others. The best way to find good solvents is to talk to other chromatographers.

Even the best solvents need to be filtered. I have received HPLC-grade acetonitrile, from what I considered to be the best manufacturer of that time, that left black residue on a 0.54-μm filter. There is a second reason to filter solvents. Vacuum filtration through a 0.54-μm filter on a sintered glass support is an excellent way to do a rough degassing of your solvents. Because of filter

and check valve arrangements, some pumps cavitate and have problems running solvents containing dissolved gases.

There are numerous filter types available for solvent filtration. The cellulose acetate filters should be used with aqueous samples containing less than 10% organic solvents. With much more organic in the solvent, the filter will begin to dissolve and contaminate your sample. Teflon filters are used for organic solvent with less than 75% water. The two types are easily told apart; the Teflon tends to wrinkle very easily, while the cellulose is more rigid. If you are using the Teflon filter with high percentages of water in the solvent, wet the filter first with the pure organic solvent, then with the aqueous solvent before beginning filtration. If you fail to do this it will take hours to filter a liter of 25% acetonitrile in water. Nylon filters for solvent filtration can be used with either aqueous or organic solvents. They work very well as a universal filter, but use with very acidic or basic solutions should be avoided as they break down the filter.

If you're still having pumping problems after vacuum filtration, try placing the filtrate in an ultrasonication bath for 15 min (organic solvents) or 35 min (aqueous solvents). Ultrasonic baths large enough to accept a 1-L flask are in common use in biochemistry labs and are very suitable for HPLC solvent degassing. Stay away from the insertion probe type of sonicator; they throw solvent and simply make a mess. Ultrasonication is much better than heating for degassing mixed solvents. There is much less chance of fractional distillation with solvent compositional change when placing mixtures in an ultrasonic bath. One manufacturer actually made a system that was designed to remove dissolved gas by heating mobile phase under a partial vacuum. Obviously they never used rotary vacuum flash evaporators in their labs, at least not intentionally!

Other techniques recommended for solvent degassing involve bubbling gases (nitrogen or helium) through the solvent. Helium sparging is partially effective, but expensive when used continuously. It is required in some low-pressure mixing gradient systems, as will be described later. The only other time I use any of these techniques is in deoxygenating solvent for use with amine or anionic exchange columns, which tend to oxidize (see Fig. 6.4).

Water is the major offender for column contamination problems. I have diagnosed many problems, which customers have initially blamed on detector, pumps, and injectors, that turned out to be due to water impurities. Complex gradient separations are especially susceptible to water contamination effects.

In one case, the customer was running PTH amino acid separation, a complex gradient run on a reverse-phase column. He would wash his column with acetonitrile, then water, and run standards. Everything looked fine. Five or six injections later his unknown results began to look weird. He ran his standards again only to find the last two compounds were gone. He blamed the problem on the detector. I said it looked like bad water. He exploded, told me that his water was triple distilled and good enough for enzyme reactions. It was good enough for HPLC, he said. Over the following 6 mo we replaced

every component in that system as each in turn was blamed for the chromatography problem. Eventually, the customer borrowed HPLC-grade water from another institution, washed his column with acetonitrile, then with water. The problem disappeared and never came back—until he went back to his own water. Nonpolar impurities co-distilling with the water were accumulating at the head of the column and retaining the late runners in the column.

While HPLC grade water is commercially available, I have found it to be expensive and to have limited shelf life. The best technique for purifying water seems to be to pass it through a bed of either reverse-phase packing material or of activated charcoal, as in a Milli-Q system. Even triple distillation tends to co-distill volatile impurities unless done using a fractionation apparatus.

I have used an HPLC and an analytical C_{18} column at 1.0 mL/min overnight to purify a liter of solvent for the next day's demonstration run. The next morning, I simply washed the column with acetonitrile, then with water, equilibrated the column with mobile phase, and ran my separation. It might be better to reserve a column strictly for water purification if you are going to use this technique regularly.

An even better solution is to use vacuum filtration through a bed of reverse-phase packing. Numerous small C_{18} SFE cartridges are available that are used for sample clean-up and for trace enrichment. They are a tremendous boon to the chromatographer for sample preparation, but also can be of help in water clean-up. These SFE cartridges are a dry pack of large pore size C_{18} packing and must be wetted before use with organic solvent, then with water or an organic solution. You wash first with 2 mL of methanol or acetonitrile and then with 2 mL of water before applying dissolved sample. If you forget and try to pass water or aqueous solutions through them, you well get high resistance and nonpolars will not stick. SFE cartridges contain from 0.5 to 1.0 g of packing and will hold approximately 25–50 mg of nonpolar impurities. If care is taken not to break their bed, they can be washed with acetonitrile and water for reuse. Eventually, long eluting impurities will build up and the SFE must be discarded. I have used them about six times, cleaning about a liter of single distilled water on each pass. If larger quantities of water are required, commercially available reverse phase, vacuum cartridge systems using large-pore, reverse-phase packing designed to purify gallons of water at a time are available.

The most common choice for large laboratories are mixed bed, activated charcoal, and ion exchange systems that produce water on demand. These systems usually have a couple of ion-exchange cartridges and one activated charcoal filter in series. They work very well, but I prefer to have the charcoal as the last filter in the purification bank. After all, we are trying to remove organics. I find that the ion-exchange resins break down after about 6 mo and begin to appear in the water. The system uses an ion conductivity sensor as an indicator of water purity, but water that passes this test still may be unsuitable for HPLC use.

3.1.4 Water Purity Test

The final step is to check the purity of the solvents. Again, I have found the C_{18} column to be an excellent tool for this purpose. Select either 254 nm or the UV wavelength you will be using for the chromatogram. Wash the column with acetonitrile until a flat UV baseline is established and then pump water though the column at 1.0 mL/min for 30 min. This allows nonpolar impurities to accumulate on the column. The final step is to switch back to acetonitrile. I prefer to do this by running a gradient to 100% acetonitrile over 20 min. If no peaks appear after 5 min at final conditions, the water is good. The chromatogram (Fig. 3.4) gives you an idea of the expected baseline appearance.

Peaks that appear during the first acetonitrile washout are ignored as impurities already on the column. Watch the baseline on switching to water. At 254 nm, the baseline should gradually elevate. If instead it drops, you may have impurities in your acetonitrile. If the baseline makes a very sharp step up before leveling off, you may have a large amount of polar impurities in the water. Polar impurities probably will not bother you on reverse-phase columns but might have some long-term accumulation effects. Peaks appearing during the acetonitrile gradient come from nonpolar impurities in the water that accumulated on the column and are now eluting.

I have done this with water from a Milli-Q system in need of regeneration. Even though their indicator glow light shows no evidence of charged material being released from the ion exchanger, peaks that will effect reverse-phase chromatography show up at around the 70% acetonitrile portion of the gradient run.

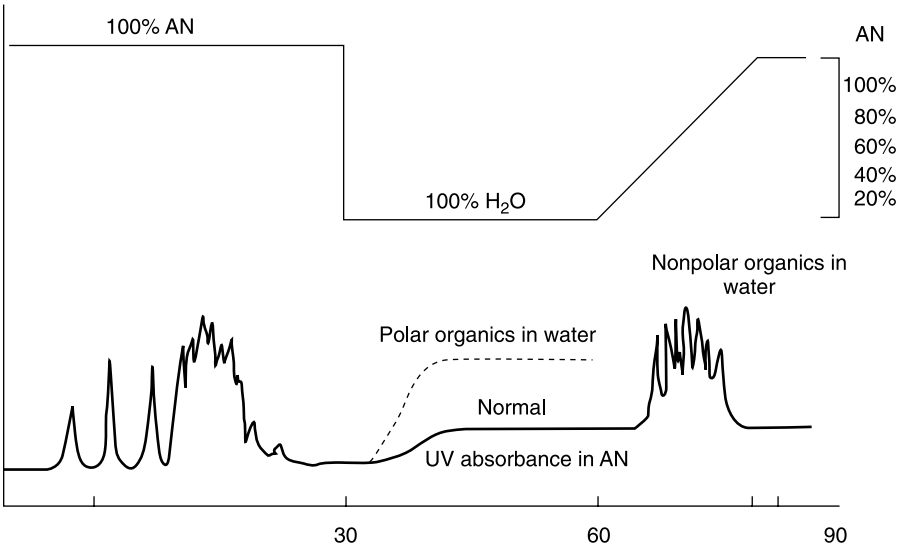

Figure 3.4 Water purity test.

If your water passes this test at the wavelength you will be using for your chromatography, you are ready to use it to equilibrate the column. The next step is to flush out the dry system and prepare to add the column.

3.1.5 Start-up System Flushing

Fill the solvent reservoir with degassed, filtered solvent by pouring it down the wall of the flask to avoid remixing air into it. I usually start pumps up with 40–50% methanol in water. Even if the pump was shut down and allowed to stand in buffer, there is a good chance this will clear it. It is also a good idea to loosen the compression fitting holding the tubing in the outlet check valve at the top of the pump head to relieve any system back-pressure. This is an especially important step to use if the column is still connected. When running with a column blank, as we are, it is less important.

The first step is to insure that the pump is primed. This may mean pushing solvent from an inlet manifold valve through the inlet check valve and into the pumping chamber. A few pumps on the market, like the old Waters M6000, use spring-loaded check valves, so you may have to really work to get solvent into the chamber. With other pumps, you open a flush valve and use a large priming syringe to pull solvent through the pumphead. The next step is either to turn the pump flow to maximum speed or uses the priming function of the pump, which does the same thing.

As soon as the pump begins to pump solvent by itself, tighten down the outlet compression fitting and drop the flow rate to about 1 mL/min. The pump is ready to run and should be allowed to pump into a beaker for a few minutes to wash out any machining oils, if new, or soluble residues or dissolved buffer if old.

Before we move on, let us talk about shutting down a pump. The pump seal around the plunger is lubricated by the contents of the pumping chamber. There is always a microevaporation through this seal/plunger combination, whether the pump is running or not. Buffers and other mobile phases containing dissolved solids should not be left in a pump when it is to be turned off overnight. This evaporation causes crystallization on the sapphire plunger and can result in either breakage or seal damage on starting up the pump. Solvents containing dissolved solids should always be washed out before shut down. I prefer to wash out and leave a pump in 25–50% methanol/water to prevent bacteria growth in the fluidics system.

Occasionally, I have had to leave buffer in a pump overnight. In such a case, I leave the pump running slowly (0.1 mL/min.) and leave enough solvent in the reservoir so that it can run all night. This has the additional value of washing the column overnight. If the column is clean and doesn't require further washing, you can throw the detector outlet into your inlet reservoir and recycle the solvent, ensuring you will not run out.

Now we can move past the flush valve to the next major system component, the injector. Whichever position you find the injector handle in, leave it

there! *Never turn the handle on a dry injector.* The injector seal is hardened Teflon facing against a metal surface and can tear if not lubricated with solvent. Once solvent is flowing through the injector to lubricate the seal, turn the handle to the inject position so that the sample loop is washed. Watch the pressure gauge on the pump; a plugged sample loop will cause the pressure to jump. If this happens, go to the troubleshooting section in Appendix E.

3.1.6 Column Preparation and Equilibration

The next step is to hook up the column. Stop the pump flow. I assume you have a C_{18} column compatible with 40% methanol/water (otherwise, select a solvent appropriate for your column). Disconnect the column bridge, remove the column fittings from the ends of the stored column, and connect the inlet end of the column to the line coming from the injector. The inlet end is almost always marked; check for an arrow or a tag pointing the direction of flow. I have always preferred to hook up a column with some solvent running. Turn the flow rate on the pump down to 0.2 mL/min. Fill the space in the end of the column fitting, then screw in the compression fitting at the end of the injector line. Place a beaker at the outlet end of the column to catch wash out solvent. Wash the column with start-up solvent if it is an old column that might have been stored in buffer. (Storing a column in buffer is a very bad technique, but you never know if you weren't the last person to use the column! It is a good idea to label a column with the last solvent used in the column before you put it away.)

Next, change the solvent in the reservoir to 70% acetonitrile in water, turn the pump on, and flush the column with the new solvent. Turn the flow rate up to 1.0 mL/min while catching the column effluent in a beaker. Check the back-up line for leaks; if you see any, tighten the appropriate fittings until the leaks just stop. You will always have leaks! If you do not, you are probably overtightening your fittings. Leaks are messy, but are probably a sign of successful technique (leaks, not streams).

Check the pump pressure. The pump pressure gauge and the baseline trace are the two major tools for diagnosing system problems. If the column was shipped in isopropanol or methanol it should start high (3,000–4,000 psi) then slowly drop to around 2,000–3,000 psi.

Stop the flow and connect the column outlet with a short piece of 0.10-in tubing to the inlet of the detector flow cell. Resume flow to the column. Turn the detector on and start the recorder chart speed or computer data acquisition at 0.5 cm/min. You should have a flat baseline. If the baseline continues to drift up or down, the column still hasn't finished its wash out and equilibration, or the detector has not fully warmed up.

By the way, I must hasten to add that we really haven't reached a true equilibration at this point. The experts have informed me that it takes about 24 hr to reach a true equilibration on a reverse-phase packing. However, after six column volumes we have reached a reproducible equilibration point good enough for our purposes.

We are now ready to prepare for injecting a sample. Let's turn our flow rate down to 0.1 mL/min and get our sample ready.

3.2 SAMPLE PREPARATION AND COLUMN CALIBRATION

The worst thing a chromatographer can do is to grab a column out of its box, slap it into his HPLC, and shoot a sample. Before we begin, it's important to make sure the sample is clean. We will talk about removing soluble contaminants later. Here we're going to be dealing with suspended solids or particulates. Second, we need to know the initial condition of the column, so that we may return to it when we begin to develop problems. In other words, we need to need to do column quality assurance, or QA.

3.2.1 Sample Clean-up

The generally recommended procedure for cleaning samples is to filter them through a 0.54-μm filter in a Sweeny filter holder or using a disposable plastic filter cartridge. The same types of filter materials are available as those discussed in solvent filtration: Teflon, nylon and cellulose. In-line filters are available that fasten between the syringe barrel and the injection needle. These are useful if you are not sample limited or are doing repeat injections of the same material. I have found that most chromatographers won't bother with the time, cost, and sample loss that this entails, although I am finding an increase in the use of syringe in-line filters.

Sample clarification is, however, important! The column frit pore size is usually 2.0 μm; any larger particulates build up and plug the frit. Being a lazy chromatographer, but not a stupid one, I decided to use a different clarification procedure. I place the sample in a microcentrifuge tube and sediment solids by spinning at maximum speed in a clinical centrifuge ($700 \times g$) for 1–2 min. I pull a sample carefully from the supernatant and shoot that as my sample. It has the advantage of spinning down most of the solids, can be used on a number of samples at the same time, works even with very small samples, and is fast and inexpensive, if you already have the centrifuge. While it may not be as efficient as filtration, most chromatographers are willing to use it on every sample. It greatly extends column life between clean-ups.

A third alternative combines the two techniques. A commercially available filter/reservoir fits in a microcentrifuge tube. Spinning the unit filters the sample in the reservoir. It is more efficient than simple centrifugation, but takes longer to assemble and costs more.

Like the oil filter advertisement says, "you can pay me now, or pay me later." If you don't take time to remove particulates, you will spend much more time and effort cleaning the column. The choice is yours.

3.2.2 Plate Counts

Once the shipping solvent is washed out of the column, it is important to determine whether the column survived shipping and to determine its running conditions. Most good chromatography laboratories have established a quality control test for newly purchased columns. A stable test mixture of known running characteristics has been prepared and stored to test new columns.

One commercially available standard used for testing C_{18} columns is a solution of acetophenone, nitrobenzene, benzene, and toluene in methanol (many chromatographers like to add a basic component, such as aniline, to the test mixture as a check against tailing problems). To adjust for extinction coefficient differences, add 10 μg of each of the first two ingredients and 30 μg of the last two compound in 2 mL of MeOH. Inject 20 μL of the mixture into the column equilibrated with 70% MeOH in water and read at 254 nm on the UV detector. This is a convenient mixture since α's between pairs of peaks double as you go to larger retention volumes. Be sure to keep this mixture tightly stoppered. The last two compounds will evaporate from the mixture on access to air. For use at low wavelengths, dissolve these same four ingredients in acetonitrile and run in 60% acetonitrile in water.

Using this or similar mixtures, inject a sample into an equilibrated column, elute the resolved bands, and record them on the recorder. Calculate plate counts for the first and last peak using the "5 σ" method mentioned in Section 4.1.1. Log these numbers in the form $V_4/V_1 = 1.1/6.5$; $N_4/N_1 = 7{,}500/3{,}600$. When we see changes in a separation we have been running, we can reequilibrate the column in 70% MeOH/water and rerun our standards. Changes in these ratios will be useful in troubleshooting column problems later on.

Obviously, this mixture will not be as useful on other types of columns, although I have used this mixture on C_8 columns. Each column type should have its own known standards mixture. They should be stable against both chemical and bacterial changes. With them, you always have a touchstone to return to in case of problems.

3.3 YOUR FIRST CHROMATOGRAM

Now that we have our system set up and the column equilibrated and standardized, we are ready to carry out an HPLC separation on a real sample. We might add an internal standard (if necessary, to correct for injection variations), dilute our sample to a usable concentration, and prepare it for injection. After injection, we will record the chromatogram making sure that it stays on scale. Then, from the trace we obtain, we will calculate elution volumes either by measuring peak heights or by calculating peak areas by triangulation.

We can compare these values of areas or peak heights with known values for standard compounds. From elution volumes or retention times, we can

begin to identify compounds. Comparing peak areas or heights to those derived from standard concentrations, we can calculate the amounts of material under each peak.

3.3.1 Reproducible Injection Techniques

From the last section, it becomes obvious that we must first make a decision about what we are trying to accomplish. We can do scouting, trying to identify compounds by their retention times. Or, we can try to quantitate peaks by comparison to known amounts of standards.

In scouting, we may be running very expensive sample and have to simply guess at the amount to inject. In this case, I would pull up >10 μL of the supernatant in a 25-μL syringe, turn the syringe point up, and pull the barrel back far enough so I could see the meniscus just below the point where the needle joins the barrel. (Injectors such as the Rheodyne injector use a blunt-tip syringe needle. Sharpened needles cut and ruin the Teflon port liner.) I would check for bubbles at the face end of the barrel, on the inside wall, and at the meniscus. Small bubbles can generally be dislodged by gently snapping the outside wall of the syringe with your finger. Slowly push the barrel forward to the 10-μL mark, then quickly wipe the outside of the needle past the tip with a tissue. Place the syringe needle into the injector syringe port, make sure the injector handle is in the *load* position, and slowly push the sample into the loop to insure that the sample goes in as a plug.

If the syringe is new or dry, you may find a large, tenacious bubble clinging to the barrel face. It can be dislodged by rapidly expelling the sample from the syringe back into the sample tube (try not to remix the pellet into the sample) and then slowly pull up a new sample. Repeat the check for bubbles, expel the excess sample, and wipe before injecting. Don't let the tissue linger at the tip; it can wick up extra sample and give irreproducible sampling.

When working with sample we don't mind wasting, the simplest way to achieve reproducible injections is to overfill the loop. With a 20-μL loop, we need to flush with at least 30 μL of sample to insure complete displacement of mobile phase from the loop.

Quantitative sampling is handled a little differently. We usually know the expected concentration level and retention times. After clarification, we add a known amount of the sample solution and an internal standard to a volumetric flask and dilute. The sample is pulled into the syringe for injection as above.

Internal standards are used for many reasons in chemistry. Here we are using it to correct for differences in sampling volumes. It takes much practice for a person to accurately deliver the same size sample every time. It is nearly impossible for two people to accurately deliver the same sample each time if they are partially injecting a loop. If we add a known amount of internal standard to both our sample and our known standard mixture, we can calculate peak heights or areas relative to that of the internal standard. Variations in the injection size of the sample do not affect these relative areas.

To make the injection, we turn the handle of the injector to the load position (see Fig. 9.9). Push the syringe needle into the needle port and slowly push the barrel forward so the sample goes in as a plug. Leave the needle in the injector port to prevent siphoning of the sample out the waste port. The handle is thrown quickly to the inject position. This last step is done quickly to prevent pressure build up while the ports are blocked in shifting from one position to the other. *Remember*: Load slowly, inject quickly.

Mark the injection point on the chromatogram. Some detectors, computer systems, or integrators will do this for you automatically. It is good laboratory practice to mark the injection with the operator's initials, time, date, sample number and injection volume, mobile phase composition, flow rate, detector wavelength and attenuation, and chart speed. If a gradient is being run, mark the starting composition, gradient start and end, and final composition. You can annotate later injections only with conditions that change, such as sample identification number and injection size. If you tend to cut your chromatograms apart, however, you may lose critical information if you fail to annotate every run with full information. There are commercially available rubber inkpad stamps that provide spaces for the necessary information. Do not rely on your memory to come up with the data at some future time.

3.3.2 Simple Scouting for a Mobile Phase

My scouting gradient technique was developed when I had to make separations in a customer's laboratory to sell an HPLC system. I only had a few hours to make a separation to convince the customer that he should consider buying a system. But, it provides useful insight for developing a method to use in your laboratory.

The first step is to determine a starting point. If I am handed a mixture of a completely unknown nature, I will probably first try to get more information. I will try to determine the mixture's solubility in organic solvents, the effect of acid on the solubility, and something about the molecular weights and isoelectric points if it is a mixture of proteins.

If this information is not available, I will try to separate the mixture using a C_{18} column in acetonitrile and water. Something like 70% of the separations in the literature are now made on a C_{18} silica-based column. Acetonitrile is my solvent of choice because of its low wavelength transparency, its polarity, and its intermediate position between methanol and tetrahydrofuran. Generally, I will use 254 nm for the detector because the majority of the literature separations can be made at this wavelength (see the Separations Guide in Appendix A).

If I know that the compound is not soluble in aqueous solvents, I will probably select a silica column and a chloroform/hexane mobile phase. Separations of proteins will take me first to a TSK-3000sw column and a 100 mM Tris-phosphate pH 7.2 mobile phase unless I am separating soluble enzymes; then I use a TSK-2000sw column.

For illustration purposes, we will take the most common case. We will start with a 15-cm long C_{18} column, 254 nm, and acetonitrile/water in a scouting gradient. Scouting gradients are run much more rapidly than analytical gradients. A mixture of the compounds to be separated is dissolved in 25% acetonitrile in water. A sample is injected into an HPLC equilibrated in the same mobile phase and a 20-min gradient is run to 100% acetonitrile.

Examination of the chromatogram while the separation is occurring lets us select conditions for a starting isocratic run. Since we were running very rapidly, conditions inside the column were not in equilibration. We use the gradient position of the first peak maximum as a guide to an isocratic mobile phase. Find the solvent composition from the controller %B output corresponding to the first peak and drop back to 10% less acetonitrile for a 25-cm column (7% less for our 15-cm column). Using the gradient controller to dial-a-mix the solvent, we equilibrate the column for 15 min at this acetonitrile concentration and reinject our standards.

We have our conditions if all the peaks are accounted for and separated. If not, we can do k' development, control pH by buffering, or change the stronger solvent or the type of column to produce an α change. We have a starting point, and that is half of the battle.

If you do not have a gradient system, I have developed a fast isocratic scouting technique. You select the same column and detector wavelength, but equilibrate the column in 80% acetonitrile in water for our first injection. A strong solvent composition is selected to blow everything off quickly. Look at the peaks; if they are resolved, quit. If they are still unresolved, mix the mobile phase with an equal volume of water making 40% acetonitrile, reequilibrate, and shoot again. This time, the peaks should be much farther apart. If not, do another equal dilution with water to 20%, reequilibrate, and reinject the sample.

If the first peak from the 40% run takes more than 20 min or the peaks are too far apart, wash everything off with 100% acetonitrile. Mix mobile phase 80% and 40% in equal volumes to get 60%, reequilibrate, and shoot again. I usually found that I could get acceptable chromatography by the third run or I needed to make a solvent α change by going to methanol/water.

Normal phase silica column scouting is run the same way. Start gradients at 25% chloroform/hexane and run to 100% chloroform in 20 min. For isocratic scouting, start at 80% chloroform/hexane and make dilutions with hexane. We will cover methods development in more detail in Chapter 11.

3.3.3 Examining the Chromatogram

I usually run scouting samples at an initial UV attenuation of 0.2 AUFS (absorbance units full scale) or refractive index attenuation of 8×. This way, I can increase attenuation if the peaks start to go off scale or decrease attenuation if they are too small. An integrator or a computer system will see everything from the baseline up to full attenuation, but you've got to be reasonably

close if you are using a strip chart recorder. Otherwise, you will lose peak information.

I would rather blow my first sample off scale and have to dilute the second one. At least I know I got the sample in and what the next step should be. If I shoot too little, I wait and wait for something to happen and waste a lot of valuable time. Besides, I've found that the first shot of the day is usually a "column tranquilizer." It seldom agrees with other samples of the day. Two and three agree, but not necessarily with number one. I've discussed this problem with other chromatographers and many have observed the same thing. If this bothers you, remember that chromatography is still art as well as science. Shoot the first sample and go and have some coffee. Then, you can get down to work.

I'm often asked if peak heights or peak areas give more accurate results. The answer to this question is yes. When working with mixtures of pure compounds with very little overlap, peak areas give more accurate results. However, my clinical friends, who must quantitate on peaks from complex mixtures with overlapping peaks, insist that peak heights are more accurate.

3.3.4 Basic Calculations of Results

In peak height measurements, we measure the vertical displacement from the baseline and compare that to the peak height of a known standard amount. Peak areas are a little more complicated. They are usually done by triangulation; assume a right triangle and multiply the peak height times the half peak width. The areas of each peak are summed to give a total area. Dividing this into the area of each peak gives a relative area percentage for each peak. Like peak heights, peak areas can be compared to peak areas for known standard to allow calculation of the amount of compounds present.

Another, more accurate method is to copy the chromatogram, cut out the peaks, and weigh them. Of course, if you have an integrator or a data processing computer system, it will do the job for you. They can usually be set to do either peak heights or areas. They also can be calibrated for standard runs and will calculate actual amounts relative to these earlier runs. Some also can be calibrated with compound names related to peak retentions to provide annotated outputs.

Integrating systems are designed to make the chromatographer's life easier, but they can complicate it if not properly used. They usually have an auto/zero function, which, when selected, looks at the baseline before injection and sets various integration parameters. This is designed to prevent integration of very small or extraneous peaks or of baseline noise. On most integrators, autozero must be requested by the operator and should be used every time a detector attenuation change is made. Be aware that you are letting a machine make decisions for you. It is possible to override the machines, and, sometimes, it is possible to produce a more accurate analysis by doing so.

Once we have returned to the baseline from one chromatogram, we are ready to make our next injection. When we have finished for the day, shut off

the detector (lamps have finite lifetimes) and the strip chart recorder paper drive. If we are pumping solvents containing solids, they must be washed out before shutting down the pump. The system can be store overnight or over a weekend with solvent containing more than 50% organic in the mobile phase. If you will be storing longer than a weekend, wash the system out with acetonitrile, remove and cap the column, and store it in its box labeled with the solvent and the last sample run in it.

II

HPLC OPTIMIZATION

4

SEPARATION MODELS

Three main modes of separation are used in HPLC systems. Partition separation makes up the majority, followed by size separation, and, finally, by ion exchange.

4.1 PARTITION

Separation in the column occurs when the sample in the mobile phase begins to interact with the stationary packing material. The actual mechanisms for these interactions are still being investigated. They probably involve forces generated by ordering of the charge density separations in polarized compounds such as water. Because of the electron attraction of the oxygen and the bonding angle of the hydrogens with the oxygen, the water molecule exhibits a negative end and a positive end. Water achieves a minimum energy state when it can align positive ends toward negative ends. This ordering leads to an "attractiveness" of polarized molecules for each other.

When nonpolar molecules or portions of molecules are introduced into a polar matrix, the nonpolars will orient in a manner leading to maximized polar interactions. This results in nonpolars being pushed together. The net effect is that *likes attract likes*—polars with polars, nonpolar molecules with nonpolars. Compounds with similar polarities are attracted to each other. The packing material has differential attractions for different compounds in the sample depending on their degree of polarization. Since the mobile phase is continuously being replaced, each component is washed off at a different rate. Even

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small differences in attraction for the packing surface, when repeated many thousands of times, leads to a separation.

A good model for this partition is the separation that takes place in a separatory funnel, as we discussed in Chapter 1. If you dissolve a mixture of two components (A and B) in a separatory funnel containing two immiscible liquids, an equilibrium is established for both compounds in each solvent.

If sufficient polarity differences exist between the compounds, each compound will tend to concentrate in the solvent with a similar polarity. Like attracts like. The more polar compound concentrates in the polar layer. The other member of the mixture is forced toward the nonpolar layer. The bottom layer can then be drawn off, taking with it one of the two components. The second component remains behind in the upper layer, which could be recovered next. We have thus made a separation of the two compounds.

In our example, we separated a purple mixture made up of a polar red dye and a nonpolar blue dye. Adding this mixture to a separatory funnel containing water and hexane and shaking vigorously will produce two colored layers. The upper (hexane) layer will contain the blue (nonpolar) dye. The lower (water) layer will attract the red (polar) dye. Opening the stopcock can draw off the water layer containing the red dye (see Fig. 1.2). Evaporation of the water will yield the more polar red dye. In a similar manner, we can recover the blue dye from the hexane layer left in the separatory funnel. The problem with working with separatory funnels is that the separation is not complete: each component has an equilibration concentration in each layer.

A similar separation occurs in the HPLC column. Either the mobile or stationary phase is polar and attracts the more polar component in the injected mixture. Let us assume that our column packing is polar and we are pumping a nonpolar mobile phase down the column. Both components have a partition affinity for the packing and will be retained. But the more polar of the two will be retained longer. Since equilibration is continuously being upset in favor of the moving liquid phase, the less polar component washes out faster and is eluted first from the column (see Fig. 1.3). Eventually, both compounds will wash off the column into the detector.

A number of HPLC partition columns of differing polarities are available and will be discussed later. For now, let us consider the example of a separation on a polar, hydrated silica gel column using methylene chloride in hexane as our nonpolar mobile phase.

Silica gel is hydrated silicic acid with a controlled amount of water of hydration. Each silica on the surface of the packing has one or more hydroxyl group associated with this water of hydration. The available proton on the hydroxyl group gives silica its acid nature and, along with the hydration shell, makes it a very polar surface.

The same purple mixture separated in our separatory funnel example is dissolved in the methylene chloride and shot onto the column through the injector. The two compounds to be separated are swept together onto the column. As fresh mobile phase causes them to pass down the column, the more polar component (the red dye) is more highly attracted to the polar column surface

and is retained more than the more nonpolar, blue dye. The blue dye moves a little faster and begins to pull apart from the red. Finally, the blue dye reaches the end of the column and begins to elute into the detector. The detector signals the concentration changes to the strip chart recorder as a voltage change. As the band center of the peak (B) passes the detector, the strip chart recording goes through a maxima, then returns to the baseline (Fig. 4.1). Next, the red dye completes its trip down the column and begins to elute. It also enters the detector and produces a broader peak (A) on the recorder. The peak broadening occurs because the red dye spent a longer time in the column, giving diffusion more of a chance to spread it out.

Let us now examine the chromatogram produced by this separation. Starting at the point of injection, we follow the baseline to the first deflection. After about 2 min, we see a small positive deflection immediately followed by a small negative deflection, which then returns to the baseline. The center of this peak complex is called the void volume (*Vo*). It represents the amount of mobile phase contained inside the column, but outside the packing material. It is the mobile phase volume necessary to wash out the sample solvent.

This peak occurs because the solvent composition used to dissolve the sample often differs somewhat from the mobile phase. When this sample solvent volume reaches the detector (at *Vo*), a refractive index difference upset of the baseline occurs. This *Vo* peak is very important because it allows us to normalize the separation parameters for variations in column lengths. It also gives us some assurance that the sample was actually loaded into the injector and onto the column.

The next peak is that produced by the blue dye (B); we will measure the mobile phase volume at the center of the peak and call it V_B. In the same manner, we can calculate V_A as the retention volume for the red dye. We could measure the distances Vo, V_A, V_B just as easily in minutes since injection or as centimeters of graph paper. As we will see, the separation parameters are

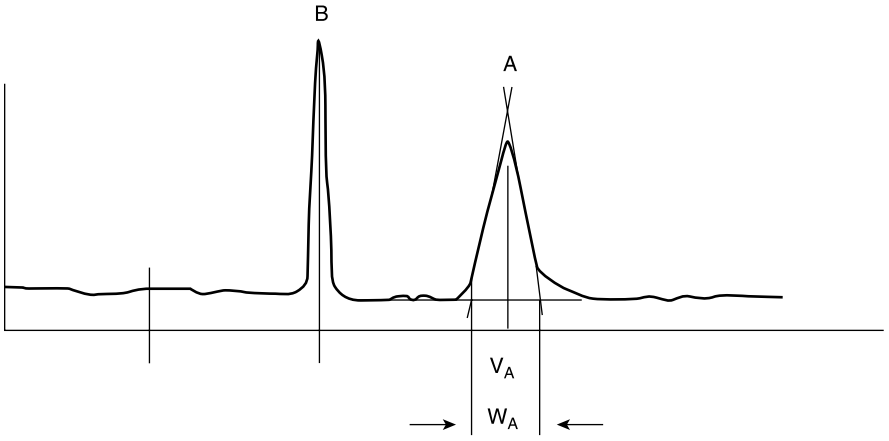

Figure 4.1 Separation model chromatogram.

$$K' = \frac{V_A - V_0}{V_0}$$

$$\alpha = \frac{V_A - V_0}{V_B - V_0}$$

$$N = 16\left(\frac{V_A}{W_A}\right)^2 = 5.42\left(\frac{V_A}{W_{0.5}}\right)^2$$

Figure 4.2 Separation factors.

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

Figure 4.3 Resolution equation.

dimensionless. Thinking in mobile phase volumes eliminates the necessity of considering strip chart and pumping speeds. In the literature, you may see V_B described as tr_B., the retention time of B, or as the retention length of B in centimeters. These are both referring to as V_B.

4.1.1 Separation Parameters

From these volumes, we can calculate three factors, k', α, and N (Fig. 4.2), which will then be used to describe a resolution equation (Fig. 4.3). This equation predicts the effect of variations in these factors in controlling resolution within the HPLC column. They are presented here to discuss the variables controlling each of them, their limits, and how you can use them to achieve your separations in a rational manner.

They also serve as a common language when discussing separation problems. With these quantities in hand, it is generally unnecessary to detail other operating conditions. Finally, their most important use is as a diagnostic tool for column problems.

The first factor, the retention factor (k'), is the relative retention of each peak on the column. In our example, k'_B, the retention factor for the blue peak is determined by dividing the difference between V_B and V_o by V_o. It effectively tells us how long it takes the center of peak B to come off the column relative to V_o. We can derive a similar factor for the red dye (k'_A) or for any peak in a multi-peak mixture.

The next factor, the separation factor (α), represents the relative separation between any two peaks' centers on a chromatogram. It is defined as the retention factor of the longer retaining peak divided by the retention factor of the faster peak. Any pair of peaks in the chromatogram will have their own α.

The final factor, the efficiency factor (N), measures the degree of sharpness of a given peak. It is determined by the retention volume of the peak (i.e., V_B) by the peak width. Two different widths are commonly used for this calcula-

tion, the width at one half the peak height and the width 10% up the peak, the 5 σ width. The half-height width is easier to measure, but corrects poorly for peak tailing. Efficiencies calculated from it are optimistically high and are unresponsive to column changes.

For our purposes, the 5 σ width is more useful for determining V_W. It is determined by drawing tangents to both sides of the peak and measuring the distance between the intersection of these with the base line. Using this definition of peak width, the calculation of N equals 16 times the square of V_B/V_W. Different peaks in a mixture will give different efficiency values.

All of these are combined in the resolution equation (R_S), which predicts how each factor will affect the separation. The derivation of the equation is not important to our work, but can be found in the Synder and Kirkland reference in Appendix G. In practice, the values used for the factors are empirically derived from chromatograms. For most uses, fairly crude measurements are sufficient, but care should be taken with peak widths in calculating efficiencies.

Generally, k's range from 1 to 8 for analytical separations and 4 to 12 for preparative. α's range from 1 to 2; at $\alpha = 1$, peaks completely overlap, much above $\alpha = 2$ and the separation can be made in a separatory funnel. For N, values may range from hundreds (poor resolutions) to tens of thousands (good resolution).

The resolution equation shown in Figure 4.3 can provide direction for starting separation scouting. *Note*: N is present as a square root term; large changes produce a small effect on resolution. k' is present in a convergent term. At low k's, a one unit change in k' produces a relatively large effect. At high k's, a one unit change has little effect. This is why changes in k' above 8 have little effect except to lengthen the time of the run. Changes in α produce the greatest changes in resolution, but the exact effect that a given change in experimental conditions will have on the α value of a set of peaks is often difficult to predict. An α change in methods development is often saved as the court of last resort. It usually must be followed by further k' or N modifications.

Therefore, as we begin to develop a separation we will check column efficiency, knowing that we can use it to produce small changes. We will make changes in retention until we reach high values of k'. Then, if we still have not achieved our separation, we will do something to change α.

Now, let us look at the variable controlling the various factors in the equation. We will return to the resolution equation when we get into column diagnostics and healing (Chapter 6) and, again, in scouting and methods development (Chapter 12).

4.1.2 Efficiency Factor

The efficiency factor, N (Fig. 4.4), measures peak sharpness. The sharper the peak, the better the separation, and the higher the efficiency of the column and the system.

$$N = 16\left(V_X / W_X\right)^2$$

Figure 4.4 Efficiency factor equation.

It is important, first, to realize that efficiency is not a function solely of the column. Bad extracolumn parameters, such as detector cell volume or tubing diameters, can make the best column in the world look terrible. Second, efficiency measurements are very poor ways of comparing or purchasing columns unless all other parameters are constant. Many columns are bought and sold because they have a "higher plate count" than someone else's column. The efficiency calculations could have been made with different equations, on different compounds, on different machines, at different flow rates, all of which will have a profound effect on efficiency. The only valid use of plate counts that I have found is in column comparisons where all other variables are equal, or in following column aging over a period of days or months.

Let us look at an efficiency measurement. Efficiency, N, is usually reported in plates, a dimensionless term that is a throwback to the days of open column, flooded plate distillations. The more plates in the distillation column, the more equilibrations have occurred, and the better the separation that was produced. In an HPLC column, the larger the plate count, the sharper the peaks are, and the smaller the amount of overlap that occurs between them.

For accurate measurement, it is important to spread the peak without changing variables affecting N. Increasing the chart speed to 2–5 times normal run speed will usually do this, but remember to correct V_B for the increase. Early eluting peaks with a k' of 1–3 should show a plate count between 6,000 and 10,000 for a 10-μm packing in a typical 25 cm $\times$ 4 mm column.

Variables affecting changes in N have a square root effect on resolution. Some are beyond the chromatographer's control, such as particle homogeneity, particle shape, and how well the column was packed. Particle size is a Gaussian distribution around the stated diameter. Different processing produces different distribution curves. Early packing produced by grinding and screening yielded very irregular-shaped particles with lower efficiencies than modern spherical particles. Packing is still very much of an art. Wall and bed voids act as turbulent mixers and are present to some degree in all columns. Spherical packing and high-pressure packing seem to greatly reduce voiding and increase column life. Other variables, such as particle diameter and column length, are user selected when the column is purchased. General analytical plates/meter for differing packings are shown in Table 4.1. These values are offered simply as a guide. Values of theoretical plates and optimum flow rate will vary for spherical packings and columns from different manufacturers. Column back-pressures increase with smaller particle size and higher flow rates.

Column length is usually optimized around a tradeoff between efficiency and run time. Doubling the column length increases back-pressure and run

Table 4.1 Relationship of efficiency to flow rate

Efficiency Changes with Particle Size		
Packing diameter (μm)	Plates/meter	Flow rate (mL/min)
10	30,000	1.0
5	50,000	1.5
3	100,000	2.5

times 2-fold while increasing efficiency only by 1.4-fold due to increased diffusion.

Finally, we have variables affecting efficiency that can be controlled at the time of the run. These are pump flow rate, extracolumn volumes in the instrument used, and the method of calculation. Flow rate is the major efficiency variable that I use during methods development. Generally, halving the flow rate will increase separation around 40%. I do much of my scouting at 2.0 mL/min, knowing that I can improve separation by dropping to 1.0 mL/min. Plotting of efficiency versus flow rate shows that each diameter of packing has its own optimum flow rate. Efficiency decreases at higher flow rates. In the microparticulate packings, large packing diameters show a more rapid loss of efficiency with increasing flow rate than do smaller packings.

Decreasing extracolumn volumes is critical to HPLC success. The most important volumes are those immediately adjacent to the column: zero-dead-volume end-fittings, inlet and outlet tubing diameters, and detector cell volumes. From the time the sample enters the injector until it exits the detector, nothing must add increased mixing space. Tubing from injector to column must be 0.010 in for 5-μm and 10-μm packings with tubing lengths no more than 4–6 in for the 5-μm. Use 0.007-in tubing about 3 in long or less for 3-μm packing. Zero-dead-volume endcaps and connectors must be prepared correctly, so that tubing ends butt firmly against the fitting. We covered the preparation of compression fittings in Chapter 3, but if you find efficiency drops after you change a fitting, check the dead-volume fit. For detector cells, the rule of thumb is 8–12 μL; anything larger acts increasingly as a mixer for your already separated bands.

Tubing volumes outside the critical injector-detector range are important only if you are doing recycling or collecting samples. Pump-to-injector tubing is generally 0.020-in; vents, flush valve, etc. may use 0.04-in. Be sure you know what these look like and do not confuse them with injector tubing. In telling tubing apart, 0.02-in and 0.01-in are the most difficult to tell apart. If you have to look twice to make sure there really is a hole, it is probably 0.01-in. If you are in doubt, put them next to each other. By comparison, 0.04-in tubing looks like a sewer pipe.

There are many methods used to calculate efficiency. All methods give the same results with ideal, Gaussian peaks. Real chromatography peaks tend to tail on the backside of the peak (away from the injection mark on the

chromatogram). When column problems occur they often tend to show up as increased tailing. Calculation methods that use a peak width high on the peak miss these changes and give artificially high efficiencies. The 5σ method described above is excellent for detecting early appearance of tailing. If you're planning on using a calculation using half-peak width, make sure there is some method of measuring and correcting for peak asymmetry.

The retention factor, k', also called the capacity factor, is the usual starting point for methods development. The retention factor, as its name implies, is basically a measure of how long each compound stays on the column; V_O used to determine k' is usually only roughly measured; k' is a simply a multiple of the V_O distance (see Fig. 4.5).

The major usable variable controlling k' is solvent polarity. While temperature and column polarity also effect retention times, they do not show the same direct, linear relationship for all peaks and are usually classed under the separation factor (α).

Increasing the polarity difference between the stationary and mobile phases increases the retention of compounds with polarities most like the column. Compounds stick tighter and peaks will broaden through diffusion. Decreasing the polarity difference will make things come off faster and shoved together. Peaks will be less resolved and sharper.

For example, for a polar silica column equilibrated with a mobile phase of methylene chloride in hexane (nonpolar), you would dilute with more hexane to increase the k' of relatively polar components. Adding methylene chloride, the more polar of the two solvents, would decrease k's causing all components to wash off faster. With k' changes, peak position changes are proportional and in the same direction. The order of resolved peaks will remain the same; unresolved peaks should begin to pull apart.

If in our model system, we had used 80% methylene chloride/hexane and the red peak had partially overlapped the backside of the blue peak, we would attempt to resolve it by reequilibrating in 40% methylene chloride/hexane and reinjecting. We could expect that we should see two well-resolved peaks; if not, we could go to a 20% mixture. More than likely, we would have overshot on the first change and would have to fine-tune back toward the 80% mixture. Simply by modifying the solvent polarity, we are able to increase or decrease k' and contract or spread our separation. This k' development is our usual starting point in methods development.

So far, I have referred only to "normal-phase" separations on polar columns. However, around 80% of the separations in the literature are made on "reversed-phase" columns. To understand these terms, we need a little history.

$$K = \left(V_X - V_O\right) \Big/ V_O$$

Figure 4.5 Retention factor equation.

Early "high-pressure" packings were cross-linked ion exchange resins and polymeric size separation, gel permeation packings. The first high-pressure columns for partition separations were packed with the same material as is used in open columns or for preparing TLC plates. These were 35- to 60-μm diameter silica, a very polar packing material. To achieve separation, nonpolar solvents were used for elutions. These solvents were flammable, volatile, toxic, or expensive. After a few years, someone decided to coat the silica with nonpolar compounds similar to those used in GLC column so that polar solvents, such as water, could be used for elution. The problem with these coatings was that they tended to wash out with the mobile phase, bleed into the detector, and contaminate the collected sample.

This was overcome by chemically bonding the coating to silica leading to the first "abnormal" packing materials. Because these packings could be run in aqueous solvents and did not require the careful drying and handling of the normal-phase columns, they quickly became very popular. Since no one wanted to admit to being an "abnormal" chromatographer, when they reached the majority they quickly renamed themselves "reversed-phase" chromatographers.

The first of the really successful coatings was a long-chain, saturated hydrocarbon with 18 carbons. These octadecyl- (ODS), RP_{18}, or C_{18} columns are still the most commonly used HPLC columns, primarily because of the versatility they have shown. Other packing materials have appeared with shorter or longer side-chains, and, with a variety of functional groups on the side-chains, greatly extended the possible separations that can be achieved with HPLC.

Retention changes work exactly the same with reverse-phase column as with normal-phase columns. Increasing the polarity difference between column and mobile phase increases the k's of the components. However, since the column is nonpolar, we now must add more of the polar solvent to make compounds stick tighter. On our reversed-phase column, our dye mixture would also elute in opposite order, the more polar red dye would have less affinity for the nonpolar column and would elute before the nonpolar blue dye. By controlling the column nature, you control the elution order. Figure 4.6 illustrates the effect of solvent polarity changes on a separation.

As we mentioned earlier, there is a limit to usefulness of k' changes. Because it is a convergent term in the resolution equation, the larger the value of k', the less the effect a polarity change has on Rs. Beyond $k' = 8$–10, changing k' has only a negligible effect, except on run time. At this point, the next step is to change resolution, Rs, by using the separation factor, α.

4.1.3 Separation (Chemistry) Factor

The separation factor, α (Fig. 4.7) is calculated by dividing the k's for the two peaks under question. It measures the separation between the two peak centers. Components with an $\alpha = 1.0$ overlap completely; beyond $\alpha = 2.0$,

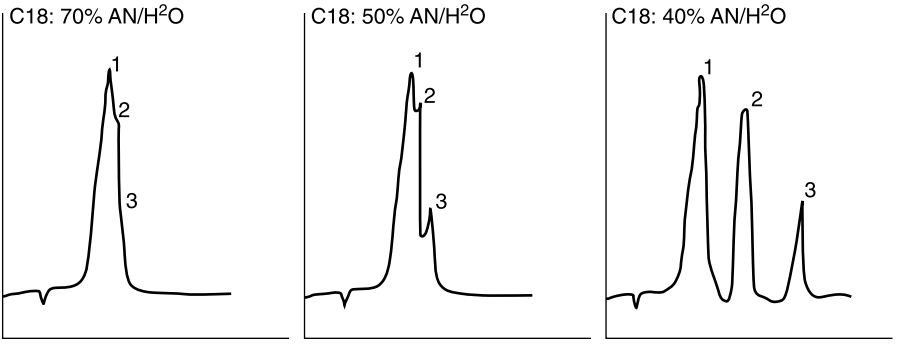

Figure 4.6 Effect of polarity changes.

$$\alpha = \left(V_X - V_O\right) \Big/ \left(V_Y - V_O\right)$$

Figure 4.7 Separation factor equation.

compounds can be separated by separatory funnel. Large αs are needed in HPLC only for preparative runs.

When we change retention with solvent polarity, all peaks show an equivalent shifting in the same direction. A variable producing an α change causes relative peak positions to shift; individual peaks exhibit different amounts of shift, both in size and direction. Thus, k' changes spread separations already present; with α changes new separations are created. With an α change, relative peak positions can even reverse.

Temperature is the first of the variables affecting separation. Increased temperature decreases retention time on the column, sharpens peaks, and produces the change in relative peak retentions typical of an α effect. At first, this appears to be the ideal variable, similar to temperature programming for GLC. However, temperature changes have some drawbacks.

First, temperature is generally limited to an effective range of 20–60°C by solvent vapor pressures. Higher temperatures can vaporize solvent in the column leading to column voiding and cavitation, similar to a vapor lock in a car's engine on a hot day. It can produce chemical changes in some compounds being separated, catalyzed by contact with the hot, acidic silica surface. Even more important is the effect temperature has on the column packing. Bonded phase columns are prepared by chemically bonding an alkyl chlorosilane to the oxygen on the silica. This process can be reversed by hydrolysis, especially under acidic conditions, leading to bonded-phase bleeding and column performance changes. Heat accelerates the process. If you're only getting 3 mo life from your columns, this might not be an important consideration. But, one of the goals of this text is to show you how to extend column life.

Recent changes in column stability with zirconium-based and hybrid silica columns have lead to resurgence in the use of column jackets to elevated temperature to speed analysis time. The problem of sample degradation at these higher temperatures remains a continuing problem as it does in GC separations.

The separation factor (α) is also referred to as the chemistry factor. It can be modified by changes in the chemistry of the components that make up the chromatographic system: column, solvent, and sample. Changing the column surface chemistry from the very nonpolar C_{18} to C_8 obviously increases the column polarity as the compounds are drawn closer to the silica surface. We would predict that nonpolar compounds would elute faster, and so they do. However, observation of the peaks shows peak shifting typical of an α variable. If we substitute a phenylethyl group for octyl, we maintain the same polarity, but now we see dramatic changes in selectivity. The so-called phenyl column has an affinity for aromatics and double bonds. It will separate fatty acids on the basis of the number of double bonds as well as chain length. Octyl columns separate only on chain length differences.

The most common variable used to control α is the "stronger" solvent in the mobile phase. The stronger solvent is the mobile phase component most like the column in polarity. Changing the chemical nature of this stronger solvent will produce shifts in the relative peak positions. For instance, if we are unable to achieve the desired separation on a C_{18} column using acetonitrile in water, we can produce an α effect by shifting to methanol in water: an opposite effect occurs on switching to tetrahydrofuran in water (Fig. 4.8).

This is true even if we adjust the polarity of the new mixtures to match that of the previous mobile phase. We can produce other α changes by adding mobile phase modifiers to our solvents. Buffers, chelators, ion pairing reagents, and organic modifiers can all be used to change or fine-tune the separation. We will cover use of all of these in detail in a Chapter 7.

The final α modifier, preparing derivatives of a mixture, is our court of last resort. If two compounds cannot be separated by changing N, k', or the

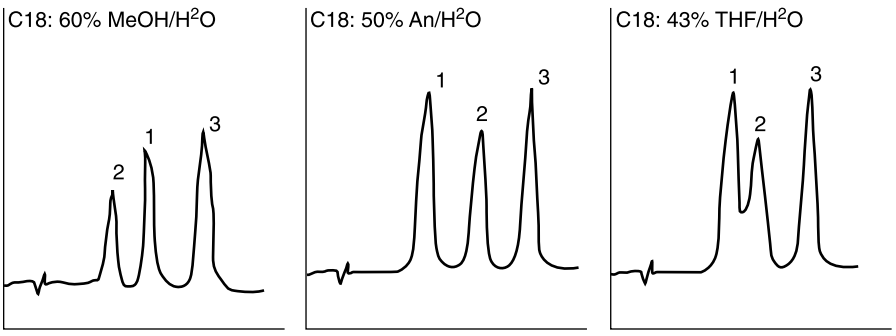

Figure 4.8 Effect of "stronger" solvent changes.

chemistry of the column or mobile phase, then changing their chemical nature by making derivatives should lead to compounds that can be separated. We use this only as a last gasp separation technique. Usually, we can separate most compounds directly. Derivatives are more commonly used in HPLC to change a mixture's solubility or to produce compounds with strong extinction coefficients to increase detection sensitivity.

4.2 ION EXCHANGE CHROMATOGRAPHY

So far, we have dealt only with partition chromatography in which compounds equilibrate between the mobile phase and the column based on differences in their polarity. Ion-exchange chromatography uses the type and degree of ionization of the column and compounds to achieve a separation. Here, opposites rather than likes attract; compounds with charges opposite to that on the columns are attracted and held by the column. Elution is achieved by competitive displacement; an excess of an ion with the same charge as the bound compound pushes it off the column. The tighter the ionic bonding to the column, the longer the compound stays on the column.

Ion-exchange columns are made of a number of backbone materials: silica and zirconium, like the reverse phase columns, and heavily cross-linked, organic polymers. Bound to these are organic bonded phases containing functional groups that either have permanent ionic charges or in which ionic charges can be induced with pH changes.

Two warnings about using polymeric columns: Early polymeric column for ion exchange would not tolerate much pressure or organic solvents. Recent columns are more heavily cross-linked and show more pressure tolerance, but be sure to check the manufacturer's column shipping notes for use limitations. Few will tolerate pressures above 2,000 psi without collapsing. Some organic solvents can cause the column bed to swell or shrink on changing solvents, which can lead to bed collapse or voiding.

Charged functional groups, which give these columns their separating character, are of two types: anionic and cationic. Anionic packing materials have an affinity for anions (negatively charged ions) and have positively charged functional groups on their surfaces, usually organic amines. Cationic packings attract cations (positive charges) with negative functionalities, usually organic acids and sulfonates. Cationic and anionic olumns can both be subdivided into either strong or weak types. Strong columns have functional groups that possess either permanent charges (i.e., quaternary amines) or have charges present through the full pH range used for HPLC (i.e., sulfonic acids). Weak columns have function groups with inducible charges. At one pH they are uncharged, at a different pH they are charged. Examples are organic acids, which are uncharged at pH 2.0, but form cations at pH 6.5, and organic primary amines, which are positively charged below pH 8.0, but exist in the free amine form above pH 12.

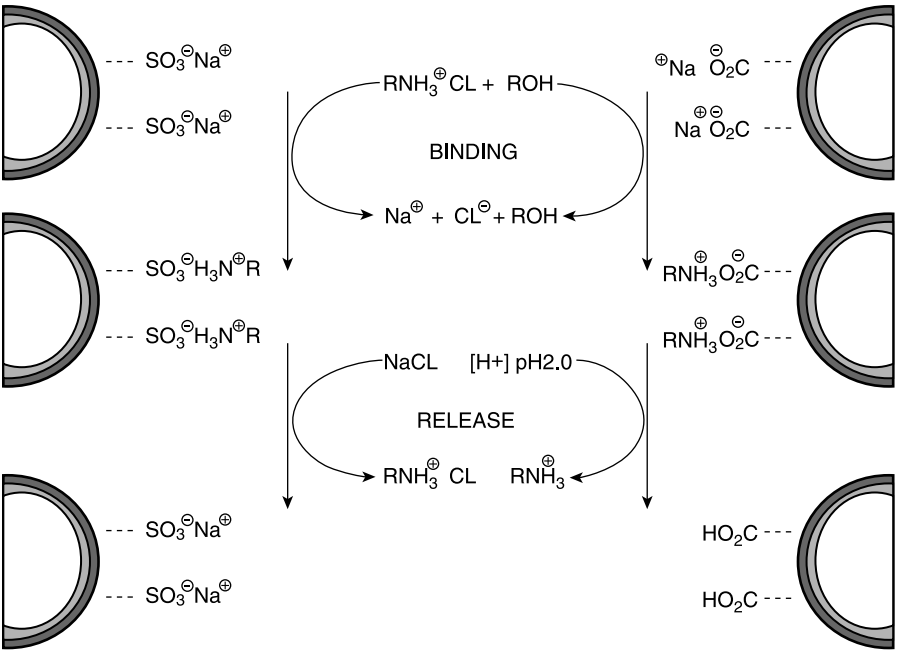

Figure 4.9 Cationic-exchange separation model.

Let us examine a silica-based cationic (sulfonate) ion exchange separation (Fig. 4.9). The column is equilibrated in 50 mM sodium acetate. An injection of amines and an alcohol in the mobile phase is made. The same mobile phase, or one containing increased amounts of sodium acetate, is used to elute fractions.

The alcohol will come off in the void volume of the column since it has no attraction to the column. The amines will be retained, because at the pH of the acetate solution they are protonated and have a positive charge. As more mobile phase passes the through the column, its sodium ions begin to compete for the sulfonate sites with the bound amines. Through a mass effect, the amines are displaced down the column until, finally, they elute into the detector. The amine that has the strongest charge and binds the tightest is eluted last.

4.3 SIZE EXCLUSION CHROMATOGRAPHY

The first commercial HPLC system was sold to do gel permeation (GPC) or size separation chromatography. It is the simplest type of chromatography, theoretically involving a pure mechanical separation based on molecular size.

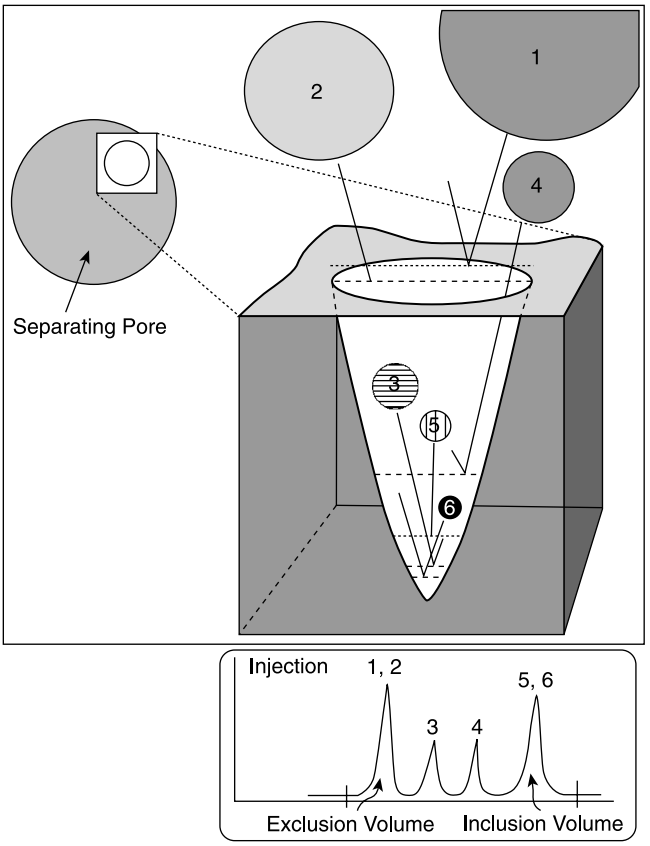

Figure 4.10 Size-separation model.

The column packing material surface is visualized as beads containing tapered pits or pores. As the mobile phase sweeps the injection passed these pits, the dissolved compounds penetrate, if their largest diameter (Stokes radius) is small enough to fit (Fig. 4.10). If not, they wash down the column with the injection front and elute as a peak at the column void volume, which is called the *exclusion volume*.

Returning to the compounds that entered the pit, we find that large particle can not penetrate as deeply down the pore as can smaller compounds. The smaller the diameter, the deeper the penetration, and the longer the compound takes to elute. The largest compounds wash out quicker, follow a shorter path, and elute just later than the totally excluded compounds. Traveling down the column, these resolving compounds wash in and out of many pores magnifying the resolution achieved by differences in the path lengths they follow. Finally, we reach a point where all compounds of a certain diameter or smaller reach the pore bottom, wash out, and elute in a single peak. This is referred

to as the *inclusion volume*. If the exclusion volume is found at V_O, the inclusion volume appears at approximately 2 *Vo*.

From this, we can see we have three types of peaks: 1) the exclusion peak, containing all molecules of a certain size or larger; 2) resolved peaks of intermediate diameter; and 3) the inclusion peak containing all compounds of a given diameter and smaller. In a crude mixture of compounds, we are forced to suspect that both the exclusion and inclusion peaks contain multiple components.

Just as it is possible to prepare a column with a single pore size, It is possible to prepare columns with differing pore size. Each would have its own particular ratio of exclusion/inclusion diameters. A column bank of columns containing different pore packing can be used to separate a mixture with a wide range of compound sizes. Columns of varying exclusion/inclusion limits can be connected with the smallest exclusion limit column first in the series. If the columns are selected so the first's exclusion limit overlaps the second's inclusion limit and so forth, the column bank produced has the first column's inclusion limit and the last column's exclusion limit. Again, remember the pressure problem when stacking columns; pressure increases proportionally to the number of columns. You may have to run very slowly if you are using pressure fragile columns.

GPC columns are referred to as molecular weight columns, but they actually separate molecules according to their largest dimension. True molecular weight measurements would be independent of shape. As long as we work with simple, spherical compounds, there is a direct relation between exclusion volume and molecular weight within the resolved range. Columns can be calibrated with standards of known molecular weight and used for molecular weight determinations. These measurements break down at higher molecular weights with compounds with nonspherical shapes (i.e., proteins), which change shape and apparent size with changes in the mobile phase. Solvent conditions that force all molecules into long, rigid shapes aid in molecular weight determinations (i.e., 0.1% sodium dodecyl sulfate [SDS] is used for protein molecular weights).

Size separation columns are available with silica, zirconium, and heavily cross-linked organic polymer backbones. The polymer columns show the same pressure and solvent fragility described for ion exchange columns. Silica size columns must be protected from pH changes like partition columns, which must be used with a pH between 2.5 and 7.5. Zirconium columns are not pH or temperature sensitive, but possess chelation properties that must be chemically masked to prevent interference with the size separation.

4.4 AFFINITY CHROMATOGRAPHY

Much less commonly used than partition, ion exchange, and size columns, affinity columns are of growing interest in the HPLC purification of proteins

because of their very high specificity. A molecule with a target site or recognizer is bound to the surface of the affinity packing, sometimes through a 6-carbon spacer. This forms a tight complex with one, and usually only one site, on the compound to be purified. The analogy used in affinity separations is the idea of the *lock and key*. The target site on the compound to be separated is the *key* and the recognizer on the affinity packing is the *lock* that it fits. When a solution containing the target compound is passed down the affinity column, only that material with the key functionality is held up and retained on the column. Everything else comes out in the breakthrough volume. The target compound can then be eluted with a change in pH, with high salt concentration, or eluted with a molecule similar to the recognizer lock function.

In practice, affinity column recognition specificity is never as complete as described in theory. Usually a range or class of similar compounds can be attracted and retained. The recognizer must be bound to the column for each target compound and after that point the column must be dedicated for that separation. Usually there is no possibility of removing the recognizer and reusing the column for a different separation.

The biggest attraction of this type of column is that often it is able to achieve nearly a total purification of the target from a very complex mixture in a single pass down the column. Like the ion-exchange column, this type of separation benefits in preparative mode from broad, short columns with a large surface area. Its weakness lies in the difficulty of finding and binding the specific recognizer for our target, and in developing optimum eluting conditions.

5

COLUMN PREPARATION

The power of HPLC is rooted in the variety of separations that can be achieved with little, if any, sample preparation. HPLC columns are often described as the "heart of the separation." Controlling a separation means understanding and controlling the chemistry and physics going on inside of the column. To do so, it is necessary to understand how packings are prepared and how columns are packed. This will lead us to methods to keep columns up and running, to an understanding of when to select a given column, and to techniques for getting the most from that column (see Table 5.1).

5.1 COLUMN VARIATIONS

The first packing materials used in a HPLC were beads of organic gel permeation resins used for size separations. These were commercially available resins and no attempt was made to optimize them for high pressure, except to select for a high degree of cross-linkage to prevent crushing.

A year later silica-based fully porous 35–60-μm diameter beads were slurry packed in a tube and used for separation. This was the same material that had been used for open column or thin-layer chromatography. The only gain over these earlier techniques was in developmental time. Almost immediately, research was begun to optimize the packing in order to improve the separation.

It was soon found that a large amount of band spreading occurred in this material because of the variety of paths a particle could follow going through the mixture of particle sizes in the packing. Size screening of the packing

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Table 5.1 Silica bonded-phase columns

Column	Phase	Solvents	Application
C_{18}	Octyldecyl	AN, MeOH, H_2O	General nonpolars
C_8	Octyl	AN, MeOH, H_2O	General nonpolars
Phenyl	Styryl	AN, MeOH, H_2O	Fatty acids, double bonds
Cyano	Cyanopropyl	AN, MeOH, THF, H_2O	Ketone, aldehydes
Amino	Aminopropyl	H_2O, AN, MeOH, THF, $CHCl_3$,, CH_2Cl_2	Sugars, anions
Diol	Dihydroxyhexyl	AN, MeOH, THF, H_2O	Proteins
SAX	Aromatic	Salt buffers	Anions
	Quaternary amine	AN, MeOH, H_2O	
SCX	Aromatic	Salt buffers	Cations
	Sulfonic acid	AN, MeOH, H_2O	
DEAE	Alkyl ether	Salt buffers	Proteins, cations
	Ethyl 2° amine	AN, MeOH, H_2O	
CM	Alkyl ether	Salt buffers	Proteins, cations
	Acetic acid	AN, MeOH, H_2O	
SI	(none)	Hexane, methylene	Polar organics, positional isomers
	Silanols	Chloride, chloroform	

allows separation of a fraction with an approximate diameter of 35 μm. This *porous* packing gave a better separation and, because of its consistent particle size, high porosity, and corresponding high load capacity, is still used today for preparative separations.

The next advance came with the discovery that intraparticle path variations were contributing to band spreading. With large, fully porous materials, compounds could follow a separation path either through the diameter or barely skimming the particle surface. It was like having a mixture of particles with diameters from 35 μm on down. The more uniform the path follow, the higher the expected efficiency of the separation would be. To achieve this, a crust of porous silica was coated on the outside of a solid, glassy core forming *pellicular* packing. This was the first of the true analytical packings. Its 35-μm diameter made it easy to handle and pack, its uniform separation path gave it good efficiency, but it had very poor loading characteristics for preparative work. This packing is still used for packing guard columns to protect 10-μm analytical columns from contamination.

The next major step was to *microporous* analytical packings. Grinding and selectively screening the 35–60-μm prepared these fully porous 10-μm packing materials. Although irregular in shape, they had very high efficiency and very good load characteristics. They suffer from two basic problems: high backpressure and fines. Because of their small diameters, they pack very tightly and provide considerable flow resistance. Modern HPLC pumps capable of 6,000–10,000 psi appeared in response to these packings. The small size and irregular shapes also made it difficult to pack these materials without trapping solvent in pockets in the bed and along the wall. The voids formed led to effi-

ciency loss by acting as turbulent remixers and premature death of the column by channeling. Fines were carefully washed out of before packing columns from these materials, but reappeared to plug the outlet filter during use under HPLC pressures. The packing suffered from microfractures and ground off fines as the bed suffered movement during pressure changes.

The most recent improvement has been the fully *spherical* microporous packings. Under an electron microscope, these packings appear as true spheres, either 1.7, 3, or 5 μm in diameter. Not all particles in a batch have exactly the listed diameter: they show a distribution around that size. Individual spherical particles show a single uniform diameter while the irregular micro-packing shows a major and a minor axis. Irregulars also show fissures and grooves while the spheres appear as featureless snowballs. The spherical packing gives a more uniform bed, a slightly higher back-pressure, and has no tendency to void unless solvent etched. They are the packings of choice for new methods development.

Little was known about the process used to prepare these packings until one manufacturer gave a clue in a technical brochure. Molten silica was cooled at a controlled rate in a polymerizing organic matrix. The plastic formed was then sintered off, leaving the microporous spheres behind. Obviously, a delicate process is needed to control diameter, shape, and porosity during preparation.

Normal-phase silica packing requires only drying at a uniform temperature to be ready for packing. At 250°C, the fully hydrated silica is produced, while at 300°C water is lost between adjacent silica molecules forming the anhydride form normally packed in normal-phase columns.

The various bonded-phase column packings require a bit more processing. The first step, *silylation*, involves reacting fully hydroxylated silica with a chlorodimethylalkylsilane and heating to drive off HCl. Variations in the chain length and functional groups on the alkyl group produce the wide variety of bonded-phase columns. If we stop here, we would have a column that gives good separations for acidic and neutral compounds, but that gives very poor, tailing separations of amines and bases. Steric hindrance prevents complete bonding of all the free silanol sites; about 10% of the available sites are still free (Fig. 5.1).

The next step is a process called *end-capping*. This involves bonding of the remaining silanols with a smaller compound, chlorotrimethylsilane. After this treatment, free silanols are <1% and the column can be used for amine separations without peak broadening. The process by which these bonded groups are attached is reversible in the presence of water at either low or high pH. In the past, dichloro- and chloroalkylsilanes have been used for silylations producing cross-linked or polymeric coatings. Controlling the degree of silica hydration also controls the amount of coating that attaches.

Hybrid bonded-phase columns are being produced with carbon chains cross-linked chemically to the silica surface to reduce the amounts of free silanols and to increase the stability of the surface in the presence of high pH media. Hybrid silica column with a bridged organo-silica coating (Fig. 5.2)

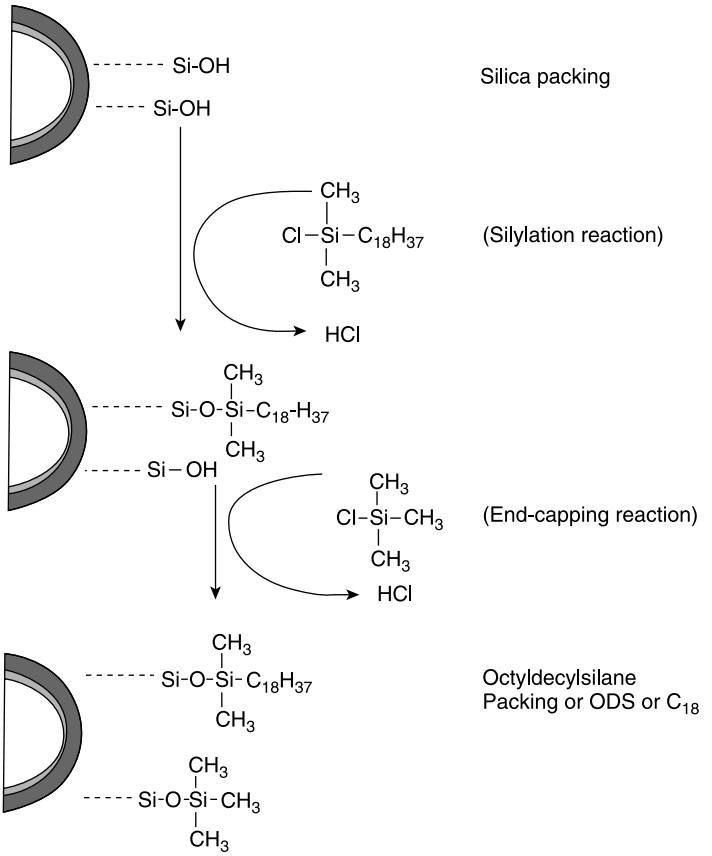

Figure 5.1 Bonded-phase preparation.

provide a surface coating that makes these columns much more resistant to dissolving at higher pH and able to retain their separation characteristic for a much longer time.

When you consider the different columns that can be produced by controlling hydration, bonding agent, coating levels, and end-capping, it is not hard to understand the variations in C_{18} columns coming from different manufacturers.

5.2 PACKING MATERIALS AND HARDWARE

Column packing is as much art as it is science. Even the professionals in the field cannot routinely prepare columns that will give the same plate count column to column. They quality control the columns with a set of standards, and columns that deviate by more than a set amount are either dumped and repacked (not cost effective) or are sold as specialty columns. Columns that exceed QC specifications are almost as bad as poor efficiency columns;

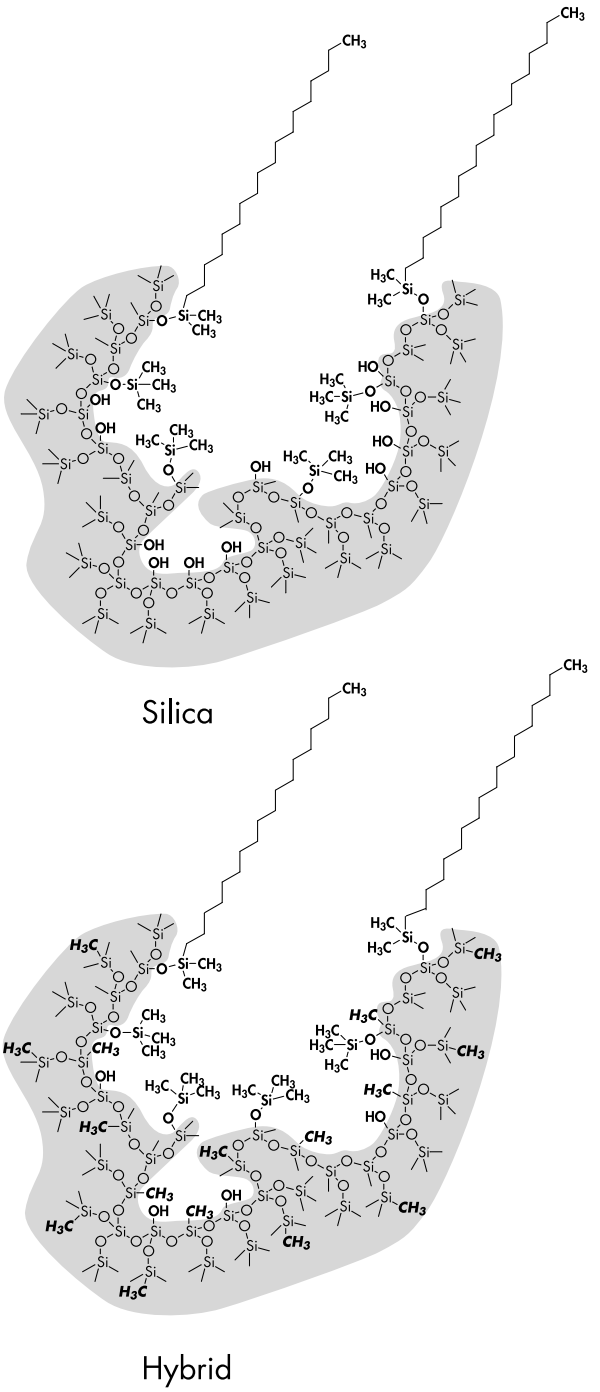

Figure 5.2 Silica and hybrid: bonded and end-capped. (© 2006 Waters Corporation. Used with permission.)

methods developed for "standard" columns will not work on these "super" columns. To use them, you have to repeat at least part of your methods development, which means lost time. Such columns are sold as special-purpose bonded-phase columns with a different set of QC specifications.

You may find that for teaching purposes that packing your own columns may be cost effective. An analytical column holds about 3 g of packing and the column itself can be dumped, cleaned, and reused. However, I would recommend not packing your own research columns. I will show you ways of extending the lifetimes of your columns enough that commercial columns could be cost effective.

Column packing is, in theory, very simple, but proves more difficult in practice. The packing material is slurried in a viscous solvent, then driven with a high-pressure pump into a column. Solvent passes out of the column through the end fitting's fritted filter, while solids build up and pack down on the frit. As the column fills with a packed bed, back-pressure increases until the column is filled. Once the column is filled, the slurry reservoir is removed, excess packing is scraped off even with the column mouth, and the inlet frit and end-cap are attached. Care must be taken to insure that no packing material is left in the threads of the end-cap. Silica is an excellent abrasive and will score the stainless steel when tightening the end-cap, leading to leaking when the column is used for HPLC.

A number of commercial column packing apparatuses are available. One type, the ascending type, is a stirred can that pumps slurry upward into the pressure line and then down into the column. The descending type of packer is simply a slurry reservoir that attaches in place of the inlet end-cap and frit and is equipped with a pump connection at the top (Fig. 5.3). Manufacturers use 20,000-psi pumps to drive slurry into the column, but most laboratory packing apparatuses rely on pumps that reach a maximum of only 6,000–10,000 psi. The pumps are run fully open until the pressure stabilizes.

Once packed, the column needs to be checked for efficiency using column standards. We've discussed storable column standards, but if you'll be using the column with amines, it might be a good idea to add fresh amine of known running characteristic to the mixture. Amine tailing is a very good check for voiding or end-capping problems, but amines air oxidize and are not stable for long storage.

5.3 COLUMN SELECTION

Selecting a column for an HPLC separation is a matter of asking yourself a series of questions (Fig. 5.4). You first must determine how much material you wish to separate in a single injection (preparative *vs.* semipreparative *vs.* analytical). The next question involves the separation mode to be employed (size exclusion *vs.* ion exchange *vs.* partition). Finally, there is the question of solubility controlling solvent and column selection in all modes.

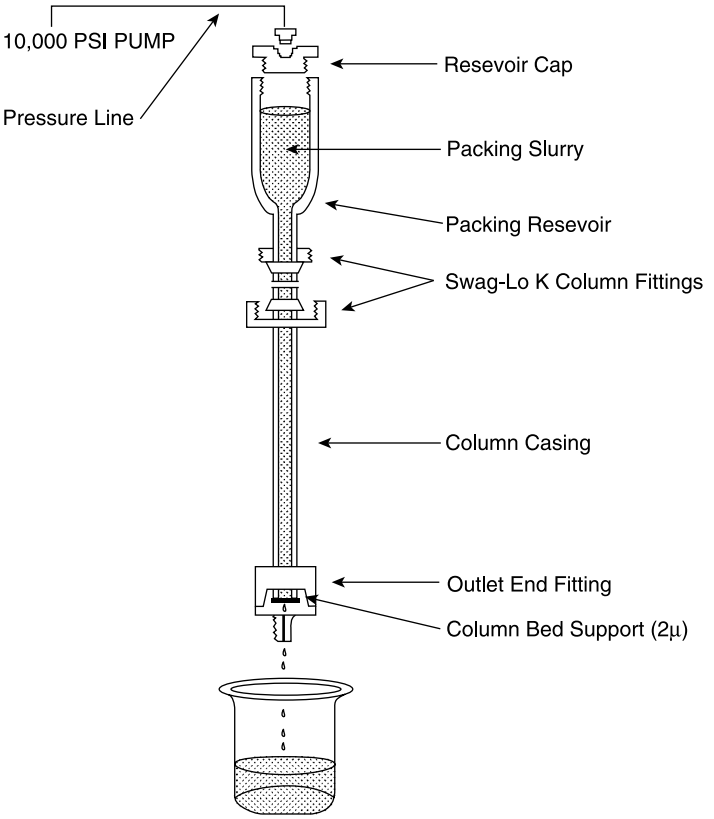

Figure 5.3 Column packing apparatus.

If your selection is size separation, do the molecules you are trying to separate vary by size or molecular weight? If the differences are in size, how large is the range of differences and how close in size is each pair of compounds that must be separated? Size columns are rated by inclusion/exclusion range and the separating molecules must fall in this range to be resolved. If you are separating strictly on molecular weight differences, you must take steps to unravel any molecular structure that will prevent true molecular weight comparison, such as the use of SDS to overcome protein folding. Generally, it is difficult to separate two compounds that differ by less than 10% in molecular weight.

Do they differ by charge or have a charge that can be influenced by adjacent substituents? It is very easy to separate a charged molecule from an uncharged molecule or a molecule of a differing charge on an ion exchange column. It is simply a matter of selecting a column that has a charge opposite the compound in question (anion *vs.* cation). With compounds that have the same type of charge, the separation is made based on the way the electron density of the charge is modified by steric difference and functional groups near the charge

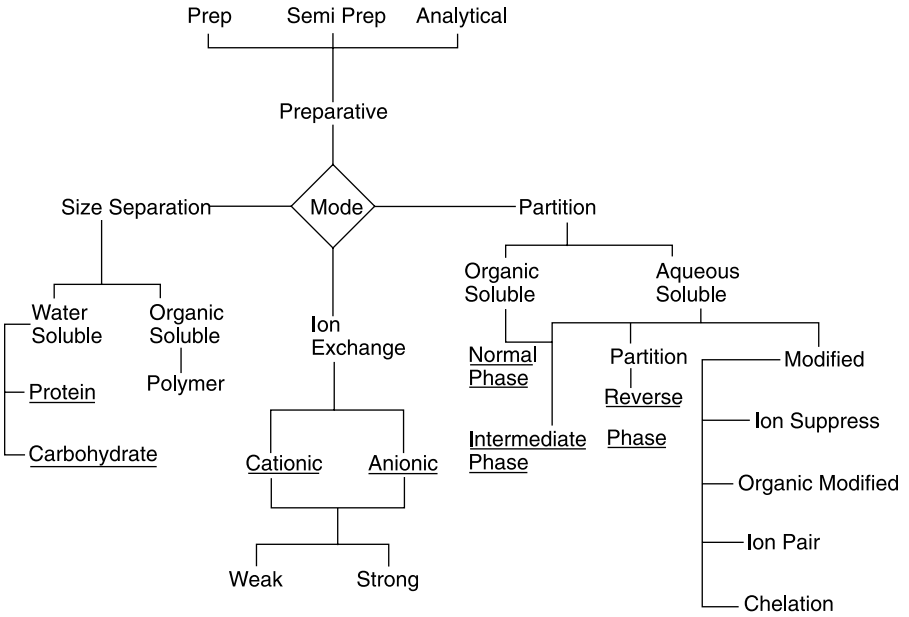

Figure 5.4 Column selection.

site. Often to get resolution, we need to control the column charge strength, the pH, or use eluting salt gradients to remove selectively the components of the mixture.

Are the primary differences in polarity? Partition columns are available that vary in polarity from nonpolar (octyldecyl), through intermediate polarity (octyl and cyanopropyl), to polar (silica). Some columns have similar polarities, but differ in their specificity. C_{18} and the "phenyl" column have similar polarities, but C_{18} separates on carbon chain length, while phenyl separates fatty acids on both carbon number and number of double bonds. Phenyl columns also resolve aromatic compounds from aliphatic compounds of similar carbon number. In another example of similar polarities, C_8 is a carbon number separator while cyanopropyl selects for functional groups.

Assuming we have selected the proper mode of chromatography, will the mixture dissolve in the mobile phase? Ion-exchange columns must be run in polar-charged solvents. Size separation columns are not, in theory, affected by solvent polarity, and size columns for use in both polar and nonpolar solvents are available. In partition chromatography, we have nonpolar columns that can be run in polar or aqueous solvents, and polar columns that are only run in anhydrous, nonpolar solvents. Intermediate columns such as cyanopropyl or diol can be run in either polar or nonpolar solvents, although often with differing specificity. An amino column (actually a propylamino) acts in methylene chloride/hexane like a less polar silica column but in acetonitrile/water

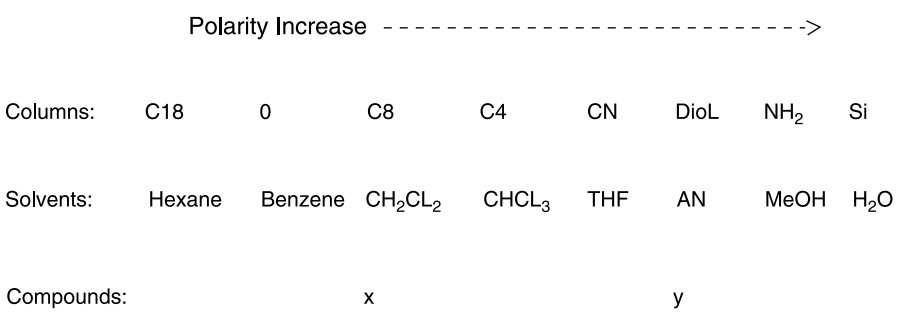

Figure 5.5 Polarity trends—columns and solvents.

can be used to separate carbohydrates and small polysaccharides by carbon numbers.

When I make a diagram of column polarities *versus* solvent polarities, I tend to think of the columns as being a continuous series of increasing polarity from C_{18} to silica: C_{18}, phenyl, C_8, cyano, C_3, diol, amino, and silica (Fig. 5.5). Under that, I have their solvents in opposite order of polarity from hexane under C_{18} to water under silica: hexane, benzene, methylene chloride, chloroform, THF, acetonitrile, i-PrOH, MeOH, and water. The cyano column and THF are about equivalent polarity. In setting up a separation system, we cross over; nonpolar columns require polar mobile phase and vice versa to achieve a polarity difference.

To make a separation, I look at the polarity of the compound I want (*X*) and its impurity (*Y*). Like attracts like. Let's assume that compound *X* is more nonpolar then its impurity *Y*. On a C_{18} column, the nonpolar compound sticks tightest to the nonpolar column and elutes last; the more polar impurity comes off first. Running the same separation on a silica column in a nonpolar solvent, we should expect a reversal. The polar impurity *Y* sticks to the polar column, while the nonpolar compound *X* washes out first in the nonpolar solvent. By thinking about the polarities involved in the separation, we can control the separation.

We are not limited to a single column type or chromatograph mode in our attempt to achieve a separation. We can use a technique called *sequential analysis* (Fig. 5.6). For example, we can make a size separation, then take a size fraction and do a partition separation. This is commonly used in separating a complex biological mixture where a single column would be overwhelmed. Separation on first a C_{18} and then a silica column is often used to confirm purity of a compound. If it passes separation as a single peak on two different types of columns, it's a fairly good bet that it's pure. Even better is to confirm identity, by using two differing separation modes, such as partition and ion exchange.

There is one more type of column you might want to select for your separation, the *protective* column. This column is designed to protect the column

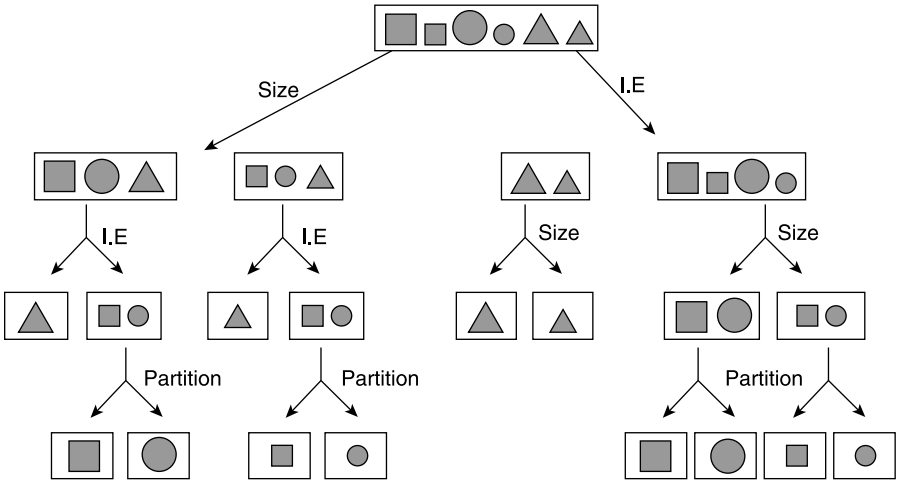

Figure 5.6 Sequential analysis.

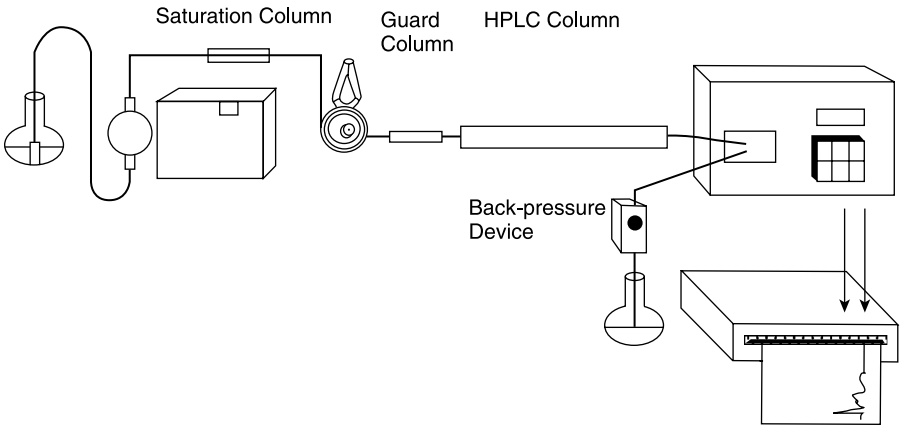

Figure 5.7 Protective columns.

bed of the analytical column, and, as such, it is a sacrificial column. There are two basic types of in-line protective columns: the guard column and the saturation column (Fig. 5.7). The disposable cartridge column or sample preparation column (SPE), used in sample preparation, also serve a role in column protection and will be covered in Chapter 12.

A guard columns is a mini analytical column pressure packed with the same type of material used in the analytical column. It is connected in the path from the injector and collects anything that normally would be deposited on the main column. It must be cleaned or replaced periodically because contamination will eventually bleed through. Since guard columns usually are only about

1–3 cm in length, they can be inverted and backwashed without causing them to void. *Please do not wash the guard column down the main column.* Disconnect and reverse it, reconnect and use the pump to pump a strong solvent through it into a beaker. This may seem obvious, but I had to troubleshoot a persistent detector problem that turned out to be caused by a chromatographer who washed a guard column into his main column.

Since the guard column is placed in the injector/column path, it does contribute to the separation. Methods development should be completed with the guard column in place. The increased separating length usually overcomes the effect of extra tubing as long as the connecting tube between the guard and analytical columns is kept as short and as fine as possible. The wrong diameter tubing can really mess up a separation. Changing guard columns in the middle of a series of runs generally has little effect on the separation. However, it is usually a good idea to follow the change with a standard QA run as a check.

The other type of protective, in-line column is the saturation column. This column is used when operating conditions tend to dissolve the main column bed (i.e., high pH, high temperature, etc.). In theory, the packing in the saturation column dissolves first and protects the main column packing. As long as the same bonded phase is used in the pre-column, the column running character does not seem to change. Using this technique, I had a customer who ran taurine separations at pH 12 for a year on the same C_{18} column. Care must be taken that the saturation column does not break through; erosion of the main column will begin immediately if this happens. A guard column will serve as a saturation column, but is not recommended, since the pre-column bed is consumed and band spreading will occur. Usually, the saturation column is placed in the flow from pump to injector. At this point, the column to be used can be slurry packed with no regard given to packing efficiency. I have even seen columns dry packed with tamping, wetted with solvent, and placed in line as a saturation column. I'm not entirely satisfied with the explanations as to why this technique works. I offer it to you as a tool that other chromatographers have used to produce separations at pH high enough to separate many amines in their free amine form. Silica appears as a solid on evaporating fractions and, occasionally, coats out on detector windows. I would recommend using this technique as an analytical tool only when other methods have failed.

6

COLUMN AGING, DIAGNOSIS, AND HEALING

HPLC columns have a reputation of being fragile things that only have a limited lifetime and, therefore, are expensive to buy and maintain. Much of this reputation is undeserved and in this section we will explore the aging of columns, the symptoms of aging, and methods of regenerating columns and extending their operating life. The typical new chromatographer gets about 3 months life from a column; an experienced operator gets about 9 months. I hope to help you extend column life to 1–2 years.

I know this is possible from a bonded-phase column because I had a customer who averaged this on his columns. He ran a clinical laboratory and rotated C_{18} columns through a series of four separations, each less demanding than the one before it. When the column failed on separation 1, it was washed and reequilibrated for a less demanding separation 2 and so on.

Over the years, I have collected hints, ideas, and tips that were not then available, allowing us to get the same performance from each column without rotation. The key to treating column problems is to know when problems are occurring, catch them as early as possible, and treat them. The main tool for early detection of problems is column QA with standards described and illustrated in Figure 6.1.

There are five basic types of "killers" of column efficiency: 1) effects that remove the bonded phase; 2) effects that dissolve the column surface, or the packing itself; 3) materials that bind to the column; 4) things that cause pressure increases; and 5) column channeling. There are definite symptoms of each of these and either treatments or preventions for each type of killer (Fig. 6.2).

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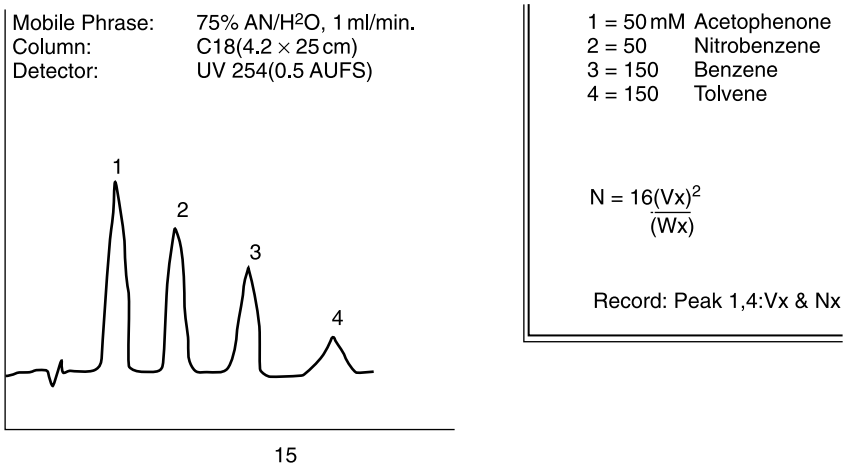

Figure 6.1 QA with column standards.

(1) Bonded Phase Loss
- a] low pH (2.0)
- b] high temperature (>ambient)

(2) End Voids—dissolved column packing
- a] high pH (8.0)
- b] high salt (>200 mM)

(3) Bound Compounds
- a] nonpolar organics
- b] inorganic cations
- c] ionic detergents/proteins

(4) Pressure Increases
- a] Column: inlet frit, outlet, bed
- b] System

(5) Center voids—channeling

Figure 6.2 Column killers.

The best way to follow column changes is by way of column standard plate counts. For discussion purposes, we will use the four-standard mixture of acetophenone, nitrobenzene, benzene, and toluene described in the discussion on efficiency factor (Chapter 4). Our column will be a C_{18} reverse-phase column run in 70% acetonitrile/water at 254 nm. In an initial run, we obtain four peaks whose interpeak α's double between each pair. After we discuss reverse phase, we will see how these killers affect normal phase columns.

6.1 PACKING DEGRADING—BONDED-PHASE LOSS

Column degradation can be caused by too low pH or too high temperature. Columns should be operated in a pH range of 2.5–7.5 at ambient temperature.

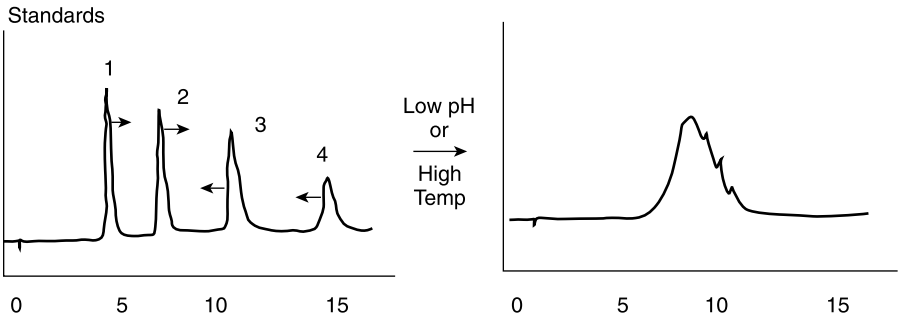

Figure 6.3 Effect of bonded-phase loss.

Below pH 2.0, bonded phase comes off and free silanols are formed, making the column more polar and increasing the cationic exchange character of the surface. Our four-standard separation tends to collapse on the center of the four peaks (Fig. 6.3). More polar peaks retain longer, less polar peaks come off faster, and all peaks broaden and tail. Finally, we end with a single, very broad, badly tailing peak. This problem cannot be healed, only prevented. Attempts have been made to pass a solution of chlorotrimethylsilane down a degraded column in an attempt to heal it, but very little success in restoring activity was achieved. Control pH with buffers so that it does not fall below 2.0. There has been limited success using saturation columns where pHs below 2.0 must be used, but window coating with bonded phase is a common problem.

Elevated temperature can produce two different effects. Basically, it increases the solubility of the silica packing and, thereby, accelerates end void production like high pH. At low pH, it also accelerates bonded-phase removal, rapidly producing the four-standard peak effect seen at low pH. It is hard to believe that one manufacturer actually recommends temperature programming as a tool for gradient chromatography using silica columns. It might work for silica columns in nonaqueous solvents, but I do not recommend it for bonded phase silica columns unless you're planning on buying a lot of columns. Zirconium columns on the other hand show neither pH- nor temperature-dependent bonded phase loss or loss of packing material. The bonded phase in these columns is usually chemically bonded to the zirconium surface using diazo compounds, and these columns can be used in column heaters to speed separation time.

One special problem already alluded to is the oxidation of bonded phase containing amino groups, such as the propylamino group or DEAE packings. These amines will oxidize, ruining the separation you are trying to make, and turn the column bed yellow, brown, and eventually black just like a bottle of amine solution sitting on a shelf exposed to light and air. Dumping out the packing shows it to be darkened all the way down the column, even though fresh column material was white when it was packed.

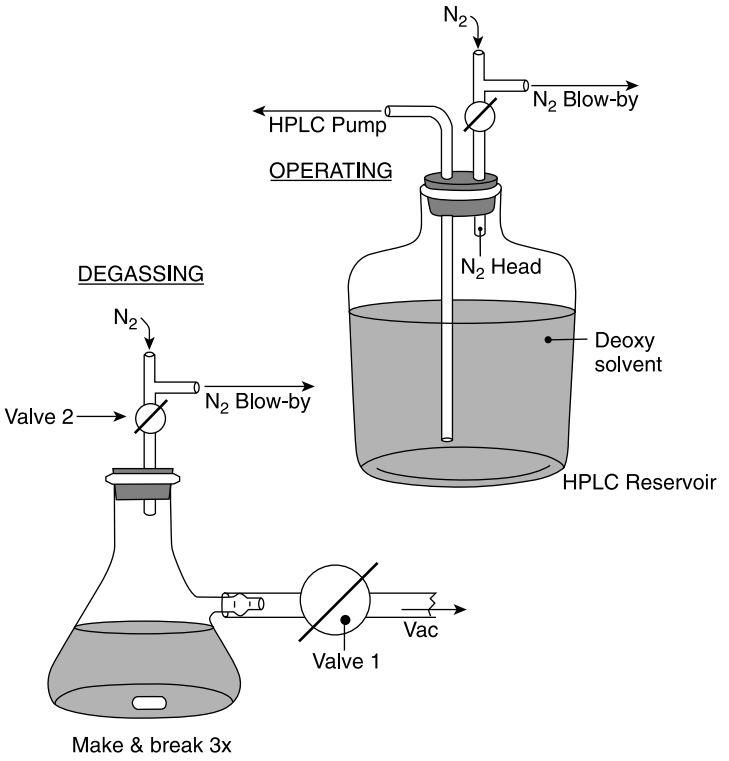

Figure 6.4 Solvent degassing apparatus.

I was able to solve this problem for a customer by giving him a new column and having him prepare and run only deoxygenated solvent as the mobile phase for his amino column. Solvent deoxygenation is done in the vacuum degassing apparatus shown in Figure 6.4. Solvent is vacuum degassed until large bubble formation stops, the vacuum valve is turned to the off position, the nitrogen blow-by turned on, and inlet valve 2 is slowly opened allowing the vacuum to be broken with nitrogen (Fig. 6.4a). Vacuum is pulled and broken in this fashion three times. Next, a nitrogen purge is placed in the solvent reservoir bottle and oxygen is displaced with nitrogen. Deoxygenated solvent from the first apparatus in Figure 6.4a is poured down the side of the purged reservoir bottle and the nitrogen blow-by top is fitted to the top, as in Figure 6.4b. The pump line is connected to the HPLC pump inlet, the nitrogen blow-by turned on, the demand valve 2 is turned on and the pump started, the system is purged up to the column, and the amino column is installed and equilibrated. At the end of the run, the pump flow and the demand valve 2 are turned off at the same time until they are needed for the next run. When not in use, the amino column is stored in deoxygenated solvent. The customer in

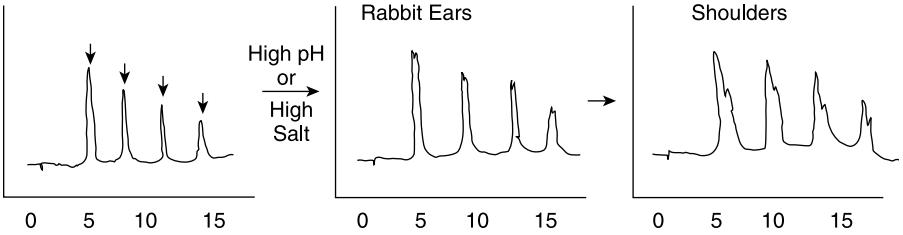

Figure 6.5 Effect of dissolving packing-end void.

question got 14 months on this column and the column bed was dirty, but not oxidized, when I unpacked it to check it.

6.2 DISSOLVED PACKING MATERIAL—END VOIDS

At high pH, above 8.0, silica begins to dissolve, forming an end void rapidly, even if protected with a bonded phase. To be safe, it is best to keep pH below 7.5, unless the column is protected with a saturation column. The four-standard separation shows a progression of "rabbit ear" fine peak splitting, to a shoulder, to peak broadening on all four peaks (Fig. 6.5). If the column end cap is opened and the frit removed at these three stages, increasing amount of pitting and bed settling can be seen at the top of the column. At the rabbit ears stage, a fine pit directly in the bed center can be seen. By the shoulder stage, the pit has spread and the bed dips down on one side. By the time the shoulder disappears, enough of the bed has eroded so that a millimeter or so of packing is missing across the whole surface. Even through the peaks change appearance on pitting, the k' remains unchanged for the peaks. This allows us to distinguish between end voiding and organic contamination, which we will discuss later.

These end voids can be repaired; fresh packing material can be worked into a paste with mobile phase and pushed into the moistened pit with the flat of a spatula. Overfill the column head, strike it off with a card, replace the end frit, and retighten the end cap. *Be sure not to leave silica in the threads.* Wet the threads with MeOH, use a Moore pipette to dry, and then blow the threads clean. Reequilibrate the column with solvent and rerun the standards. If the pit is very deep, it may be necessary to repeat the repacking and pumping. Eventually, all peaks should be needle sharp again. Packing material is available from some manufacturers in small quantities. A gram should top up a lot of columns. Try and use the same size and type of material used originally in the column. If you can't get 3-μm packing material, use 5-μm packing from the same manufacturer. (The outlet end of "used" columns, discarded by chromatographers who don't know how to repair them, is an excellent source of clean packing.) If all else fails, pack them with glass beads of the same diameter.

Salt solutions with concentrations above 200 mM tend to erode column beds by increasing the ionization and, therefore, the solubility of the silica. The effect is similar to high pH end voiding and can be treated in the same way. One customer, who ran high salt gradient ion-exchange columns, solved his severe end-voiding problem by amputation. He opened the end-cap, cut the column below the end void with a tubing cutter, put a new column ferrule on with a crimper, and replaced the endcap. The new column was shorter and had less resolving power, but still worked. He continued cutting the column, until it reached 3 cm, then used it for a guard column. I don't recall using a saturation column to prevent salt erosion, but it should work. This effect occurs with nonhalide salts as well as halides, the extractor seems to be salt positive ion.

6.3 BOUND MATERIAL

The third type of column killer is material stuck to or coated on the surface of the packing that changes the column's running characteristics. The binding materials fall into three broad classes: organics, inorganic cations, and charged organics.

Uncharged, nonpolar organics sticking to the column tend to specifically affect the later running peaks in a separation. In the four-standard mixture run, it is the benzene and toluene peaks that broaden, shorten, and disappear (Fig. 6.6).

Contaminated water is a notorious source of this problem, and is the usual place to look for the culprit. One of the quirks of human nature is that people refuse to admit that their water could possibly be contaminated. I have seen triple-distilled water, which worked fine for enzyme reactions, fail miserably for HPLC. I once spent 9 months convincing a friend and customer that his PTH amino acid gradient separation was losing its last two peaks because of bad water. After washing the column and switching to HPLC-grade water, the problem disappeared, never to return. Unextracted injection samples are the second source of organic contamination. If you find your baseline rising and falling when you are just pumping mobile phase through your column, there is probably nothing wrong with either your detector or the pump. When a base-

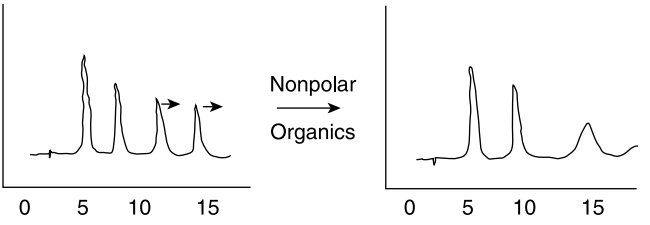

Figure 6.6 Effects of bound nonpolar material.

line goes up and down, it almost always means that a peak has just come off the column, no matter how broad the peak or how close the peak maximum is to the original baseline. Garbage on the column eventually washes off. As it starts to come off, the baseline goes up. When it finally finishes, the baseline goes down.

I have had many people threaten to return whole HPLC systems because of "bad pumps" or "bad detectors," who were simply suffering from dirty columns. It's always the detector first and then the pump that gets blamed. And, the poor service people who are hardware oriented, as they usually are, will make multiple trips without finding the problem. I encourage service people to carry a C_{18} column, used only with standards, and a vial containing four-standard mix in their service bag. The first thing they should do is remove the customer's column and run the four standards on their column. It's very embarrassing when the "detector" or "pump" problems go away, but it saves the company or the customer a lot of money.

The problem in this case is usually nonpolar organics (the polar organics do not stick to nonpolar columns, but wash through the column leading to a peak or an elevated baseline). Washing the column will remove nonpolar organics; the only question is how strong a solvent we need to elute our particular contaminant. *If we are running a buffered mobile phase, we first must wash out the buffer.* I usually keep a bottle of the same mobile phase minus the buffer on the shelf for wash out at day end before shutdown. Once we're in aqueous organic solvent, I switch to acetonitrile and wash the column for at least six column volumes (about 20 mL for a 25-cm analytical column). Watch the UV monitor for eluting peaks and a return to baseline. Reequilibrate with 70% methanol/water and run the four-standards mix. If it looks good, go back through the intermediate solvent to the buffered mobile phase, equilibrate, and return to your separation.

Be sure not to jump from buffer to pure organic or from organic to buffer. This can lead to buffer precipitation, plugging, and pressure problems. Always use a wash out, intermediate solvent or wash out with water. Allow six column volumes for reequilibration; true equilibration takes as much as 24 hours, but this six-volume equilibration is reproducible and sufficient.

If the late-running peaks still run late or are spread, further washing is necessary. Directly from the four-standard mobile phase, I wash with 20% dimethyl sulfoxide in methanol. You may have to drop the flow rate initially to keep pressure below 4,000 psi because of the mixture's high viscosity. The UV monitor will be of no use for monitoring peaks and a return to baseline since DMSO has very high absorption. After six column volumes, wash with standards solvent and reequilibrate and run the four-standard mix.

The last resort is to wash all the way to hexane and back. Since aqueous solutions and hexane are immiscible, it is necessary to go through a bridging solvent(s). This means washing with one or more solvents miscible with both water and hexane. Common bridges are acetonitrile and chloroform, tetrahydrofuran (THF), and isopropanol (*i*-PrOH). THF is probably the easiest and

best bridge; its low viscosity allows rapid pumping. However, many people fear peroxide formation in THF and prefer to wash first with acetonitrile, then with chloroform, and finally with hexane, then reverse the process. Since each solvent must wash out the previous solvent completely, this is a very time-consuming wash. The isopropanol wash is also time consuming because of this solvent's high viscosity in water mixtures and it must be thorough because *i*-PrOH does not bridge as well as the other solvents. In any of theses cases, you wash with each bridging solvent in turn (six column volumes) until you reach hexane. You then reverse the process, returning to the mobile phase for your column standards. The last step is to run the four-standard mix, then return and reequilibrate for the next sample.

It's better to pick a time convenient for you than to have to do this process on an emergency basis in the middle of a critical separation. I would have a tested column ready as a replacement. Replace the dirty column after washing out the buffer, cap it, and, then, wash the old column off-line when you have more time. You never seem to wash everything off the column. After you've used a column for a while, you often will find a brown or black residue at the column head under the column frit on opening even a freshly washed column. Don't worry about it if the column standards run correctly.

Washing with a bridging solvent seems to correct about 80% of column problems, but it can't cure the "disappearing peak" phenomena. In this case, the majority of the peaks in your analytical separation remain unchanged, but a critical peak, usually in the middle of other peaks, will change retention time. Over a period of weeks or months it will merge with another peak, until it cannot be separated. Washing with solvent does not cure the problem. The change appears to be an "α" change that points to a change in the chemistry of the system. After much work in the applications laboratory, the problem has been tracked down to metal cation chelation. Speculation is that magnesium and calcium ions from water storage bottles bind to free silanol sites on the packing, which changes its running character.

As we mentioned above, even end-capped packings have some free silanols, either left over from incomplete binding or by hydrolysis of the bonded phase. These give a reverse-phase separation a mixed mode nature. Most of the separation is due to the nonpolar partitioning bonded phase, but some of it comes from these ionizable, polar silanols. Metal cations form a pair bond couple and lock the silanol into the ionized form; the partition separation changes.

This answer suggests the treatment. Compounds that chelate metal ions should restore activity. EDTA proved unsuccessful because of steric factors, but oxalate succeeds in about 90% of the cases. The wash solvent is made by adjusting the pH of 100 mM oxalic acid to pH 4.0 with 1 N sodium hydroxide. The column is washed with six column volumes of this oxalate solution, then with water until the effluent pH reaches the neutrality of your lab water. Do not over wash with this solution. Oxalate will attack the stainless steel tubing, extracting iron if you were to wash longer. I know this from experience; a student in one of my classes did not listen when I told him to use only six

column volumes. He washed a column with oxalate overnight and had a reddish-brown waste container solution when he returned in the morning.

The last type of bound material is charged organic cations. They are usually of two sources: proteins and ion pairing reagents. They generally cannot be completely removed once they are on the column. The best treatments are either preventing them from reaching the column or dedicating a column to their use. If you must try and wash either type off the column, try using 70% acetonitrile containing 0.1% trifloroacetic acid. Silanol has a pK_A around 1.8 and must be in the free acid form to release the cations. This solvent is used to solubilize peptides and small proteins and might work. But realize, you are walking a tightrope between removing the cation and the bonded phase.

Proteins are best removed from sample before injection, and various techniques will be described in the sample preparation section for doing so (Chapter 12). If you must shoot crude sample-containing protein, use a guard column and change it often. A new guard column might be less expensive than your time needed to clean it and, certainly, will be less expensive than a new column.

Ion pair reagents are used in separating charged compounds. They are charged molecules themselves and used in fairly high concentration. Restoring a C_{18} column to initial conditions after using ion-pairing reagents takes days of washing. These columns are usually dedicated to ion pairing runs. After use they are washed with solvent to insure that the column's end-frits are free of solid, the column is capped and stored until the next use.

6.4 PRESSURE INCREASES

The next column killer class is pressure increases. Most columns and packings can tolerate pressure of 12,000 psi and higher. Most new columns do not exceed 2,500 psi when running the four-standard mix. If pressure rises to 4,000 psi, you have a problem that should be dealt with. I'm talking about a change that takes place gradually or all at once and remains high. Be aware that gradient mixtures of some solvents like methanol and water go through a pressure maximum that will approach 4,000 psi at a 1.5 mL/min flow rate. This change will reverse if the gradient conditions are changed. Column pressure problems will not reverse until the material plugging the column is removed.

The first step is to locate the point of the pressure increase. Since most problems are column problems, we can simplify our task by "eating the elephant one bite at a time." Remove the column from the system and turn on the pump. If the pressure problem goes away, it was in the column. If not, it's in the system leading up to the column. I'll deal here only with the column pressure problems, the system problems will be dealt with in Chapter 10 on troubleshooting.

There are three areas in a column where pressure increase can occur: the inlet frit, the outlet frit, and the column bed. The most likely source of

problems is the inlet frit. It is the only filter between injected samples and the column bed and is designed to collect anything bigger than 2 μm in size. When it does so, pressure increases. The more garbage it collects, the higher the pressure. You are left with the oil filter alternative: "Pay me now or pay me later!" You can filter or spin particulates out of the sample or be prepared to remove, replace, or clean the filter. Inlet filters can also be plugged by buffer precipitation caused by suddenly going from buffer to organic or vice versa. In either case, if the frit plugs it can usually be fixed. Step one is to replace the frit by opening the end-cap, removing the old frit, and putting a new one on the column top. *Did you remember to get replacement frits for your column?* Sometimes, usually late at night, you will find you do not have a new frit available. Instead, you have friends who just borrowed your last frit because they forgot to get them when they ordered a new column.

In either case, if the frit is plugged it can usually be fixed. Open the end-cap and carefully remove the frit with the column in an upright position. (If you point it the wrong way you end up with white powder on your shoes.) Put the frit in a covered flask with 20% nitric acid (6 N) and sonicate it for 1–2 min. Carefully discard the acid, add distilled water, and resonicate. Keep washing with water until the water's pH reaches lab neutral. Replace the frit, blow the end-cap thread clean with a pipette to remove silica particles that can score column treads, and retighten the endcap.

If you reconnect the column and start the pump and the pressure persists, then you need to remove the outlet end frit in the same way. (Remember the white packing on the shoes?) Outlet pressure is due to fines in the column collecting in this filter; usually only a problem if you are using the original irregular-shaped microporous columns. Sonicating with 10% sodium hydroxide can clean them since they are silica. Wash the base out repeatedly with water, replace the frit, and run the column.

If increased pressure still continues, then the column bed is plugged. If you can get any flow through the column, you may be able to wash out the problem. You must analyze the source of the problem. Bed plugs come from two things: precipitated buffer and precipitated sample. The latter only usually happens if you are doing preparative work with nearly saturated solutions. A column is a concentrator. It can supersaturate the solution at the head of the column, causing material to precipitate. If flow can be maintained, it can be washed out with the stronger solvent of your mobile phase. Precipitated buffer occurs when you switch rapidly from buffer to an incompatible solvent. The crystalline buffer can end up in the frit or in the bed. In either case it can often be cleared with long washing with water.

If flow is completely blocked, you will have to open the column, remove the frit, and bore out the plug with a flat ended spatula. Be very careful, the plug is generally only 1–2 mm deep. The packing can be washed with solvent or water, drained, and pasted back into the column like you are healing an end-void.

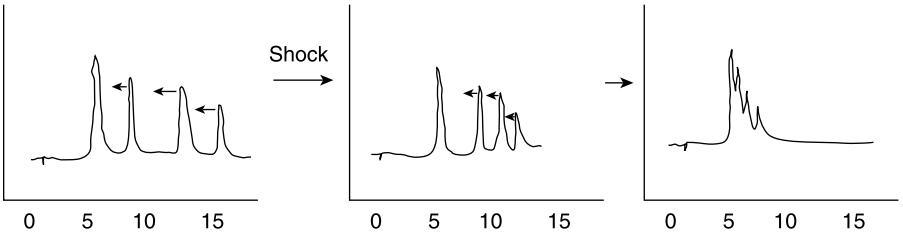

Figure 6.7 Effect of channeling—center void.

6.5 COLUMN CHANNELING—CENTER-VOIDS

The final type of column killer is the center void. When I first enter the field, this was always thought to be fatal, especially using the irregular 10-μm columns. The symptoms are that of the collapsing chromatogram. Everything can be proceeding normally when, suddenly, you notice that the retention times are becoming less for your late running peaks (Fig. 6.7). When you repeat the separation, the problem becomes worse. As it proceeds, even the early running peaks become involved. Finally, everything is coming off in the void volume. If you call the company, they will tell you that the column has been voided and needs to be replaced. If it is a brand new column that was shipped to you in this condition, they will probably offer to replace it. If not, they might tell you that it would make great column packing for end-voided column repair. They will give you Standard Lecture #1 on protecting columns: "Don't jar the column, shock it through pressure changes or by jumping to immiscible solvents, do not reverse the column flow, or jump flow rates suddenly." All of these can cause voiding in perfectly good columns. In other words, tough luck. I have given this lecture many times myself, but no longer.

I'm here to give hope to the masses. Voids can be healed! Well, not all of them, but many. The healing technique was discovered by accident, an example of serendipity at work. A novice chromatographer trying to get a job running an HPLC was told to run a standard mixture on a new C_{18} column and return to the interview with a chromatogram of the separation. He proudly returned with a single peak, only to be told there were four compounds in the mixture. The interviewer told him the column was probably voided and needed to be returned to the manufacturer and asked the prospect to remove it and pack it up for return. He also warned not to do anything to invalidate the warrantee and gave Standard Lecture #1. The person being interviewed managed to drop the column on the floor as he was removing it, in a panic he hooked it up backwards and decided that he might as well get some experience. Next, he accidentally turned the flow to 10 mL/min instead of 1 mL/min. After he got the flow under control, he shot a four-standard mixture only to find that it came off as four peaks instead of the expected single peak of a voided column.

He had healed the center void. The interviewer recognized what had happened and passed the word in the company. The trick was tried on other voided columns and proved successful in 13 of 14 tried. When I first heard of the technique used to heal columns I was skeptical. I went to my demonstration system, ran my four-standard mix, and got four peaks. I intentionally voided the column by running at high pressure, then suddenly dropping the pressure, and rerunning my standards. The third time I did this I got a single peak, center-voided chromatogram. Following the healing protocol, I removed the column and banged it on the counter, I hooked it back up, ran at high flow rates, then ran my standards, and got four well-resolved peaks.

The present method recommended by this major industrial account is to disconnect the center-voided column, grasp it in one hand, and rap the counter with it twice, reverse the column, and do the same with the other end. *Obviously, not hard enough to bend the column!* Hook it up backwards and run it at high flow rate for a minute or two. Then run the four-standard mixture. The column is run reversed from then on. It is possible that an end void may be formed yielding rabbit-eared split peaks, and must be repacked, but the column bed should be restored.

A center void probably occurs because small wall and bed voids link up under pressure changes and shock to form a channel. The channel is a path of least resistance and diverts the flow from the bed. This effectively removes this part of the column from the separation and gives a shorter column and shorter retention times. Eventually, the whole column is channeled and you have a center void the length of the column. If you have ever run a gravity-fed, packed glass column, you have probably seen this channeling phenomena if you accidentally let the column run dry.

This crazy sounding repair technique probably works because a column is more densely packed at the outlet than at the inlet end. This represents a packing reservoir that can be loosened and washed into the center void. I would not recommend using it on anything but a hopelessly center-voided column. By the same token, I would not recommend reversing the flow of a column needlessly. Occasionally, reversal has caused columns to void. If you have already used up the "packing reservoir" by reversing the column it may not be available to fix a void if it does occur. This is what happened in the unrepairable 14th column mentioned earlier. It had already been reversed before the void opened and the repair technique failed on the second reversal. Why waste a resource?

6.6 NORMAL PHASE, ION EXCHANGE, AND SIZE COLUMNS

Most of the mentioned troubleshooting tools will work with other silica-based columns. With normal-phase columns, you obviously need not worry about bonded-phase removal, but silica still dissolved at high pH and high salt concentrations. Polar materials like some proteins adhere very tightly and require

high acid concentration and low pH to be washed off. My customers were able to run normal-phase separation of phospholipids using a mixture of MeOH, acetonitrile, and sulfuric acid, conditions that would be totally unacceptable on a bonded-phase column. Particulates in normal-phase column can result in pressure problems and are removed from the frit after it has been removed from the column in the same manner as with bonded-phase columns. Center and end voids can be repaired in the same fashion.

Ion-exchange columns on silica show exactly the same problems as other bonded-phase columns: bonded-phase loss, column packing loss, bound organics, pressure problems, and end and center voids. In addition, they exhibit binding problems specific to their function. Strong ion exchangers can bind almost irreversibly to strong ions of the opposite charge (i.e., quaternary amine columns with sulfonic acids such as taurine). To break this electrostatic attraction it is necessary to either neutralize one or the other of the ions or displace the bound ion with very high concentrations of a counter ion, such as salt. Neither the quaternary amine nor the sulfonic acid can be neutralized in our example without wrecking the bonded phase or dissolving the silica. Salt (1–2 M) dissolves the packing material and attacks the stainless steel in both the column and the pump heads when used as a wash over time, but it is the only solution to this problem. A better answer is not to use strong ion exchangers: weak ion exchangers, for instance, a DEAE column that used secondary and tertiary amines, can be cleaned by using high pH washes and a saturation column. Remember that when you are using amine columns you must use deoxygenated solvent or face amine oxidation. In recent years, a number of ion exchangers on very rigid polymer supports and zirconium have emerged. They can tolerate reasonably high pressures and pHs from 2 to 13, and even briefly 1 to 14. They are ideal solutions to the problems seen with silica-based columns and should displace silica in ion exchange in the near future.

Size-separation columns on silica show all of the bonded-phase problems and can be treated in much the same fashion. They also show problems specifically related to their operation. Pore size is critical to their function. Anything that blocks pores changes their operation. Adhering materials, such as nonpolar contaminants, proteins, and detergents, can have major effects on exclusion/inclusion ratios. Pressure fragility is very common, especially on the larger pore size columns used for large molecular weight exclusion/inclusion separations. The TSK6000sw column appears to be packed with particles that resemble very fragile Christmas tree ornaments and should be handled accordingly. We had a major outbreak of crushed TSK3000sw columns a few years ago. In one case, a customer called me and said the first time he ran the column the pressure shot up to 4,000 psi and stopped the pump. I exchanged columns with him, pulled the end-cap on his old column, and tried to feel the column head with a glass rod. There was almost nothing in the column: I almost lost the glass rod. The column had been crushed and packed into disk at the outlet end of the column. A TSK3000sw column is usually not that fragile! I inquired around the country and we found 12 columns from the same lot that

had had the same problem. The manufacturer finally admitted they had shipped columns across the Northern pacific in the dead of winter in 10% MeOH. The mobile phase had frozen, expanded, and crushed the column packing. They began shipping in 50% MeOH and, to my knowledge, the problem never happened again.

6.7 ZIRCONIUM AND POLYMER COLUMNS

The main advantage of the zirconium family of columns is their stability from pH 1 to 10 and at temperatures from ambient to 200°C. Their separating character also differs from silica-based columns due to the lack of ionizable surface molecules. Silica above pH 3.0 loses a proton to form anionic silicate moieties, giving the bonded-phase silica column some anionic as well as nonpolar organic column characteristics (Fig. 6.8a).

Zirconium columns come in a variety of particle sizes and with nonpolar organic and ion exchange coatings. The nonpolar columns require 20–30% less organic solvent for eluting equivalent nonpolar compounds than required on C_{18} silica-based column. Like silica-based columns, the bonded-phase columns accumulate nonpolar organics that will change their running characteristics unless removed periodically by washing with strong solvent.

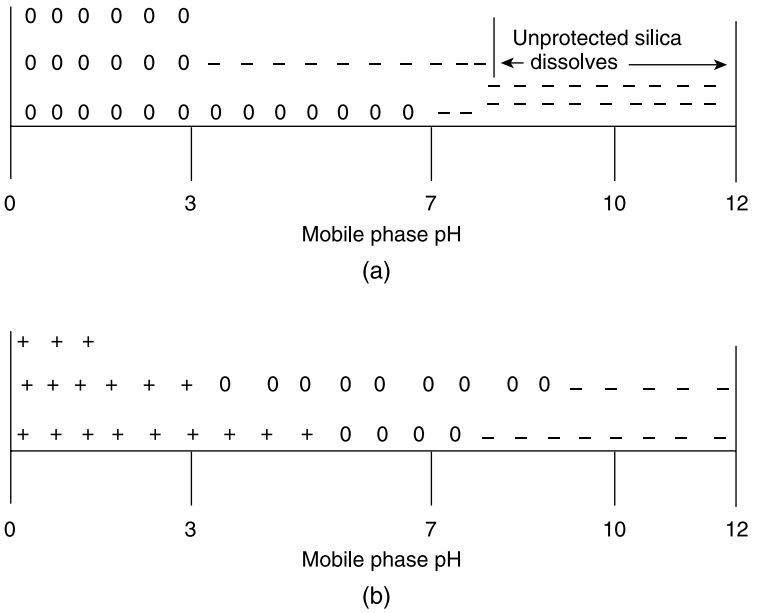

Figure 6.8 Silica (a) and zirconium (b) column charge states.

Zirconium columns combine cation exchange (Brønstead acid) effects at low pH along with their nonpolar retention character, like silica columns. At high pH, zirconium add anion exchange (Brønstead base) and act as strong metal chelators (Lewis Base) with an affinity for the free electrons pairs on compounds such as amines. These Brønstead acids and bases ionize at both high and low pH (Fig. 6.8b), unlike silica that ionizes at low pH, but dissolves at pH > 8.0. Since zirconium also acts as a chelator for Lewis acids, columns recommended for LC/MS generally come with a covalently attached chelating agent, such as ethylene tetramethlenephosphonic acid, EDTPA, to tie up the Lewis Base sites on the zirconium surface. This bound chelator allows separation similar to silica at acid pH, but also allows amines to be run at high pH. Chelators in the mobile phase that might interfere with the blocking EDTPA molecule should be avoided.

Polymeric columns are a very mixed bag of physical structures and bonded phases. Traditional polymeric columns used for size and carbohydrate separations by HPLC are physically fragile supports that crush easily and must be run under carefully controlled pressures. Columns were sometimes destroyed by simply starting up with cold solvent if the operator had failed to set pressure protection. Warming the solvent reservoir in a warm water bath was sufficient to reduce viscosity and allow column operation. Modern polymeric columns are much heavier cross-linked materials and resist moderate pressure, but recommended pressure settings still need to be observed. Nonpolar polymeric column are usually easier to wash than silica because they do not have the secondary ion exchange character. Acetonitrile or tetrahydrofuran are sufficiently nonpolar to remove most contaminates and both have lower viscosity than water mixtures with methanol. Heating the solvent reservoir can shorten washing times by reducing both viscosity and increasing mass transfer out of the column pores. Be careful not to exceed solvent boiling points or the column may become vapor locked or voided. Strong ion exchangers on polymeric supports can be treated with high salt concentrations, at low or high pH, and at high temperatures without attacking the polymeric surface. But, be aware that high salt concentrations will still attack the iron in the stainless steel column casing, so keep wash volumes to six column volumes and follow with a water wash to neutralize.

7

PARTITION CHROMATOGRAPHY MODIFICATIONS

7.1 REVERSE-PHASE AND HYBRID SILICA

So far, we have looked at reversed-phase separation using simple solvent mixtures. Many times when we carry out separation of charged or ionizable compounds we run into two problems: tailing or poor retention.

Chromatographic peaks are asymmetric and tend to broaden or tail off on the side away from the injection point. As a result, peaks tend to contaminate longer retaining neighbors. Extreme tailing, which is always due to some type of poorly resolved equilibration within the column, must be dealt with before separations can be achieved. One of the most common causes of tailing is partial ionization, either of the column bed or of the sample in the mobile phase. For instance, at the pK_a of an acid, the carboxylate form and the free acid form are present in equal concentrations. If you buffer the mobile phase at this pK_a and try to separate this acid from other compounds, the result will be a badly tailing peak as the column tries to separate the two equilibration forms of the acid.

Another problem is compounds that are too soluble to retain on the column and elute unresolved in the void volume. Modifications of either the sample ionization or of the surface nature can increase retention and, therefore, resolution. In this section, we will study modifications of the column or mobile phase that will allow us to improve our separations.

HPLC: A Practical User's Guide, Second Edition, by Marvin C. McMaster

7.1.1 Ionization Suppression

Buffers are used in HPLC to control the ionization of one or more molecules in solution so that they will separate as sharp bands. The key to understanding ionization is to understand pH and pK_a.

The pH of a solution is simply a measure of the hydrogen ion concentration and represents the degree of harshness on either the acid or basic side. A pH of 7 is neutral and represents the mildest conditions. As we go toward lower pH, the hydrogen ion concentration increases and the solution becomes more harshly acidic. Starting at 7 and going toward higher pH, the hydrogen ion concentration decreases and the solution becomes a harsher base.

pK_a for each ionizable function on a molecule is the pH at which equal concentrations of the ionized and free form of the compound exist. Organic acids have pK_a around pH 4.5, amines have pK_a between pH 9.0 and 10.5. Below 2.5, organic acids exist mainly in the protonated, free acid form. Above 6.5, the proton is removed and, mostly, the carboxylate form is present. As a rule of thumb, try and buffer 2 pH units above or below the pK_a of the compound being separated. The worst tailing seems to occur directly at the pK_a. Also, be aware that some compounds, such as phosphate ion, have more than one ionization state and show more than one pK_a.

Since buffers control pH best at their pK_a, pick one close to your desired pH. The most common buffer used in HPLC is phosphate. It has two usable pK_a's, 2.1 and 7.1, and is UV transparent. A 100-mM solution of phosphate precipitates in solution of >50% MeOH or 70% acetonitrile. Other buffers in common use are acetate, pK_a 4.8, formate, pK_a 3.8, and chloroacetate, pK_a 2.9; all absorb in the UV below 225 nM. Sulfonate, pK_a 1.8 and 6.9, should be substituted for phosphate when analyzing mixtures of organic phosphates.

The other factor to consider is the effect of ionization on solubility. Ionized forms are more soluble in aqueous solvents. If you need to increase a compound's retention on a reverse phase column, force it into its unionized form. For small organic acids, its best to run your separation at pH 2.5 with phosphate buffers or use 100 mM acetic acid that gives you a pH of 2.9. For large acids containing massive nonpolar substituents, it might be better to operate at pH 6.5 and take advantage of the decreased retention time for faster chromatography.

Amines pose an interesting problem for ionization control because their pK_a are so high that they are usually ionized at any pH tolerated by the silica column bed. This makes them very soluble and hard to resolve on a reverse-phase column. It is possible to force them into the free amine form by using mobile phases at pH 12, but be sure to use a saturation column and change it often.

Using the HPLC system with a mass spectrometer as a detector forces the use of volatile buffers to avoid contamination of the analyzer. The buffers are still needed in many cases to control sample or column ionization to improve the chromatography, but must be removed in some way before they reach the detector flow cell. A table of volatile buffers and their pK_a's is listed in Appendix C.

7.1.2 Ion Pairing

Amines have traditionally been separated using ion pairing reagents. These are counter-charged organic molecules, such as hexane sulfonate, that are added in excess (typically 30–100 mM) to the mobile phase. One theory says that they form an "ion pair" with the amine in solution that becomes one long nonpolar pseudomolecule with a masked charge couple in the center. The pseudomolecule then partitions with the bonded phase as if the charges did not exist. Instead of eluting at the void volume like the ionized amine, the pseudomolecule is retained longer even than the free amine. An alternate theory of ion pair action says that the ion pair reagent first interacts with the bonded phase, forming a nonbonded ion-exchange column. This modified bonded phase column then interacts with the compounds in solution through a mixed partition/ion-exchange mode.

The longer the nonpolar chain of the ion pairing reagent, the longer this retention peak takes to come off (Fig. 7.1). This allows us to position the retention time of an amine in a separation by controlling chain length of the ion pair. A very interesting observation is that a 1:1 mixture of hexane sulfonate and octane sulfonate gives a single amine peak retaining half way between the peaks formed when either sulfonate is used along. This 1:1 mixture has the same retention as the same amine with heptane sulfonate. Neither of the two theories of ion-pairing interactions explains why this mixed ion-pairing reagent does not form as a pair of peaks or a badly tailing peak for each compound.

Ion-pairing reactions can also be carried out using quaternary amines as counter-charges for organic acid and organic phosphate samples. Generally, pH control is the preferred technique for acids, but, occasionally, ion pairs give better position or solubility control. Ion-pairing reagents are very difficult to wash out of a bonded-phase column and columns are usually dedicated for a particular ion-pairing reagent operation.

If ion-pairing reagents are used in gradient runs, they must be added in equal amounts to each solvent to prevent baseline drift during the run. The pairing reagent should ideally be transparent at the wavelength being used.

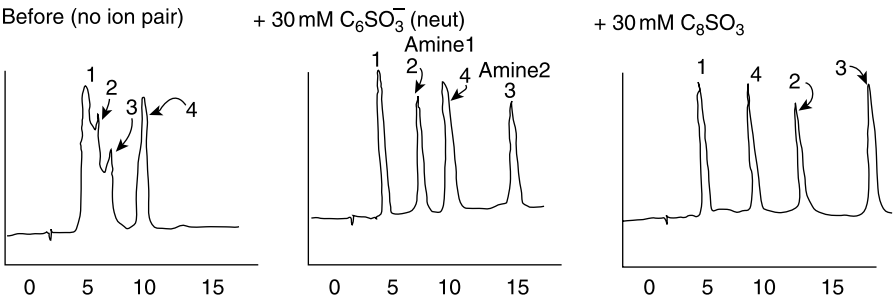

Figure 7.1 Using ion-pairing reagents.

7.1.3 Organic Modifiers

The other ionization that causes tailing in reverse phase separations is ionization of the packing surface. As I have mentioned, there is always a small percentage of free silanols in a bonded-phase packing. The older the column, the more likely that more of these free silanols will be present due to packing material hydrolytic degradation. These are available to react with amines in the mobile phase through an ion-exchange interaction. This effect can be greatly overcome by adding 5 mM nonyl amine to the mobile phase during equilibration and during chromatographic runs. The amine function of this competing base or organic modifier ties up the free silanol presenting a nonpolar surface to sample amine in solution. The competing base effect is very dramatic at low pH, but also shows some peak sharping when used at pH 10 with a saturation column.

7.1.4 Chelation

Adding a metal salt, such as nickel or cobalt, to the mobile phase can often enhance the separation of compounds that serve as ligands for chelating metals. If the ligand co-elutes from the column with a nonligand, adding a soluble chelating metal cation will increase the ligand's solubility and decrease its retention time, pulling the two compounds apart (Fig. 7.2). In a second use of chelators to make a separation, an immobilized chelating metal can be formed by first complexing it with a nonpolar molecule possessing a ligating functional group, then saturating the reverse phase column with this complex. This adhering complex can be used to make a separation by tying up the compound in the mixture, which acts as a ligand, causing it to run slower than the nonligand component, again pulling them apart, but with a reversed order of elution.

Commercial bonded-phase chelation columns have also been offered for sale. The interest in these separations has risen because the chelation with metals such as Ni and Zn is asymmetric and allows the selective separation of optical isomers, such as amino acids, peptides, proteins, and carbohydrates.

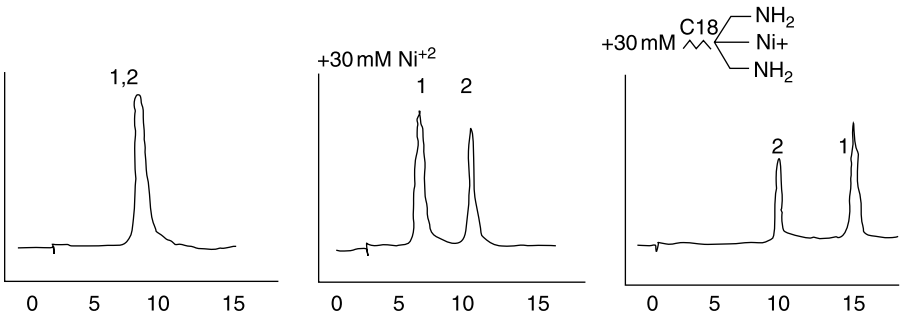

Figure 7.2 Using chelating agents.

7.2 ACIDIC PHASE SILICA

I have discussed normal phase separations on silica and hydrated silica columns in which polar compounds retain and nonpolars elute. Separations have also appeared in the literature using an acid phase silica column for the separation of phospholipids.

These represent a different type of normal phase partition separation. As much as 1–2% sulfuric or phosphoric acid is added to the mobile phase, forming a protonated hydration shell around silica. The phospholipids are soluble in nonpolar solvents, but differ from each other in polar functionalities, such as sugars, alcohols, amino acids or varying numbers of phosphate groups. By going to very harshly acidic conditions, even phosphate groups can be forced into their protonated form, allowing them to be eluted off the silica surface by nonpolar mobile phases. With no bonded phase on the silica to be affected by acid hydrolysis, pH can be kept very low. Every thing can be stripped off the column with acid water and then washed back up to nonpolar solvents.

7.3 REVERSE-PHASE ZIRCONIUM

A bonded-phase columns based on a zirconium backbone at first glance appears simply to be just another reverse-phase column with slightly differing separation characteristics, but having the serious drawback of the strong inherent chelating nature of zirconium. It would be easy to reject these columns when doing new methods development or adapting existing methods, but this would be a serious mistake.

The chelating character has been largely overcome by use of covalently bonded tetraphosphonic acid chelating agents to eliminate column bleed for LC/MS application. Unprotected bonded-phase zirconium columns offer fertile ground for research to provide unique methods for taking advantage of this column's strong chelating nature. There are covalently bonded phases available on zirconium that can simulate silica C_{18} separations with α-type peak shifting, but bonded phases are available that provide truly unique separations. Many of the column phases are bonded directly to the zirconium surface through diazo compounds creating a carbon to zirconium covalent bond much more stable than the reversible silane linkage used to prepare silica-bases bonded-phases.

Because of this linkage, the columns are stable at pH ranges of 1–10, greatly increasing column lifetime and extending the range of separation that they can produce. The stability of the covalently bound phases at elevated temperatures allows these columns to run successfully at higher temperatures in column heaters. Commercial chromatographers have used this characteristic to significantly reduce separation run times. Zirconium reverse-phase columns along with hybrid organic-coated bonded-phase silica seem to represent the major thrusts of future HPLC column technology.

7.4 PARTITION MODE SELECTION

How do you make the decision when to choose a reverse-phase instead of a normal-phase column or an intermediate-phase column such as a cyano column? Reversed-phase columns are chosen about 70% of the time, so most compounds can be separated by this partition mode. What in the make-up of the compound being separated selects one column over the other?

We have already mentioned solvent solubility. If the compounds are not soluble in nonpolar solvents, there is little chance we will be able to separate them on a normal-phase column. The operating solvent ranges are fairly wide on both columns, as we have seen, and a solvent can usually be found that dissolves our compounds and allows them to be run on the column.

Column selection often has to do with which area of a molecule contains the differentiating portion. Two compounds to be separated may vary by substitution on a benzene ring. Another pair may only vary by a polar functional group. Again, like attracts like. The working rule is to select a column in which the variable parts of two molecules point toward the column.

In the first case, the variation was in a nonpolar side-chain, say for instance, *o*- or *p*-toluene. We want the nonpolar ends of our molecules pointing toward the column bed, so we select a C_{18} reverse-phase column. We buffer the mobile phase to 7.5 to help orient the polar benzoic groups toward the mobile phase and the substitutions on the ring toward the column surface.

In the second case, we have the same nonpolar side-chain, but differing polar functions, say *p*-methylphenol and *p*-toluidine. We want the phenolic and anilinic functions toward the column, and, therefore, you would select a normal phase column. The nonpolar solvent attracts the aromatic methyl substituents, correctly orienting the molecules for separation.

One other empirical rule. For some reason, positional isomers seem to be best resolved on anhydrous silica columns. I can't offer you a good reason why this is true. Separation of *cis-/trans-* and axial/equatorial isomers seems to proceed best on these normal-phase columns.

Hydrophilic size separation columns for use with aqueous samples have recently become very popular in purifying proteins and carbohydrates. These will be covered in size separation in Chapter 8.

8

"NONPARTITION" CHROMATOGRAPHY

So far, we have dealt primarily with partition separations, which represent about 80% of HPLC runs. Now we turn to other separation modes. Size separation makes up another 15% and ion exchange the remaining 5% of HPLC separations on silica. Silica, zirconium, and polymer column supports are available for most of these separation modes. Except for the carboyhrate separation column, almost all HPLC-based ion exchange is carried out with silica-based columns. Zirconium-based ion exchanger media for HPLC is slowly winning converts because of the stability of the zirconium surface to salt corrosion and temperature-induced hydrolysis at pH above 8.0 seen with silica. These columns are much easier to wash because of the stability at low and high pH and both strong and weak amine exchangers are available. Of course, zirconium ion exchangers need to be run in the presence of chelators to overcome the Lewis acid effects when comparing them to silica ion exchangers, but these chelation effects can be utilized to provide a mixed mode ion exchange/chelation separation.

Size separation uses both silica- and polymer-based columns. Even though both of these techniques are supposed to be free of partition effects, in the real world, these are bonded-phase columns. To use them successfully, you must not only understand the basic separation mode, but also be able to correct, eliminate, or take advantage of partition effects that are sure to be present.

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8.1 ION EXCHANGE

Ion exchange relies on charged columns attracting oppositely charged molecules from the mobile phase, then releasing them in inverse order of their attracting strength. Two types of ion exchangers exist: cationic and anionic, named for the types of molecules they attract. Each can be divided into two subtypes: strong and weak exchangers, depending on the type of bound functional group. Strong groups are ionized at all working pHs, while weak groups can be either charged or uncharged depending on the pH. Three techniques can be used to remove attracted counterions:

1. competitive displacement by a mobile phase salt ion,
2. pH control of the attracted ion's charge, and
3. pH control of the column's charge.

8.1.1 Cationic: Weak and Strong

Strong cationic ion-exchange columns have an aromatic sulfonate connected through an aliphatic side-chain to the silica surface. In the pair-bonding stage, cations injected into the mobile phase are attracted to the column, while the column counterion, neutral compound, and anions are eluted in the void volume. In the second step, salt cations from the mobile phase attack and competitively displace the sample cation from the pair bond. The stronger the bond, the tighter is the hold on the sample ion on the column, and the longer the retention time. These two stages run simultaneously and repeatedly as the compounds move down the column and are eluted as separate bands. This salt displacement can be supplemented with salt gradient elution. Increased salt pushes bands off faster and somewhat sharpens them. Many ion-exchange chromatographers prefer to run isocratic, shoot the sample, then immediately shoot two injectors full of a very concentrated salt solution. They claim they obtain sharper bands and better separation.

We are not limited to using only salt displacement to remove the sample cation. If it is a weak cation, its charge can be removed by running a pH gradient to high pH. Separation will be in order of pK_b, with the lowest pK_b coming off first. Since many of the naturally occurring cations are amines, it will be necessary to go to pH around 12 and a saturation column will be required.

Strong cationic exchangers attract all cations in the mobile phase. If it is a strong cation (i.e., a quaternary amine), and the compound it attracts is a strong anion, they will form a very tight pair bond that can be broken only by long washing with high concentrations of salt, which erodes the packing. This can be avoided by using a weak cationic ion exchanger that has an alkyl carboxylate bound to the silica. The weak exchanger forms a weaker pair bond with the strong cation allowing salt elution. If necessary, this column can be stripped of cations by going to pH 2.0. The weak exchanger is now in the acid form and must be regenerated by reequilibrating at pH 6.5. The carboxylic

acid column is the functional equivalent of the carboxymethylcellulose (CM) column used for protein separation in open column work.

One other cationic column in common use is the carbohydrate column. This column has a sulfonate bound to a polymeric support and is used pair-bonded to a large cation, either calcium or lead. It probably separates carbohydrates by a partition rather than an ion-exchange mode. The column is run in water at 80°C to speed mass transfer and decrease viscosity. It is very fragile and should not be run over 1,500 psi. Even accidentally jumping the flow rate can rupture the column; this often can be avoided by heating the mobile phase to reduce its viscosity. Small amounts of organic alcohols sharpen peaks, but do not let the organic content exceed 20%. Polysaccharides come off the column first, followed by trisaccharides, the disaccharides, and, finally, simple sugars. This column will resolve among the monosaccharides and separate alcohol sugars from each other.

8.1.2 Anionic: Weak and Strong

In strong anionic ion exchangers, the quaternary amine is attached to the silica surface by an alkyl chain, and the attracted anion comes from the mobile phase. In the pair-bond stage, the anion attaches while neutrals, cations, and the column's counterion elutes in the void volume. Salt displacement release anions with the weakest ion coming off first. Strong anions poison the exchanger, but can be washed off with very strong salt. Weak anions can be removed by using pH gradients from pH 6.5 to 2.0. The free acids are eluted in order of their pK_a's with high pK_a off first.

Weak anionic exchangers use bonded phases with either primary, secondary, or tertiary amines as the function exchanger. It forms weaker bonds with strong anions and is cleanable by going to high pH using a saturation column to form the free amine form of the anion exchanger.

Weak amine columns, whether primary, secondary, or tertiary, will all oxidize in solvents containing dissolved oxygen and need to be protected by nitrogen-purged vacuum-treated solvents as mentioned in Chapter 6. It may seem inconvenient, but column life will be 3 months or less without it.

Both cationic and anionic silica columns need occasionally to be repaired. If you have the same packing material as the column, make a paste of it with mobile phase and paste it on to the column head. If the same packing is unavailable, use cyanopropyl packing for small repairs. If necessary, these columns can be washed with water, then with 20% DMSO/MeOH, with water, and, finally, reequilibrated with buffer.

Do not try to open or repack polymeric columns. They are usually under some pressure and come out of the tube like toothpaste. The column is of no use. Polymeric columns are usually packed in one solvent, then switched to a second solvent, which causes the packing to swell and squeeze out voids. They are then designed to be run in the second solvent. Polymeric ion exchangers are usually run at elevated temperature. This serves two purposes: it decreases mobile phase viscosity, thereby reducing operating pressures, and it speeds

equilibration of the sample with the ion exchanger, which is usually very slow with these columns.

8.2 SIZE EXCLUSION

Although the oldest type of columns, these are presently the most popular nonpartition columns because they can separate large biological molecules such as proteins and nucleic acids. By means of controlled pore sizes, they separate compounds by their molecular size and shape. The resolution achieved by a size resolution column is not nearly as great as that shown by ion exchange or by partition. You will need a 100% difference in molecular weights to achieve a clean separation. Partition can separate on the basis of a proton up or down out of 13 protons on a compound.

Although we often describe these as molecular weight columns, the separating parameter actually is their Stokes radius, the major axis of the molecule in its current configuration. The shape and folding of a protein molecule under differing solvent conditions affect their maximum radius and, therefore, their retention times. Only when extreme conditions are used to force all molecules into the same shape are we able to obtain a direct molecular weight relationship.

Both polymeric and silica-based columns are in common use. The polymeric columns are heavily used in the analysis of synthetic polymers and plastics where organic solvents are required. Silica-based columns with hydrophilic bonded phases are used to separate aqueous solutions of macromolecules. Finally, polymeric size-separation columns with hydrophilic phases are available for separation of polysaccharides, peptides, and very small proteins.

Size columns tend to dilute the sample shot into them, unlike partition, ion exchange, or affinity columns, all of which tend to concentrate samples placed on them. To obtain maximum effect, size columns need to be tall and thin to allow enough time for compounds to interact with column pores without diffusion upsetting the achieved separation. Since they are generally the poorest columns for achieving resolution, they have two main uses: 1) they are used as a first column to tear a mixture of compounds into size groupings before going to a concentration mode separation, and 2) they are the final column of choice to remove buffers and salts from elution fractions from other separating modes. SFE or gravity-fed desalting columns using Sephadex G-25 are a much faster and more complete method of removing salts from protein solutions than either dialysis or molecular weight filtration membranes.

8.2.1 Organic Soluble Samples

Polymer samples are size separated by dissolving them in an organic solvent, such as THF, then passing them through a linked series of sizing columns. These cross-linked polymer columns vary in pore size, with each column

graded by its inclusion/exclusion ratio. Ratios start at less than 100 and go to well over 20 million Da; a typical range for a single column would be from 30,000 to 200,000 Da. The separating range of each column is determined using polystyrene standards of known molecular weights. While each column may have a narrow separating range, connecting an ascending series together with the smallest next to the injector produces a combination with a range from the smallest packing's inclusion to the largest packing's exclusion. The range will be continuous if each column's exclusion limit overlaps its neighbor's inclusion.

Switching solvents is a very poor practice because of bed swelling and shrinking from solvent to solvent. Usually, a solvent is selected with a broad solubility range; the system is turned on and allowed to equilibrate for 24 hours and then kept in a constant flow recycle mode until needed. When a sample is to be shot, flow is switched out of recycle, the chromatogram run, and then immediately returned to recycle. The pump is left on at all times.

Since polymers are not discrete compounds, but instead a range of compounds, the chromatograms produced are not a series of peaks, but a continuum with peaks. Measurements are generally made with a refractive index detector and the amount of material present at various points in the trace is measured. Early running components are high molecular weight and give information about stretch and flexibility of the polymer. Later runners are smaller and give information about leaching, solubility, and brittleness. The chromatographer is less interested in determining molecular weight distribution than in getting a "fingerprint" of a particular polymer. This distribution fingerprint can provide information on unreacted monomer, degree of polymerization, and serve as a batch-to-batch quality control device.

Very-high-density polymers are run in a special high-temperature HPLC. This device can automatically dissolve, filter, inject and run these samples at 200°C in a solvent such as chlorobenzene.

8.2.2 Hydrophilic Protein Separation

Hydrophilic size separation columns for use with aqueous samples are very popular choices for purifying proteins and carbohydrates. Protein separation columns are available on both silica and polymeric supports. It is surprising that the best of these protein purification columns in terms of resolution and in recovery of native protein are silica-based columns. One would expect that protein release from silica would be a real problem. It certainly is in many other silica columns. These columns, however, especially the TSK family of columns, give excellent recovery of enzymatic activity. I have talked to other column manufacturers who have investigated the problem. They say that when you remove the bonded phases from these columns they appear to be identical to bonded phases from a number of other, less successful, columns designed for protein purification. All of these bonded phases are primarily diol ether polymers, very hydrophilic, but of intermediate polarity. Some modification of

the underlying silica appears to give the TSK-type columns their unique ability to release proteins placed on them.

The protein molecular weight exclusion and inclusion ranges grade these columns like other size-separation columns discussed earlier. This can be very deceptive since globular proteins and many enzymes are folded in and wrapped around themselves causing them to run smaller than you would expect from their molecular weights. This intermolecular folding is an integral part of maintaining their enzymatic behavior. Their functionality as biological catalyst is often irreversibly lost if the folding is broken with detergents.

Adding denaturing compounds such as sodium dilauryl sulfate (SDS) to the mobile phase causes the proteins to straighten out and show a fairly linear relationship with molecular weights. A column with a molecular weight range of 10,000–40,000 Da with folded proteins run in Tris buffer mobile phase will drop to a range of 8,000–25,000 Da when run in SDS-containing mobile phase.

Hydrophilic columns exist with exclusion limits of 4.5 million Da: work continues to extend the range high enough to allow separation of intact nucleic acids and very large restriction fragments, but very large silica particles to handle such large, straight molecules are very fragile and crush easily. The lower limit at the moment for smaller proteins and polypeptides is around 8,000 Da on silica. A customer of mine claims to be able to separate decapeptides using the smallest of the polymeric, hydrophilic size columns, the TSK-2000pw column. This column is sold as a carbohydrate size-separation column and he grabbed it by mistake when trying to separate these peptides from a protein mixture.

I have mentioned that solvent affects the separation of proteins. Mobile phases for recovery of native proteins and enzymes resemble enzymatic assay conditions and are selected to stabilize structures and preserve activity. I routinely make separations in 100 mM Tris-phosphate buffer at pH 7.2. Often metal ions and sulfhydryl stabilizers such as dithioglycerol are added. Chromatography is sharpened with salts, phosphates, sulfates, and, best of all, 150 mM sodium chloride. The latter is unfortunate because it erodes the packing and corrodes the exposed metal surfaces in the systems and column. We can protect the systems hardware with pacification, but there is little we can do about column corrosion short of using glass or plastic-lined columns. The packing has to fend for itself and end voids are common in these columns. Even worse, it is very difficult to buy packing material from the manufacturer for topping up the columns. Of course, you can buy a new column and sacrifice the old column to provide topping material.

One mobile phase additive—glycerol—serves a double purpose. Up to 10% glycerol is often added to stabile protein activity. It also serves to decrease partition interaction of glycoproteins with the diol column packing and make the glycoprotein come off faster than they should by size separation only. A glycoprotein can be made to run slower in a glycerol-free mobile phase, then reinjected into the same column equilibrated with a mobile phase containing 5% glycerol, which makes it elute more quickly than compounds with which

it formerly co-eluted. This can produce a two-step sequential separation for the glycoproteins on a single column. Isopropanol is also said to have a similar effect.

Crude plasma quickly fouls the protein separation column with lipids, usually after only three injections. These lipids can be removed by washing the column with water, then with 20% DMSO/MeOH, and finally with water again before returning to buffer. DMSO/MeOH is fairly viscous and you may have to drop the flow rate to avoid triggering your overpressure setting.

Enzyme purification is not the only job of these hydrophilic size-separations columns. These columns also serve as protein preparation columns and will accept as much as 100 mg of protein per injections. When this protein is to be used for structure determination, detergents can be used in the mobile phase to increase the large protein solubility. Nonionic detergents will often give enzymatic activity back on dialysis against Tris-phosphate incubation buffer, but ionic detergents seem to finish activity off by making permanent structural changes. The ionic detergent also acts very much like ion-pairing reagents in partition work and are very difficult to remove. It is generally better to dedicate a column for this work rather than take a chance on losing your next enzyme preparation to a dirty column.

Another series of hydrophilic size-separation columns are based on cross-linked polymers. They are sold as carbohydrate size-separation columns and will separate a polymer series (i.e., dimer, trimer, hexamer) from each other, but not separate the monomer isomers (i.e., glucose from galactose). These columns also work for proteins and polypeptides. They have the same diol-type bonded phases as the silica-based columns, but do not show as broad a molecular weight range or as high a resolution. Because they are polymer based, they will not take pressures over 1,500 psi and should not be cleaned with organic solvents. They show considerable promise for separation of heparin and chondroitin sulfate-type polysaccharides used with a CAD-type of detection. Again, detergents and glycerine can be used to increase solubilities and to control sample interaction with the bonded phase. Heated mobile phase speeds equilibration and improves peak shapes and resolution.

The hydrophilic silica-based diol packings have been modified by derivation through some of the diol groups with carboxymethyl and diethylaminoethyl functions to make weak anionic and cationic protein size-separation columns. These provide the HPLC equivalent of the CM- and DEAE-cellulose columns used in protein purification on open columns and are used with the same type of buffers to provide ion exchange purifications of proteins.

8.3 AFFINITY CHROMATOGRAPHY

Affinity chromatography is a technique of growing interest in HPLC because of the commercial availability of affinity columns for specific use separations

(such as protein A columns for antibody purifications). Traditionally, the affinity column was designed to pull out of a mixture only a single compound or class of compounds. An affinity separation often would act as a one-step purifier for the molecule it was designed to attract, hold, and eventually release and elute. With enzyme purification this was done by determining the substrate that served as the key for the "locking site" on the protein. The substrate was then attached chemically to the bonded phase to create the affinity packing.

Proteins and antibodies are natural substrates for affinity columns because of the nature of the enzyme recognition site and the antibody-antigen interaction sites. They have a three-dimensional shape and electrical charge distributions that interact with only specific molecules or types of molecules. Once these substrate sites are identified, molecules can be isolated or synthesized with the key characteristics and used to build affinity supports. These substrates are often bound to a 6-carbon spacer so that they protrude farther away from the packing surface toward the mobile phase and are therefore more available. Certain natural and synthetic dyes have been found to serve as substrate mimics for a class of enzymes call hydrogenases and have been used to build affinity columns for their purification.

In certain cases, affinity columns can be used to fractionate within classes of bound materials; for instance, protein A antibody columns have been used to separate the various subtypes of IgG. In this case, the packings are microporous, heavily cross-linked polymers and benefit from HPLC operating conditions. Eluting conditions are usually step gradients of buffers with different pHs. The last step of a protein G column is at a very acidic pH and the sample is eluted into a buffer solution that quickly raises the pH to prevent protein denaturation.

8.3.1 Column Packing Modification

First, the target compound to be bound to the column must be identified, isolated, and activated. In some cases, the column packing is purchased already activated. Once the target is chemically bound to the column, the packing must be slurry packed into the column. Fortunately, these columns concentrate and bind the substrate so they can be broad and narrow like an ideal ion exchange column and are fairly easily packed.

Zirconium columns kits for preparing affinity columns have been recently released by ZirChrom. They contain an activated linker that can be reacted with the target compound in the prepacked column to prepare the affinity column in situ. Generally, when an affinity column is made, the column must be dedicated to only that one separation. If you have six different affinity separations to make, you must buy six columns. But with these zirconium column kits, the affinity head can be stripped off in the column, the column cleaned, the linker reactivated, and a new affinity column created with a new affinity target without unpacking and repacking the column. This should open create new interest in affinity separations.

8.3.2 Chelation and Optically Active Columns

Vitamins are known to act as co-factors for certain enzymes and have been studied as affinity targets to be used in purification of these enzymes. Another class of general co-factors for enzymes is the metal ions that many enzymes require for their activity. They lead to general affinity packing, one with a bound group that can chelate a number of transition metal ions. Typical of these is a long-chain organic molecule with branching chains, each of which ends in an amino group. These "amine finger groups" can serve as ligands for the metal ion. A tridentate (three-finger) molecule can cup the metal and allow ready access from only one side. This type of target group has been used to resolve racemic mixtures of optically active compounds on commercially available optical activity columns. One optical isomer of an isomeric pair can approach and bind the complex better than the other, leading to a separation as the tighter-bound twin retains longer on the column. Elution can be made using salt or pH gradients to break the complex. For very tightly bound material, the natural substrates will generally exhibit a tighter fit and serve as a displacer. For the metal ion affinity column, the binding can be broken with strong metal chelators such as EDTA.

9

HARDWARE SPECIFICS

The HPLC separation occurs when a mixture of compounds dissolved in a solvent may stay either in the solvent or go onto the packing material in the column. The choice is not a simple one since compounds have an affinity for both the solvent and the packing. In fact, separation occurs because different compounds have differing partition rates. Left to themselves, each compound would reach its own equilibrium composition in the solvent. However, we upset conditions by pumping fresh solvent down the column. The result is that the components with the highest partition affinity for the column packing stick the longest and wash out last. This differential wash-out or elution of compounds is the basis for the HPLC separation, a very simple technique that often requires complex equipment for its execution.

9.1 SYSTEM PROTECTION

The simplest HPLC system is made up of a high-pressure solvent pump, an injector, a column, a detector, and a data recorder (Fig. 9.1). The high pressures referred to in the system name are of the order of 2,000–6,000 psi. Since we are working with liquids instead of gases, high pressures do not pose an explosion hazard. Leaks occur with too much pressure. The worst problems to be expected are drips, streams, and puddles.

An isocratic system is used with single solvents, a premixed solvent mixture, or step gradients. It has the advantage of needing only a single pump, no mixer, and no gradient controller. Because of this, isocratic systems are simpler and

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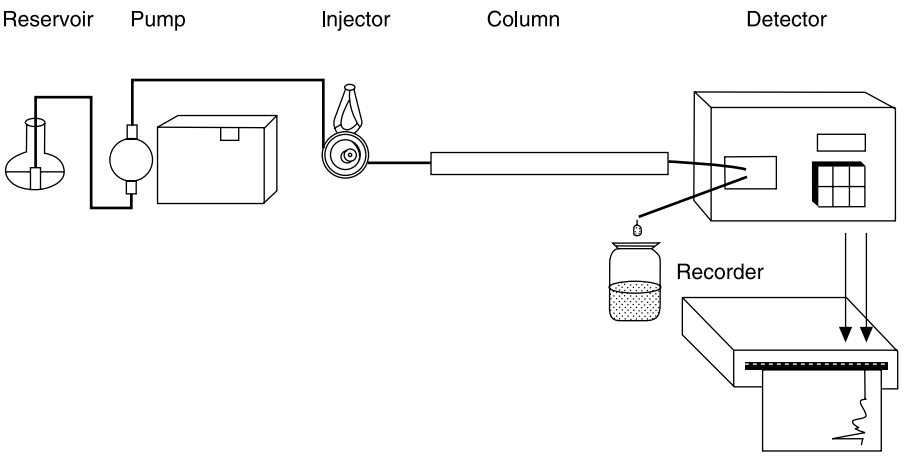


Figure 9.1 Isocratic system components.

less expensive than gradient systems. With an isocratic run there is no necessity to reequilibrate to initial conditions before making your next injection. Many HPLC separations do not require a gradient. A gradient solvent system is inherently slower than an isocratic because it must be run slowly enough to establish reproducible mobile phase conditions within the column. Isocratic chromatography is more efficient and economical because it produces more rapid analysis and faster turnaround times. However, there are separations that cannot be achieved with only an isocratic separation. At present, an informal survey of users has shown that gradients are used only 20% of the time in HPLC production laboratories, although gradient systems are purchased at a much higher rate. Certainly, for research and development labs, gradients provide a greater degree of flexibility where time constraints are not critical.

9.1.1 Filters, Guard Columns, and Saturation Columns

The tubing used to tie the system together is of extremely small internal diameter and is easily plugged. Packing beads are made of very fine particles and must be protected with a series of filters and guard columns. The usual first filter is a 10- to 30-μm pore size sinker on the end of the solvent inlet line in the solvent reservoir. This filter is probably redundant since HPLC solvents should be filtered through at least a 0.54-μm filter before use. Some pumps may also have a 5- to 10-μm filter before or in the inlet check valve system. Too many filters before the pumping chamber can lead to problems because they provide a resistance to flow and may cause bubbles to form in the pump head. If the solvent sinker gets plugged with solids or rusts, it can restrict flow and lead to pump starving. This can be determined by using a graduated cylinder to measure the pump outlet versus the selected pump flow. I also have sold a high-pressure mixing system that uses a magnetically stirred high-pressure mixing chamber that is equipped with a filter. We were not aware that the filter

was even present until we developed output pressure problems and found it when we disassembled the mixer.

We have already discussed the use of a saturation column between the pump and injector to protect the main column against column degradation caused by high pH buffers. Another function of this column is to provide filters (the column inlet and the outlet frits) before the injector. I have seen unusual pumps that flaked Teflon® off the pump seal; this ended up plugging the injector. An in-line filter would have prevented the problem, which required tearing down the injector to remove the plugging.

The only fiter between the injection sample and the column bed is the inlet filter of the column or pre-column. Because of this, it is very important to filter *all* injected samples. I had a customer who got a pressure increase every time she injected her standards. It never occurred to her that she would need to filter purified standard mixtures. When we dissolved her standards in solvent in a test tube and swirled the solution, you could see opalescence. Centrifugation brought down a white pellet. The standards had evidently been decolorized with charcoal and filtered through a Celite bed. Celite fines probably passed through the filter paper with the solution and were trapped in the compound during final solvent evaporation.

9.1.2 Inert Surfaces and Connections

The stainless steel used in HPLC lines, fittings, and other wetted surfaces is corroded by exposure to halide salts. In systems that require routine operation with salt, protection is available in two forms. If less than 200 mM salt is to be used, the pre-column and column can be removed, a column bridge can be added, and the system can be treated with 20% nitric acid followed by water washing. This *pacification* treatment protects the stainless steel surfaces for up to 1 month and is also used to remove precipitated buffer crystals and organic precipitation in lines and injector loops. If a more concentrated halide operation is foreseen, it might be worthwhile to purchase a system with inert wetted surfaces. These consist of titanium and polymer pump heads and flow cells and *PEEK*-type pressure-resistant tubing and fittings. Titanium construction adds surprisingly little to the cost of a system, but does present some problems. Titanium is very hard and brittle. Titanium lines are available, but should be avoided. They are hard to cut without breaking, and compression fittings do not bind well and can split lines while being attached. Two problems I have seen with inert systems should be avoided: First, lines going into inert flow cells are soldered into place with nickel or silver solder. These metals leach out with corrosive solutions and appear in the effluent. The second problem is with pump pressure sensors (transducers). These flow-through devices are inside the pump and are often ignored or forgotten by inert pump manufacturers. If inert transducers are not used during inert pump construction, they will corrode and can eventually collapse or begin leaking inside the pump. I have seen this happen, and it is not a pretty sight to see the whole inside of a $4,000 pump corroded with strong salt solution.

9.2 PUMPING

Pumps are basically devices for pulling in solvent, pressurizing it, and driving it out through the injector, column, and detector. As described earlier, it does this with a plunger driven through a Teflon® seal into a pumping chamber (Fig. 9.2).

Inlet and outlet check valves ensure a one-way solvent flow. Problems arise when the plunger must be pulled back to refill the chamber to ready itself for the next stroke (Fig. 9.3). The pressure drops until the plunger starts forward again. This results in pulsation, which causes variations in solvent delivery flow and, more importantly, variation in pressure to the column. The column acts as a pressure dampener, which can easily seen by watching baseline fluctuation of a system with and without a column in place.

Manufacturers have come up with additional pulse dampeners to place in the flow from the pump to reduce this pulsation. The high-pressure versions are metal cans with a long, tight coil of small-diameter stainless steel tubing coiled around itself. When a pulse comes down the line, the coil flexes then recoils through a spring effect and dampens some of the pulse. For some reason, manufacturers are often reluctant to admit that their pumps need pulse dampening. They describe them as "pressure filters" or "polishers." Generally, if your pump has a hole with two liquid lines leading into it, it is either a pressure sensor or a pulse dampener.

Pump manufacturers continue to work to reduce pulsing. The first attempt was the two-headed pump, which is connected through an output manifold or pressure transducer (Fig. 9.4). One head delivers solvent while the second refills. This improvement led to the first high-performance HPLC pumps, but

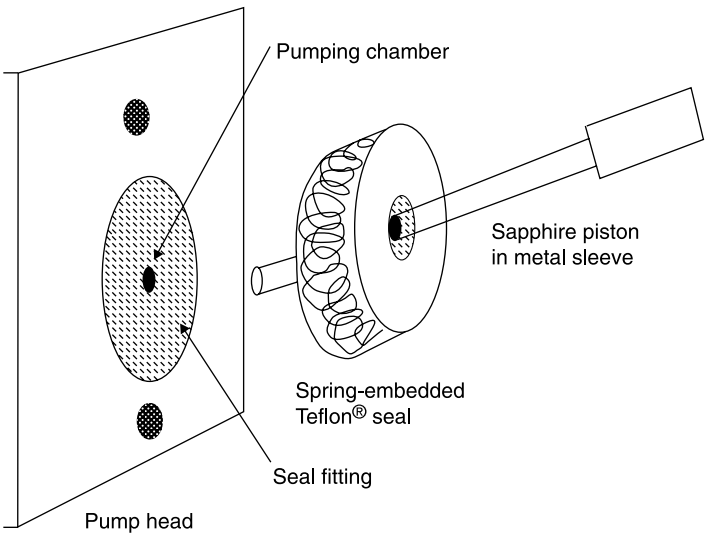


Figure 9.2 Piston and seal design.

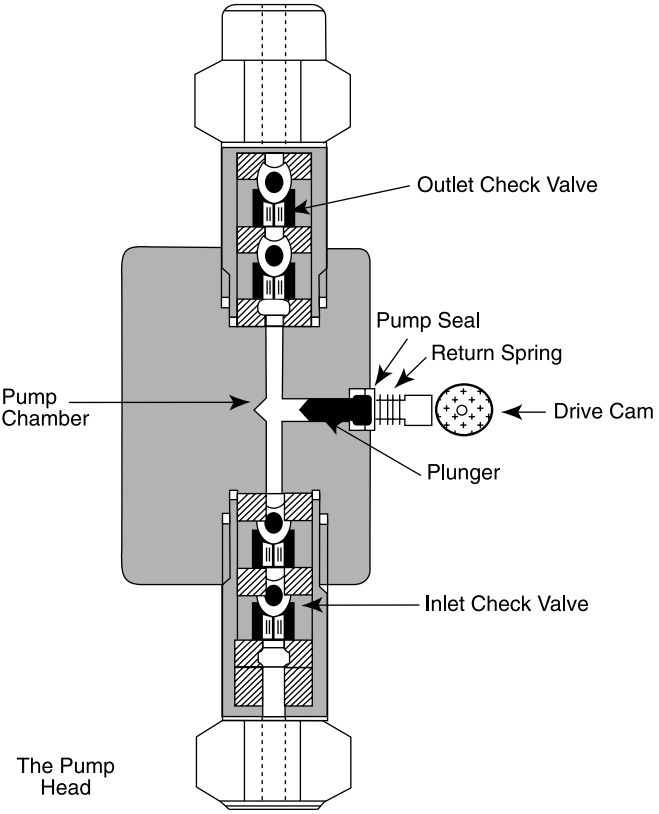


Figure 9.3 Pump head design.

it has an expensive downside: twice as many check valves, plungers, and seals, twice as much metal in the pump head, and more engineering know-how leading to a higher cost. This eventually led to the three-headed pump. If this pump had been successful, we would probably be running V8s and in-line 12 HPLC pumps by now.

The next step was the electronically compensated pump. All pumps speed the motor as resistance increases to maintain a constant solvent slow. These pumps also add a major plunger speed-up during refill and repressurization. With this modification, a pump with a single pump head and a pulse dampener could give 90% of the performance of a two-headed pump for 50% of the cost. An overall dramatic price reduction for the dual-pump HPLC system resulted.

9.2.1 High- and Low-Pressure Mixing Controllers

Gradient systems are often oversold. They have two legitimate purposes. The first is for analysis of very complex mixtures with widely differing polarities.

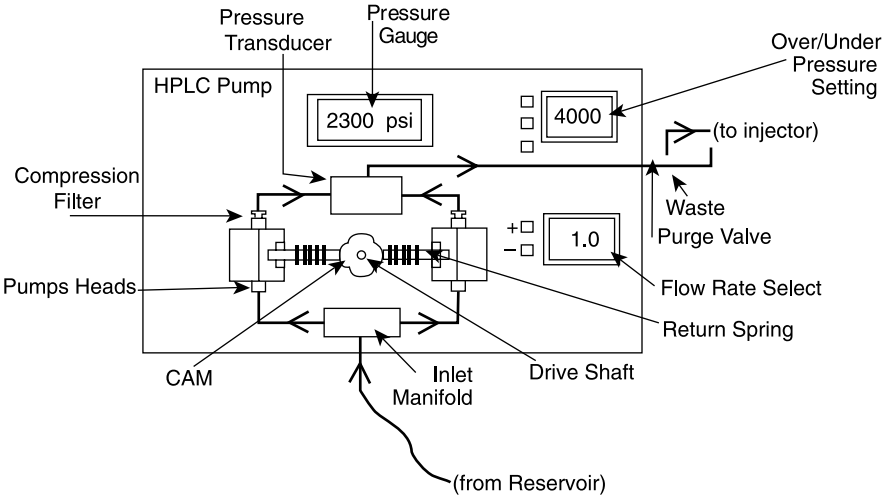


Figure 9.4 An HPLC pump.

Some of this work results from the necessity to separate polar and nonpolar impurities resulting from the sample matrix. SFE cartridge columns for preinjection sample preparation can help to eliminated many of these gradient runs. The second purpose for a gradient is for running methods scouting gradients that can be used to produce rapidly running isocratic methods. Solvent gradients are used to move late running peaks off the column and resolve areas of compressed peaks in the chromatogram. To generate a solvent gradient you speed up solvent flow from one reservoir while slowing down the feed from another, but still maintaining a constant pump flow rate. Therefore, a gradient controller is, first and foremost, a pump flow controller. There are two types of gradient systems in HPLC: the high-pressure mixing gradient and the low-pressure mixing gradient system. The first gradient systems were high-pressure mixing systems. Two pumps were used to pump the individual solvents; the controller sped or slowed the flow rates from each pump. Once the solvents were pressurized in the pump heads, they were mixed in a chamber before passing them to the injector. The remaining parts of the system were the same as the isocratic system (Fig. 9.5).

The first high-pressure mixing system I saw used a static mixer. The manifold that mixed the streams from the two pump heads on the first pump had another inlet at 90° that accepted the manifold output from the second pump. Impinging the streams of solvent from the two pumps did the mixing. You plugged control line from the flow controller into the back of both pumps and you had a gradient system to control the flow rates of each pump and the overall solvent output rate of the combined system. More sophisticated systems brought both pumps solvent output together into a 50-μL magneti-

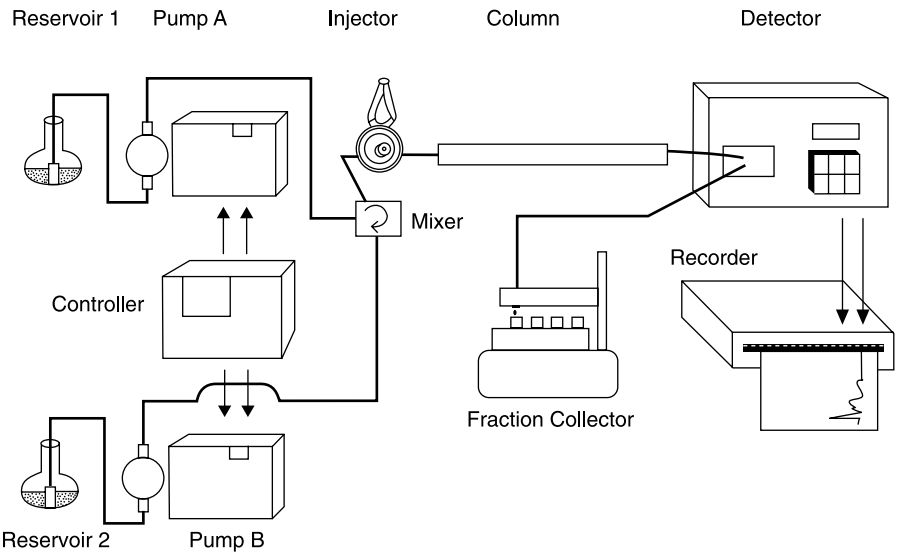


Figure 9.5 High-pressure mixing gradient system.

cally stirred dynamic mixer and then out to the injector. In either case, the mixing was done after the solvent had been pressurized in the pump heads.

The problem with the high-pressure mixing system is that you have two very expensive pumps. You could actually benefit from having a third solvent when you are automating methods development, but that meant adding even another expensive pump! There had to be a cheaper, better way of doing this. There is a cheaper way, but not necessarily better. High-pressure, dynamically mixed gradients will always give the best gradient formation.

The second gradient alternative is the low-pressure mixing system (Fig. 9.6). This system uses a single pump, a gradient controller, and a solvent selection block. Each solvent line connects to the selection block through a separate solenoid valve operated by the solvent controller. Instead of controlling a pump's flow rate, the programmer in the controller switches each valve open for a specific length of time. If we dial in 80% A and 20% B, then valve A is open 80% of the time and the other valve B is open 20% of the time. This solvent selection valve design is not limited to two valves; systems exist with three- and four-solvent selection valves.

The problem with this method of creating gradients is that it tends to put out plugs of each type of solvent in the outlet line from the selection block into the pump. There is a heat of mixing associated with combining the solvents. Plugs of solvent mean variability in the gradient unless a mixer is added before the pump. Pumps are designed not to mix solvent; they have a first-in, first-out construction. The heat of mixing is a more serious problem. Dissolved gases released from solution on heating end up as bubbles in the pump head

and can and will lead to cavitation of the pump. Cavitation is what happens in your car when it vapor locks on a hot summer day. The HPLC pump locks up in the same way. It tries to pump solvent, but all that happens is that the bubble gets smaller, then bigger and no solvent flows.

To overcome the first problem, that of mixing, manufacturers have introduced a variety of solutions. One provides a 2-mL mixing chamber after the pump head; this can be increased to 4 mL for critical gradients. That is a lot of dead volume to be added to the system before the column since it translates to time delay and imprecise gradients. A second manufacturer tried to add dynamic mixing immediately after the solvent selection block, but it just aggravated the degassing problem. A third manufacturer brought out a head and a half pump in which the second partial head piston is designed to mix the output from the first piston.

The degassing problem is generally solved by using helium-purged solvents. Solvents are continuously purged with helium, sometimes under vacuum, and then run under helium sparging or under a helium demand valve. Using this technique, large bubble outgassing is prevented and these systems have become the most economical choice of the methods development laboratory, and, in many cases, the only choice considered for general research laboratories.

9.2.2 Checking Gradient Performance

In addition to the problem of expensive use of helium, there is some question about the efficiencies and reproducibility of the gradients that these systems form. This question is easily answered experimentally. I have had customers in the past call to say that their chromatography had suddenly gotten very bad on their mixing valve gradient system. When I questioned them it became apparent that the stronger solvent solenoid valves in their pumping system had stopped working. They were running an isocratic separation in water. They needed a method of checking performance of the solenoid valve. My suggestion was to run an acetone-spiked gradient check.

The best test of gradient performance is to run acetone-spiked gradients from 0 to 5% and 95 to 100%. In a high-pressure mixing system, these stretches are the points at which one pump or the other is running the slowest, a true measure of pumping performance. In the low-pressure mixing system, these are the points at which the individual valve is open the least. Set up your system with MeOH in all solvent reservoirs and place 50 mM acetone in the A reservoir. Set your UV to 235 nM. Have your gradient programmer set for a binary gradient of 1%, 1-min steps from 0 to 10% B, then 5% steps to 95% B, and 1% steps from 95% to 100% B, then reverse and repeat the same sequence going down to 0% B (1%, 1-min steps from 100 to 95% B, etc.) Next, have the controller go through the same sequence with A to C. If you have four solenoid valves, repeat with a similar gradient from A to D (Fig. 9.7). You end up with a jagged series of steps if you trace the gradient. Ignore the gradient traces, but pay attention to the baseline traces. Since acetone absorbs at

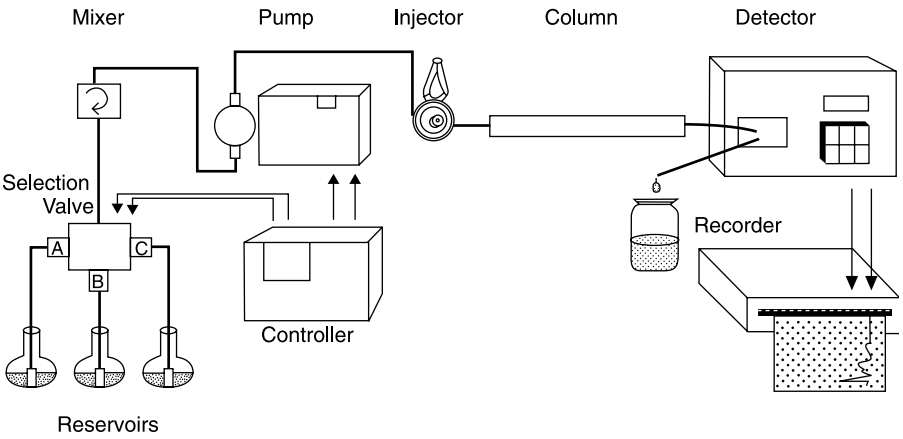


Figure 9.6 Proportioning valve gradient system.

230 nM, you will see the actual gradients you produce on the recorder. Ideally, each step should be vertical, with sharp shoulders and no overshoot. In practice, you want to see only a bit of rounding at the shoulders, a near vertical slope, and no ringing or overshoot at the high end of the slope change.

My preference today would be for a high-pressure mixing system if I had to run very complex mixtures on a routine basis because these systems give the best reproducible gradients as a rule. As a routine research instrument or a methods development system, I would prefer a low-pressure, four-solvent dynamically mixed system using a dual-headed pump. I would use the deoxygenation apparatus (Fig. 6.4) to degas my solvents with helium and run them under a helium demand valve to conserve helium.

One of the advantages of the two-pump gradient system is the ability to split it quickly into dual isocratic systems by adding a second injector, column, and detector (Fig. 9.8). A majority of QA/QC HPLC runs made are fast-running isocratic analysis once the proper operating conditions have been worked out. You could use two detectors in series and gradient to scout the separation, then add an extra injector and column, hook both systems up to your two-channel integrator, strip chart recorder, or computer system, and run parallel isocratic runs at the same time. The system can be recombined into a scouting gradient for the next scouting problem, an excellent, cost-effective use of the lab's HPLC dollars. It is actually like buying three systems in one: one gradient and two isocratics.

9.3 INJECTORS AND AUTOSAMPLERS

The next important component of the system is the injector. Since it lies between the pump and the column, it also must be able to operate at high

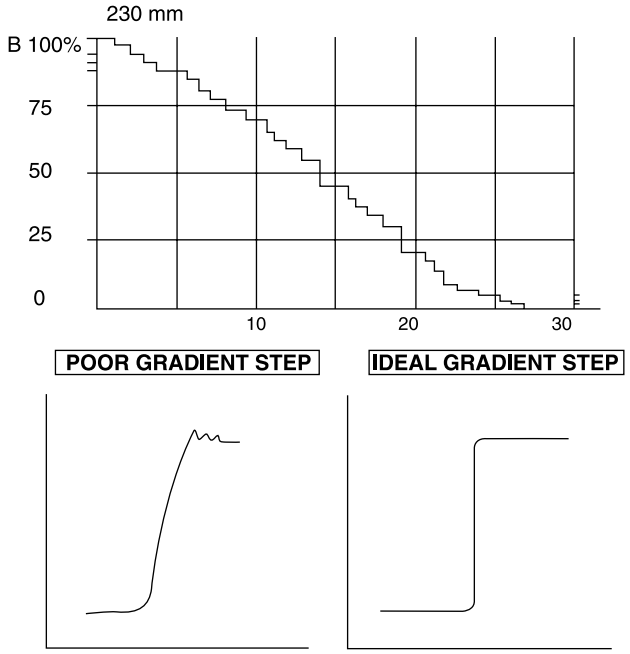


Figure 9.7 Gradient performance testing.

pressures. All injectors on the market work on the principle of the loop and valve. In the *load* position, system solvent is displaced from the injection loop with sample solution at atmospheric pressure. Sample goes into the loop at the point closest to the column and displaces loop solvent out the back end of the loop. Injection occurs by roating the injector against a Teflon® seal to the *inject* position. This places the loop containing the sample in the solvent flow from the pump to the column. Since mobile phase enters the loop at the end opposite sample injection, we achieve a last-in, first-out loading of the sample onto the column head. No dilution of the sample solution occurs within the sample loop solvent volume. The loop is pressurized and the sample is washed onto the column (Fig. 9.9).

Autosamplers take this same loop and valve principle and automate the filling and handle-turning sequence. The major differences between models on the market are in the way they get sample into the loop and the method of cleaning between injections. Most autoinjectors use a carousel loaded with sample valves to hold samples until their turn for injection occurs. Sample vials are usually capped with a screw cap fitted with a septum, although some recent autosamplers replace the carousel with microtiter plates having 96–364 wells containing the samples for use with robotic workstations. Conical vials are available for limited samples and 1-μL injections are possible with some

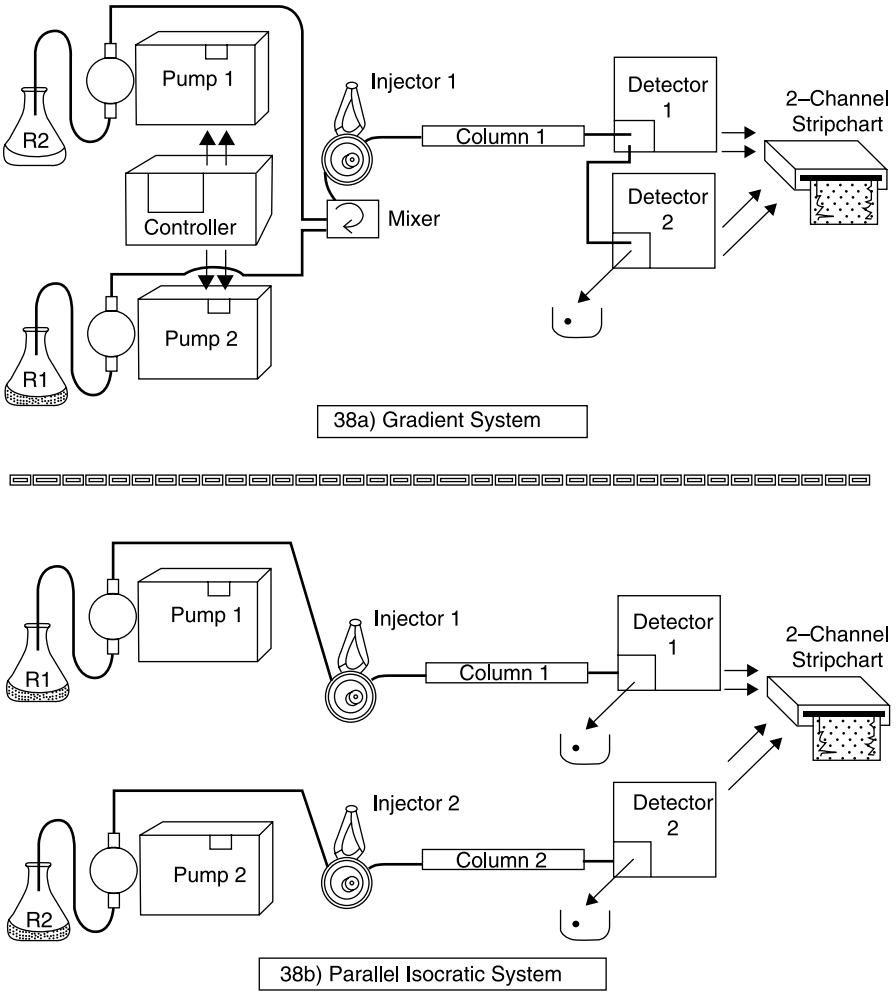


Figure 9.8 Gradient to parallel isocratic systems. (a) Gradient system; (b) Parallel isocratic system.

autosamplers. A few autosamplers are equipped with chillers to preserve sensitive samples such as proteins solutions until they are ready for injection.

Some autosampler models use air pressure or a piston to push sample out of the vial into the injection loop; others pull the sample into the loop with vacuum or a syringe barrel. Various techniques are employed to wipe or wash the outside of the injection needle before the next injection to prevent sample-to-sample contamination. Other systems just wash the inside of the needle by pulling in some of the next sample and spitting it out to a waste vial or back into the last vial used.

A series of valves is actuated to actually *load* the sample into the column. Generally, low-pressure valves in the autosampler can be turned electrically, whereas high-pressure valves must be turned using pneumatic pressure. Most autosamplers require a source of compressed gas to run these air-actuated valves.

Micro-injections in micro-flow and nano-flow systems are done with injectors in which the external sample loop is replaced with the internal fixed volume within the injector body. HPLC-on-a-chip systems also build the column into the injector body. The internal path within the injector body is abladed with a laser, packed with micro-packing material, and this serves as the separating media. The injector inlet is connected to the pumping system and the outlet to the detector. Sample is loaded into an internal loop in the *load* position, then injected onto the chip HPLC by turning the injector. Obviously, in a system like this sample size is very limited and the detector is usually a highly sensitive mass spectrometer.

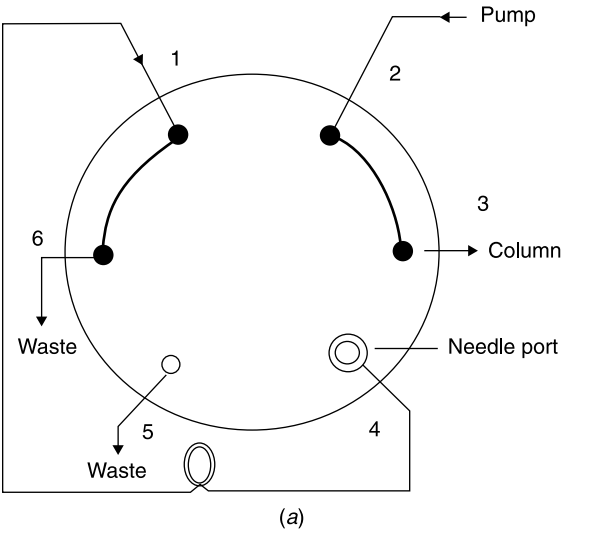
9.4 DETECTORS

The detector controls the sensitivity with which each compound can be detected and measured once separated on the column. To be effective, the detector must be capable of responding to concentration changes in all of the compounds of interest, with a sensitivity sufficient to measure the component present in the smallest concentration. Not all detectors will see every component separated by the column. Generally, the more sensitive the detector, the more specific it is and the more compounds it will miss. Detectors can be used in series to gain more information while maintaining sensitivity for detection of minor components.

There are six main types of detectors used for HPLC: refractive index (RI), ultraviolet (UV), fluorescence (FL), electrochemical (EC), conductivity (CD), and mass spectrometric (MS). Infrared and nuclear magnetic resonance detectors have been used, but they suffer from solvent limitations. Many of these detectors are affected by temperature and recent sensitivity gains have been made by actively controlling flow cell temperature, by using "cold" infrared and emitting diode light sources, and by optimizing flow cell design to decrease gradient turbulence affects.

9.4.1 Mass Dependent Detectors

The oldest detector, and the least sensitive, is the refractive index (RI) detector. Originally designed for effluent monitoring, it was adopted along with a peristaltic pump, a stop flow injector, and a metal tube packed with ion exchange resin in creating the first commercial HPLC system. Light from a source in the flow cell is directed first through the reference cell, containing trapped mobile phase, and then through the sample cell. The signal to the pho-



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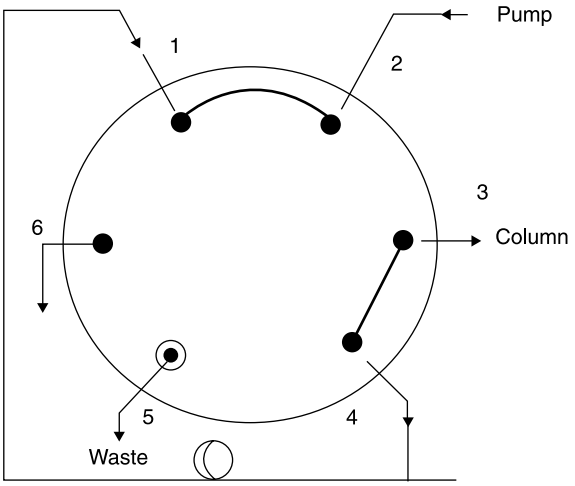

(*b*)

Figure 9.9 A loop and valve injection.

todetectors is balanced with only mobile phase passing through the sample cell (Fig. 9.10). The difference in refraction when the sample arrives in the sample cell causes the position of the beam to shift, sending more light to one photodectector than to the other, resulting in a voltage shift in the detector output.

The detector of choice for preparative work, the RI detector can be used only in isocratic systems. It is very sensitive to turbulence, temperature, or

solvent changes. Using a temperature-controlled compartment and a cold light source, such as a photodiode, it is possible to push this detector to a 50–100-ng level of detection. If has the advantage of being a mass detector: the same weight of two compounds will give the same peak areas. However, make sure that the compounds have refractive indices differing from the solvent. The glued flow cells of these detectors are fragile and will not tolerate back-pressure. Simply blocking the flow from the detector for a few seconds is often enough to build up pressure and break the cell.

A second mass detector is the conductivity (CD) detector. It is designed to measure differences in conductivity in the flow cell against a reference electrode. Buffer in the mobile phase cuts the operating range and, therefore, the sensitivity. Gradient runs of either salt gradients or aqueous organic solvents cannot be tolerated by this detector. This detector is usually seen only in water analysis of inorganic cations and anions. To handle the problems seen with buffers, it is usually run with a reverse osmosis scrubber column immediately before the detector, which helps to remove background buffer signal. The growing efficiency of these scrubber columns has allowed this detector to earn a growing application in analysis of organic compounds and polypeptides that show poor sensitivity against UV detectors.

The fasting growing application of a mass detector is in the burgeoning field of LC/MS in which the HPLC is connected through an evaporative interface into a mass spectrometer. The MS detector is by far the most sensitive, versatile, and expensive detector used in an HPLC system. The information it provides can yield a definitive identification of the separated compounds, but requires extensive data acquisition and interpretation, computer treatment, and expertise in operation. It will be covered in more detail in Chapter 15.

Light-scattering detectors have been used with HPLC systems for a number of years for determination of protein and polymer molecular weights based on the ability of these large molecules to scatter incident light proportionally to their size and concentration. Evaporative light scattering detectors (ELSD) have come into increasing use as a universal, mass-based HPLC, GPC, and SFC detector in the last two decades. Effluent is evaporated in a drift tube using a heated gas nebulizer, and light scattered off the droplets formed is measured at a specific angle of offset. These detectors find best application in analysis of things like fatty acid, carbohydrates, and polymers with little UV absorption.

A detector that recently appeared on the HPLC market may provide a nice compliment for the mass spectrometer detection. It is offered as a high-sensitivity universal detector for gradient work using volatile buffers. The Corona™ charged aerosol detector (CAD) uses something like an ion spray nebulizer to evaporate solvents and buffer, then places a charge on the multi-atom droplet that is formed by passing it over a low-voltage charged needle. The charge on the droplet is then measured with an electrometer. It is advertised to provide high-sensitivity detection of carbohydrates, phospholipids, steroids, and peptides that are difficult to measure with a UV detector (Fig. 9.11).

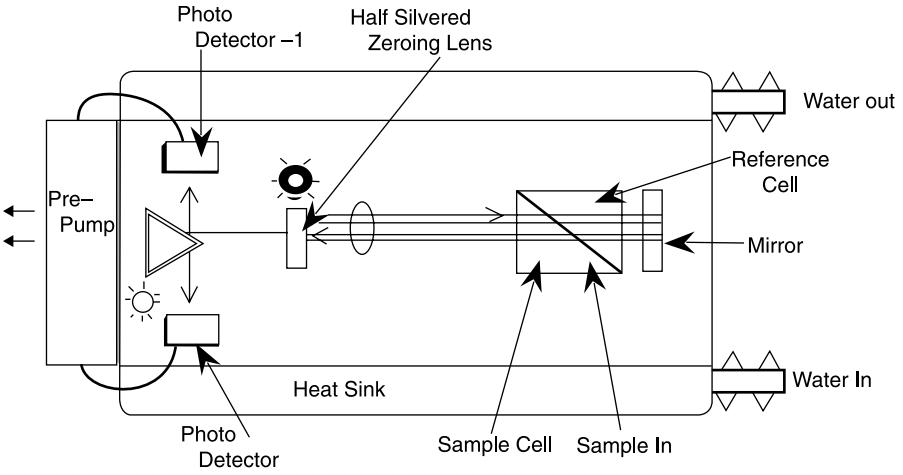

Figure 9.10 A refractive index detector light path.

9.4.2 Absorptive Detectors

The detector of choice for most separations is the UV/visible detector. These are available in two types: filter variable, fixed wavelength, and fully wavelength variable. The fixed-wavelength detector uses only a single wavelength, although this may be changed using filters or selecting lamps with different inherent wavelength output. The most common wavelength used is the 254-nm lamp. Variable UV detectors are more expensive than a fixed-wavelength detector, and cover the whole wavelength range from 195–650 nm using deuterium (UV) and tungsten (visible) lamps as its sources. Fixed-wavelength detectors within 15 nm either side of their wavelength maximum give 4–10 times the sensitivity of the variable detectors. Fixed lamp life is 2–20 times longer and replacement cost is 2–5 times less than for the variable lamp. All of these differences—cost, sensitivity, and lamp life—have narrowed rapidly in the last five years. The variable wavelength detector has become almost an HPLC necessity: it is the detector of choice in almost all laboratories. Light from the lamp passes through a concentrating lens into the flow cell and out to be detected by the photodetector. In the variable detector, the concentrated light falls on a refraction grating, which splits the light into its individual components allowing selection of a specific wavelength to be passed through the flow cell (Fig. 9.12).

UV detectors are affected both by the mass of material present and its extinction coefficient at that wavelength. Some compounds will not absorb light at the wavelength used and will be missed. At present, these detectors can detect compounds, with good extinction coefficients, down to 100 pg. They probably could do better with purer solvents. Compounds with substituted aromatic chromophores usually absorb around 254 nm. Carbonyl compounds and organic acids show “end absorption” at 220 nm; any solvent containing

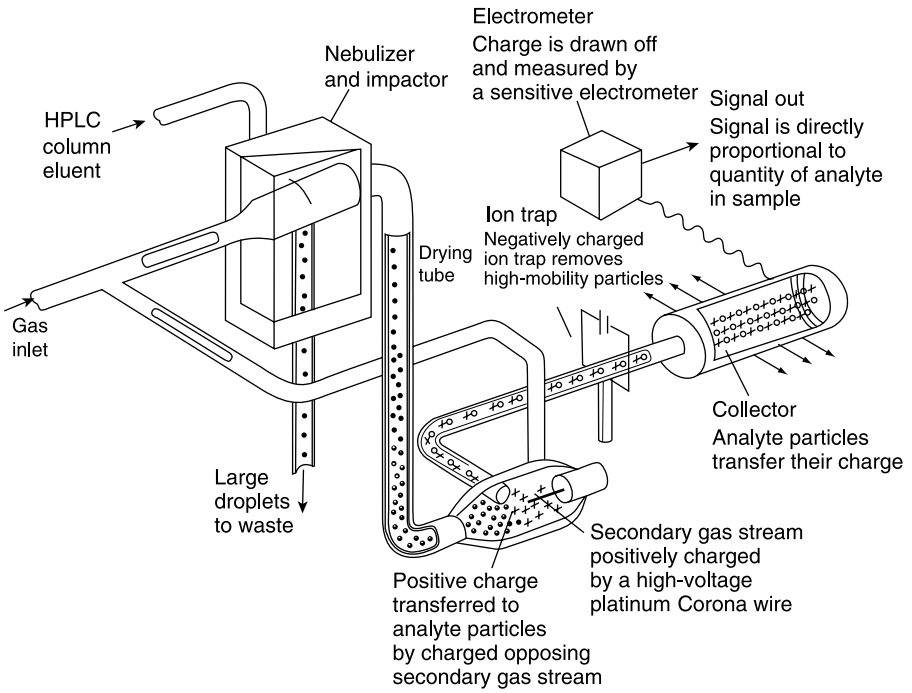

Figure 9.11 Corona charged aerosol detector (CAD). (Courtesy of ESA)

carbon-oxygen bonds absorbs too strongly to be used below 220 nm. Carbohydrates are often detected at 190 nm, but dissolved oxygen in the mobile phase begins to absorb heavily and cuts the sensitivity. UV detectors are also affected by temperature and solvent changes at very high sensitivity but are reasonably unaffected at lower sensitivities. They offer the best, most economical detection for wide ranges of concentrations or types of compounds of any of the commercial detectors.

The newest form of the UV/visible detectors is the diode array model. The diode array detector modifies the position of the refraction grating used in a variable detector, placing it *after* the flow cell, and adds an array of detection cells all looking continuously at a different wavelength of light from the grating (Fig. 9.13). The larger the array, the closer together these wavelengths can be selected. For any time point in the chromatogram, an absorption spectrum can also be displayed from the array data storage.

This may sound like the perfect detector since almost all organic compounds appear to absorb light somewhere in the UV/visible region. It does suffer from a few problems. The most serious problem is real-time data display. With all the information available, it can display only a maximum of two wave-

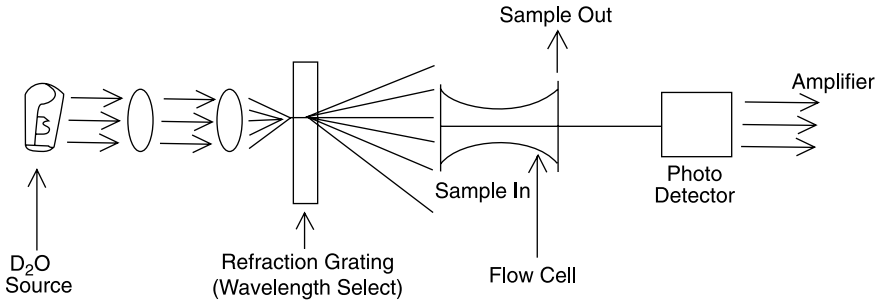

Figure 9.12 A variable UV detector light path.

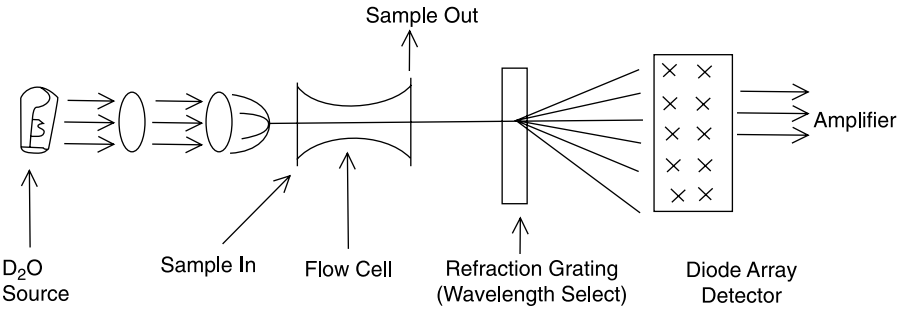

Figure 9.13 A diode array UV detector light path.

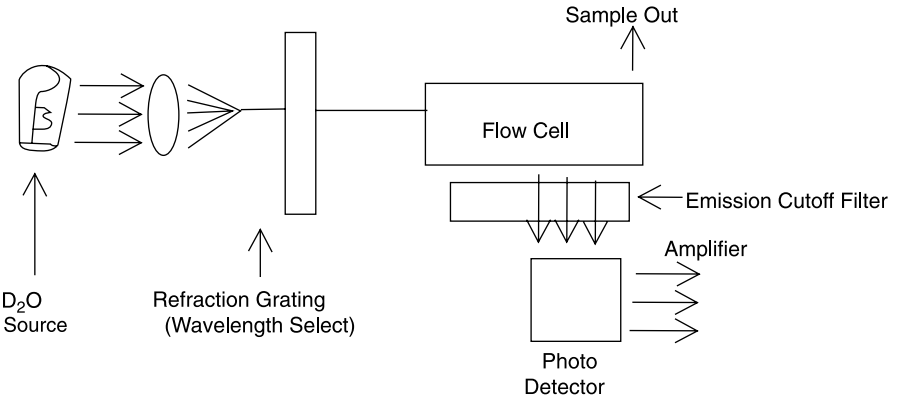

Figure 9.14 A fluorescence detector light path.

lengths at a time on a strip chart recorder or an integrator or up to four to eight small chromatograms at a time on a computer display screen. It becomes a fancy, very expensive variable UV detector. The mass of three-dimensional data (voltage–time–wavelength) output by the array can quickly overwhelm any but the most modern data storage systems. A number of three-dimensional display systems are available to provide *real-time* topological chromatogram maps of this data set, but it is very difficult to extract meaningful information. Each HPLC run requires high-powered computation and display technology (translation: high priced) similar to that needed to process LC/MS data. A secondary problem with this detector is array chatter, which limits sensitivity and generally gets worse as the system ages. The third problem is the cost of the detector and associated data-handling and storage computers, although this seems to be improving as technology advances.

The third type of absorption detectors is the fluorescence detector. Fluorometers are a more sensitive and more specific than a UV detector. A compound, to be detected by a fluorescence detector, must both absorb in the ultraviolet region and fluoresce. Compounds that meet both criteria can generally be detected at 2 to 10 times more sensitivity by a fluorometer than by a UV detector. A fluorometer uses a UV lamp as an excitation source for the sample in the flow cell (Fig. 9.14). Light absorbed at one wavelength is promoted and emitted at a higher wavelength. The photo detector is placed on the cell at a 90° angle to the incident light and a cut-off filter is used to remove light from the emitted light below a certain wavelength. Only the higher wavelength emission light escapes and is detected.

These detectors are often used to detect components of a fluorescent derivative prepared to increase the detection sensitivity of compounds with poor UV absorptions. Both variable and filter variable, fixed-wavelength fluorometers are available for HPLC, with the same limits of lamp life and sensitivity seen in comparable UV detectors.

9.4.3 Specific Detectors

The most sensitive detectors in routine use are the electrochemical (EC) detectors. They also are the most specific detectors, seeing only compounds that are oxidized or reduced at the voltage applied across the flow cell. They need isocratic mobile phases that will carry ions, and most separations are made with reverse-phase or ion-exchange columns in aqueous solvents. Current is applied across the flow cell from an operating electrode to a reference electrode. Control of the compounds to be oxidized (or reduced) is achieved by controlling the applied voltage. Increasing the voltage potential increases the types of compounds that will be oxidized and detected and making the detector less specific. EC detectors have been used to detect 5 pg of rat brain catacholamines, probably close to the current operating sensitivity limit of HPLC detectors.

Occasionally, a laboratory will need an in-line detector of radio-labeled molecules. These detectors take the flow from the column or from an initial detector, mix it with fluorescing compound, and measure the fluorescence due to radioactive breakdown. A different system uses beads in the flow cell with an immobilized fluorescing compound, but these systems suffer from ghosting and cannot be used with very "hot" labeled compounds because of secondary radiation problems. These systems are very useful with tritiated samples and less so with carbon^{14} labeled compounds. Some success has been reported with sulfur^{32} label detection.

Detectors are not limited to solo use; they can be hooked in series to get more information from the same sample. In a serial operation, be sure that the refractive index detector or electrochemical detector is the last in the line. Their flow cells are more fragile than UV and fluorescence cells and won't take the increased back-pressure. Keep the tubing diameter fine and as short as possible to avoid band spreading. You must correct for connecting tubing volume (time) delay in comparing chromatograms from the two detectors.

9.5 FRACTION COLLECTORS

Although not common in organic synthesis laboratory HPLC systems, the fraction collector is an important part of preparative and protein purification systems. It is an automated tray system that is designed to collect sample for a specific time period or collect a specific sample volume, if it has either a drop counter attachment or can sense the pump flow rate. Some fraction collectors have a peak sensing function that can be connected to the sample detector, allowing them to collect only peaks while diverting baseline effluent to waste. It is important that the exit line from the last detector be equipped with a flow-through back-pressure device so that sample can be passed to the fraction collector without too much band spreading or outgassing bubble formation in the flow cell.

Most commercial fraction collectors using a carousel tube storage arrangement with some arrangement for periodic standards check vial injections. Refrigerated housings are available for some fraction collectors allowing long-term holding of temperature sensitive samples.

9.6 DATA COLLECTION AND PROCESSING

Strip-chart recorders, integrators, and computers are all means of storing and/or calculating information generated by the detector. When a compound is detected in the flow cell, the detector sends out a signal with increasing, then decreasing voltage. The recorder, running at a constant chart speed, records this voltage change as a continuous trace versus time.

The integrator and the computer, using A/D converters, change the voltage from a continuous, analog signal to a discrete, step-wise digital signal. The integrator sums the areas under each peak and stores the areas, peak heights, and peak maxima time for each peak. A computer samples the voltage at preset time points and records the sampling rate and each voltage displacement point. Peak detection, integration, and identification are separate computer operations, usually done on the fly and sometimes annotated on the chromatogram. Computer information processing stores more information and requires more memory but allows post-run redisplay and reprocessing of the chromatography information. Because of the low cost of memory, many modern integrators have become dedicated microcomputers and allow post-run reprocessing.

Strip-chart recorders are still the least expensive option, but areas and retention times have to be manually calculated from the tracing. The more expensive integrators, using small memories, give us a time-noted trace followed by a report of areas (or peak heights) versus retention times. The computer requires much more memory to store the one point per second (or more) required for an HPLC run. However, it has much more flexibility in manipulation, redisplay, calculation, and report generation. Data processing will be covered in detail in Chapter 14.

10

TROUBLESHOOTING AND OPTIMIZATION

Troubleshooting is the secret of keeping the wolf away from your laboratory door. Even if you don't want to work on the system yourself, knowing how to quickly find the problem can save you uncounted needless service calls and shorten the time the serviceperson has to charge you for on any given call. Probably as much as 80% of the total of HPLC problems are column problems and 60% of these are due to bad water. If you still are using tap or triple-distilled water, reading the rest of this chapter will simply be a waste of time. Buy HPLC water; you will improve your chromatography and your life.

We will start by reviewing the wetted surface from reservoir to flow cell output. I will discuss the minimum tools and spare parts you should have on hand and when to use them as well as a strange-sounding technique to cut your solvent usage. A systematic approach to locating problems will be presented next. Then we'll look at how to get the most out of data acquisition hardware.

10.1 HARDWARE AND TOOLS—SYSTEM PACIFICATION

The first step in the wetted path in the HPLC is the solvent reservoir holding freshly filtered (and possibly degassed or deoxygenated) solvent. Most systems use a porous fritted "stone" (5–30-μm filter) as a solvent line sinker. The tubing to the solvent inlet is wide-diameter Teflon®. In the solvent inlet line we may have another frit and a sapphire ball/stainless steel check valve. The wetted surfaces in the pumping chamber are all stainless steel except for the plunger

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and the seal. The plunger is made from sapphire (actually it's beryl), it is very resistance to solvents and buffers. The plunger is strong along its long axis, but is fragile to sheer across its diameter. It will accumulate a film from buffers that can be cleaned with toothpaste or light rubbing with scouring compound. The seal is a marvel of technology, without which HPLC would be impossible. It is made of Teflon® containing a hardening agent, such as carbon fibers or ruby. Embedded in the seal is a spring that squeezes the seal against the plunger when the pump head is tightened to the block. The plunger is lubricated by the mobile phase in the pump head, allowing it to slide inside the Teflon® seal. In the outlet line from the pumping chamber is another sapphire ball/stainless steel check valve. The compression fitting at the top of the check valve assembly is connected to 0.02-in tubing leading toward the injector through a pulse dampener and/or a pressure transducer. All the tubing used is a special acid-washed, heavy-wall HPLC tubing.

The pump outlet line usually passes next through a pressure sensor. One type of these is an in-line, double-coiled tube called a *Bordon tube*. As pressure increases, the tube begins to straighten, blocking light falling on a sensor, which is translated into a meter deflection; the more pressure, the more deflection. From the sensor, the tubing travels on to a flush valve allowing venting through a 0.04-in tube to the atmosphere for rapid exchange of solvents. With the valve shut, the flow goes next to the injector.

The injector's wetted surfaces are stainless steel and acid-washed tubing, except for the rotor seal. The seal is a block of beryl or carbon fiber–impregnated Teflon®, like the pump seal, drilled for the loop and bypass pathways. Movement from *inject* to *load* is sealed and lubricated by the Teflon® face plate. The injector outlet is drilled to 0.01 in and equipped with a compression fitting carrying tubing of the same diameter.

The next step in the pathway is the column. The compression fitting on the inlet end-cap leads to the stainless steel frit at the top of the column. The column itself is a heavy-walled stainless steel tube filled with packing and mobile phase. The outlet end is identical to the inlet. Moving on down the wetted surface, we find 0.01-in tubing leading to the detector flow cell.

The detector inlet usually passes through a coiled stainless steel tubing heat exchanger and into the flow cell. The flow cell is the most complicated part in the system. The body is stainless steel or quartz, windows are quartz, and, if it can be taken apart for cleaning, there is usually a Teflon® gasket between the stainless steel body and the quartz window. Finally, we move out of the flow cell into the wide-diameter Teflon® tubing of the outlet tubing and into a back-pressure device in the waste vessel.

The purpose of this tour was to review for you the solvent path from the solvent's point of view. I also wanted to point out that the system is made from resistant material, except for the column bed.

Wear points (and resulting problems) are as follows:

1. Pump: check valves (buffer crystallization), seal (tearing leading to leakage), and plunger (breakage);

2. Injector: rotor seal (tearing—sample carryover) and needle seal (scoring—leakage);
3. Column: (particulates, precipitation in bed, fines);
4. Detector: flow cell (breakage and hazing) and lamp (aging).

Most of the pump problems come from buffer precipitation in going to immiscible solvents, the rest come from normal wear. Plunger breakage may come from buffer accumulation, but is usually the result of removing the pump head without drawing the plunger all the way back. Injector problems come from particulate scouring from buffer crystals and unfiltered samples. Detector problems come from buffer blockage of the outlet tube, outlet tube restrictions (such as fingers held on the end), and sample decomposition on flow cell windows.

Realizing that 80% of all system problems are column problems, it becomes clear from the above that the next major cause of problems is "doing something dumb." Buffer precipitation on the back of the plunger will usually cut seal life in half compared with use with buffer free mobile phase. Generally, buffer crystallization in lines and filters is caused by failing to go through an intermediate solvent. This leads to buffer crystals thoughout the system, which causes wear on any moving surface and plugs lines.

Since prevention is the answer, but is often ineffective, how do we deal with these problems? The first step is to get the column out of the system. Second, to treat the whole system we need to replace it with something to provide a liquid path across the missing column (Fig. 10.1). Most systems are designed to operate best under some back-pressure. I generally replace the column with a column blank. This is easily made from tubing, unions, and compression fittings. I have found that 1 foot of 0.01-in tubing equals about 50 psi back-pressure at 1 mL/min of acetonitrile. Please check this since tightening of

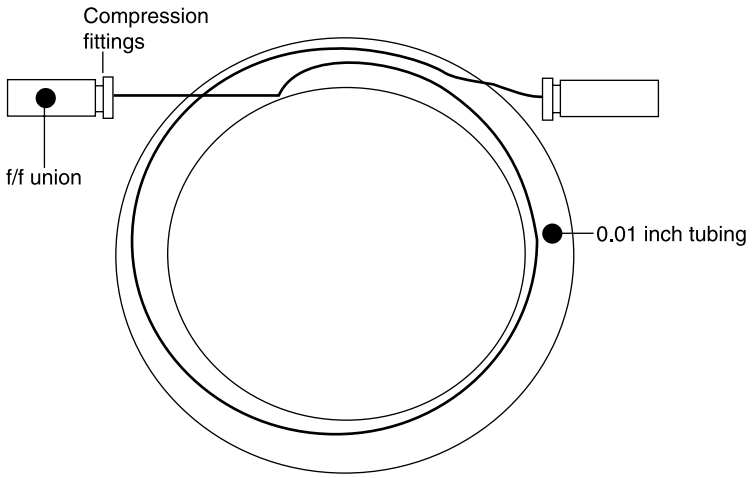

Figure 10.1 Column blank for pacification.

compression fittings will vary and can change the back-pressure. With compression fittings and unions, you should be able to match the ends of your column exactly.

With the column blank in place, you can now proceed to check out the mechanical system unhindered by overriding column effects. Check valve problems can usually be seen on the strip chart recording magnified by the low back-pressure. Injection of a standard into the system should give an instant response. This signal can be used to diagnosis detector, injector, and recorder problems. I personally feel a column blank belongs in every HPLC tool bag. It is an ideal tool for system washing with harsh reagents that would seriously damage the column.

With the column blank in place, we are ready to do a general system cleaning. The technique is called *pacification*. It involves washing the system with 20% nitric acid. It sounds harsh and is, but your system should be resistant to it. Check with your manual to ensure that all wetted surfaces are stainless steel, ruby, Teflon®, and quartz. If you can't tell from the manual, call the manufacturer. Use pacification only with UV detectors, I can't guarantee other detectors will be resistant. *Do not use pacification on mass spectrometers or their interfaces!*

Before we proceed, make sure you have *removed the column*. Trying to do pacification with a column in the system will make a mess and, at the least, ruin the column. After removing the column and inserting the column blank, wash with water for 15 min at 2 mL/min to remove any buffer. Follow this with 20% HNO_3 (6 N nitric acid) for 15 min at 2 mL/min, then wash out with water until neutral pH is reached. This last step takes a long time.

I usually recommend doing pacification on the last Friday of the month on a routine basis. Starting late in the afternoon, you wash with water, *remove the column*, replace it with the column bridge, wash with acid and then water, then let the system run over the weekend at a slow (0.1 mL/min) flow (leave at least 600 mL in the flask when you leave Friday). On Monday, check pH, replace the column, reequilibrate, and run your standards.

Pacification *after removing the column* is an excellent cleaning technique for the whole system. It tends to remove buffer precipitation from check valves, organics from the injector rotor, and deposits on detector flow cell windows. Such a treatment is recommended to protect the system from halide attack on stainless steel. Experienced protein people have told me that pacification will protect a system against 200 mM NaCl for a month. This assumes you wash out the NaCl before you shut the system down.

Again, please check your owner's manuals or talk to the manufacturer of your system before attempting pacification. All the systems I have worked with have been resistant to this treatment. They are built from stainless steel, Teflon®, sapphire, ruby, and quartz, all of which are resistant to this concentration of HNO_3 for short periods of time.

However, there are some weird and wonderful machines that may not be able to stand the treatment. Finally, I'd like to remind you one more time to

remove the column before attempting to pacify your system. These reminders may seem excessive, but I had a student pacify a C_{18} column after being warned six times in class. Please do not make the same mistake!

Other tools you will need for HPLC are fairly standard for a lab with a few exceptions. I keep two of the small open wrenches supplied with an HPLC system or a column for use with compression fittings, 1/4-in, a 5/8-in box wrench, and a 6-in crescent wrench for column work, a file, a Terry® tool tubing cutter, a couple of pairs of blunt nose pliers for tube flexing during cutting, a reversible screw driver, a jeweler's screwdriver for phoenix block's screws, a universal Allen wrench set, an adjustable-head dental mirror (for seeing behind boxes to hook up leads), and a stopwatch for timing flow. The key is to lock up your tool kit to prevent tool evaporation.

Spare parts that are needed are compression fittings and ferrules, plunger and injector rotor seals, an extra plunger and seals, column filters, and injector needle port seals. If you do not use pacification, you might want to keep a set of check valves on hand. I always have one coil each of 0.01- and 0.02-in tubing in addition to my column blank. A replacement solvent inlet line with a porous stone is useful in case of corrosion. If you filter solvents, you need cellulose, nylon, and Teflon® filters. You also need a back-up lamp for your detector.

If it takes a while to get replacements, double the amounts of the preceding parts and add a detector flow cell, another C_{18} column, and a full pump head. If you are going to Antarctica for the season, an extra injector, pump, detector, strip chart recorder, and a case each of strip chart paper and pens might be nice. One of my customers found that his back-order time in Little America (Antarctica) was 14 months.

10.2 REVERSE ORDER DIAGNOSIS

The most commonly reported symptom of a bad system is baseline drift; the second is a noisy baseline. Baselines that drift up and down are almost always due to peaks coming off the column. Baselines that drift up continuously can be garbage, a bad detector lamp, or decomposition on the flow cell window. Noise can come from almost anywhere in the system.

When you have a problem, do not guess as to where it might be. Start looking for problems from the strip chart end of the system. Prove that the strip chart is good, then use it to check the detector, and use the detector to check the injector and finally the pump. Using this systematic, reverse-order analysis will save you time and frustration.

As we said in the column section, the first step is to remove the column and see if the problem goes away. Pressure problems can be determined by looking at the pump pressure. Other problems can be diagnosed from the detector's digital display and the recorder baseline. Of course, with the column gone, there is no path to the detector. Here is where the column blank shows

its versatility. When we remove the column, we replace it with the column blank. Now we can run the pump, make injections, and see the effects on the detector/strip chart. The 5 feet of tubing allows the pump to run with enough back-pressure to close check valves, but not as much as the column; pumping problems are magnified and injections fly through the detector.

The strip chart is the most ignored part of the system, but it can contribute its share of problems. Usually people will spend $20,000 for an HPLC, then grab some old strip chart off the shelf, blow of the dust, hook it up, and wonder why things look terrible. I had a customer who did exactly that, then called complaining that his pumps were varying by 10% in flow rate and his chromatograms were not reproducible. I went into his lab, disconnected the HPLC, and timed the strip chart bed speed at 0.5 cm/min. We found it varying by 10% on either side of this speed. It had been used at 1–2 cm/min on a GC for years and somewhere a spring was overstretched.

There are two modules in a strip chart: the electronics and the mechanical. The trick is to look at one at a time. Disconnect the detector leads, turn off the bed, and watch the pen. Does it sit quietly or chatter up and down? Noise at this point comes from the strip chart electronics. If it's quiet, turn on the bed and let the pen trace to see if the baseline is flat. If so, short across the leads and make sure the pen deflects without sticking (this would show up as a plateau in a chromatogram). Lubrication or drive wire replacement would fix the problem. Do not get much oil on the slide bar; it just traps dust. Spray some WD40 on a Kimwipe. Wipe the bar, then wipe off the excess. Next, use a stopwatch and time the bed. Is it accurate at 0.5 cm/min where you will be using it? If it passes these tests, we're ready to hook up the detector leads and move on.

The detector also has two modules: the electronic, including the lamp, and the flow cell. Flush the flow cell with strong solvent, then turn off the flow, and observe the strip chart signal. An old lamp and a noisy baseline might indicate a lamp replacement is needed. Tungsten lamps used in fixed-wavelength UV detectors are good for 1,000 hrs; old variable lamps for 250 hrs or less depending on the wavelength. If you change the lamp and the baseline stays noisy, suspect the electronics. If the baseline continuously rises, you may have a compound coated on the flow cell window that is decomposing under UV excitation. Some older model UV detectors, like refractive index detectors, are very temperature sensitive and the baseline will follow air conditioner cycles, daily window temperature effects, or drafts. The baseline effects are fairly long and you may have to shield the detector or thermostat it.

If the detector passes the static test, turn on the flow and watch the baseline. Noisy baseline at this point is probably coming from before the detector. Realize that a reciprocating pump is noisy and it now lacks pulse dampening from the column. While we're here, shoot a sample; response should be instantaneous, straight up and down. I have not tried it, but you might be able to quantitate lamp strength by shooting a known standard over a time period and measure the deflection.

The next component to check is the injector. Usually, only three things happen: leaks and plugs, which are immediately obvious, loop contamination, and carry over. Plugging can be localized by working backwards from the column connection port through the loop connections, with the injector first in the *inject* position, then in the *load* position with the pump running until the pressure drops. If the problem is in the loop, reverse the loop and use pump pressure to blow the plug out into a beaker.

Once flow is cleared, shoot the injector, that is, just throw the handle into the *inject* position. Do you see a small positive peak? Next, shoot a normal injection size of a strong solvent to see if it gives a peak. If both show peaks, you may have a sample loop that needs cleaning. Next, shoot a sample of a good UV absorber, and then shoot the injector without putting in new sample. Do you get a "volunteer peak" without shooting a new sample? A scratch on the injector rotor face can cause sample carry over and may be causing this ghost peak. This indicates that the rotor seal needs to be replaced.

The last module to be inspected is the pump. Leaking is the most obvious problem. There is always some flow around the plunger, but this evaporates with volatile solvents. With buffers, this leads to a weeping down the face of the pump, which is messy, but also harmless if washed off periodically. When the seal goes, solvent comes out rapidly and pressure cannot be maintained. Stop the pump with the plunger back, loosen the screws holding the head in place, and carefully slide the head off the plunger. Dig out the old seal, clean the plunger with mild abrasive if it is needed, and replace the seal and the pump head.

If the pump does not deliver solvent, do not immediately assume that the plunger is broken. Open the compression fitting at the top of the check valve to release pressure. Pressurize the inlet line with a syringe full of solvent and turn the pump on. Often, an air bubble will cause the pump to cavitate and stall. This is often a problem with pumps having fine filters in the inlet check valve. This technique should cause air bubbles to come out of the outlet fitting. Simply reseat the compression fitting and go back to work.

If the head does not leak and is delivering solvent, catch solvent in a graduated cylinder and time the flow with a stopwatch. If you get much less than the calibrated flow, your outlet check valve may be dirty, allowing back flow. Place the inlet solvent sinker in a graduated cylinder partially filled solvent and watch the surface of your reservoir. If it rises and falls repeatedly during pumping and you can get pumping pressure, you may have a dirty inlet check valve. Both of these problems can be treated with pacification if caught early enough; otherwise, they may require replacement of the check valve(s).

You now have the tools to find most of the problems in the system. Remember that 80% of the problems are column problems and about 60% of those come from dirty water. If the problem fails to leave with removing the column and putting in a column blank, then do reverse-order diagnosis. It will be amazing how much time you save.

10.3 INTRODUCTION TO DATA ACQUISITION

Inexpensive integrator systems have made the chromatographer's life easier in one sense, but complicated it in another. The computer extends this dichotomy. I'll discuss briefly the variables that must be controlled in an integrator and how to use it in a research versus a clinical environment. I'll also explain when and why you might want to hook up to a computer, if that decision was not made for you when you bought your system.

The output from a detector is a voltage that varies with changes in concentration and the compounds' extinction coefficient. In a strip chart recorder, this voltage change is plotted as a vertical displacement versus time at a constant chart speed. We can measure a peak's area from the vertical maximum and the peak width. The integrator plots the same output, but time-stamps the peak maxima. Internally, for each peak, it stores in memory two numbers: the peak area (summed from all the points from leaving baseline to finding it again) and the maxima. At the end of the chromatogram, it reports these areas versus time, sums all areas, and reports each as a percent of the total.

The computer handles data differently. It measures the voltage at a preset time interval and stores each displacement as a digital word, usually at a rate of 10–20 points/sec. The computer requires much more memory than the integrator to store a single chromatogram, however, it can use this raw data to report tables of peak maxima, areas, and heights versus time and recalculate, redisplay, and compare chromatograms.

If you're using the integrator for scouting or research runs, where run-to-run reproducibility is not critical, it is very easy to initialize conditions. Most integrators have a slope test or calibrate button. When you push the button, the integrator looks at the baseline for about a minute and sets all its variables. The only time you need to recalibrate is when you change the detector sensitivity. Integration is started either by a signal from the injector or by a time programmable delay triggered by the injector or the start button.

When you are in a clinical environment where reproducibility is critical, you need to know and understand the variables that the calibration selects. An integrator should be able to integrate close neighbors about four times more accurately than you can by hand. The integrator sets three variable levels: peak width, slope rejection, and noise rejections. It also makes decisions on how to integrate unresolved peaks. As peaks widen (or narrow) in later parts of the chromatogram, the integrator doubles (or halves) the peak width value to include the whole peak.

To achieve maximum reproducibility you must make these decisions instead of leaving them to the machine. You will have to do a methods development project. Turn off the automatics, shoot a sample, adjust a variable, and then repeat until you have it right. When setting variables manually, we first set the peak width followed by slope rejection and then noise rejection. If peak width is correct, the print gap, left when the integrator prints the retention time, will fall half way down the backside of the peak. If it falls closer to the

top or on the front side, the peak width is too narrow. Normally, 5 msec is the preset and will handle fast eluting peaks. The integrator is designed to automatically double this setting as late running peaks broaden by diffusion. If you are running gradients, peaks may sharpen instead of broadening and the peak width value may have to be shortened. You can usually override this function and program timed peak doubling (or halving), so each chromatogram is handled the same.

When the machine automatically looks at the baseline, it finds the largest peak and uses its front slope to set slope rejection. Any slope greater than that is a peak, is time stamped, and is integrated. Once the slope detect is set, the integrator averages the heights of the baseline's peak top level and uses this to set noise rejection. If slope rejection is too high, the integration start and stop marks will fall upon the peak shoulders. If noise rejection is too low, you get an almost continuous printout of retention times even though peaks may not be seen.

Unresolved peaks pose a real problem for the integrator. Depending on their size and the baseline slope, the integrator may do a vertical drop to baseline from the minimum between peaks, do tangential skim, or draw the baseline valley to valley and integrate. It may do it differently from run to run. You can make a decision on each peak pair and build a time program forcing the same type of integration each time.

The basic difference between the integrator and the computer is that the latter stores more information and can be more flexible with how it handles it. The computer, with the right software, can regenerate all or part of the run with all the stored points that make up the chromatogram, blow it up or shrink it down, leave out extraneous peaks, or do a point-by-point comparison with other curves. Flexibility adds complications; setting up and using the computer is more complicated and costly than an integrator.

10.4 SOLVENT CONSERVATION

At the start of the chapter, I promised to describe a technique for solvent use reduction. A customer in a government laboratory that I called on cut his solvent use by a factor of 5. He started with 5 L of fresh mobile phase, pumped it through the column, made his injection, and recorded his chromatogram while recycling his waste liquid to his stirred solvent reservoir. He autozeroed his detector baseline before each new injection. If you have an injection-sensing switch connected to your injector or autosampler, it can be used to autozero the detector automatically. After pumping a total of 25 L of mobile phase through the column, he discarded his solvent and started again with a fresh batch.

This sounds very unsanitary, but it works well. It can only be used for isocratic runs, not for gradients. Injection samples (nanograms per microliter) introduced into the 5-L reservoir only slowly increase the baseline absorption levels.

Commercial mobile phase recyclers use peak detection from either a computer or an integrator to signal baseline acquisition. A waste/recycle valve is triggered by this signal to discard peak containing mobile phase and recycle the baseline. There is little solvent recovery for chromatograms containing many closely spaced peaks, but for normal chromatography or in cases where the HPLC is always left running this might represent 40–50% solvent recovery. Estimates have appeared in the literature that claim that for a quality control instrument run on a single shift this would mean a savings of about 60 L per year at a savings of about $3,000/yr in solvent, man power, and environmental impact. Obviously, the savings would be higher in stat instruments running in clinical laboratories and in quality control laboratories that operate on a three-shift schedule.

III

HPLC UTILIZATION

11

PREPARATIVE CHROMATOGRAPHY

The preparative use of HPLC is often overlooked in the rush to analyze. There are two outputs. The detector's electrical signal to the strip chart or computer and the liquid output normally sent to waste. Most columns and detectors are nondestructive; aqueous samples can be run without derivatization. The same system used for analysis can be used for all but the largest preparative runs. Usually, all that is required is a change of columns and a slight modification of the running conditions to accommodate the increased sample concentration and load (Table 11.1).

Speed, load, and resolution are the three trade-off considerations that must be balanced to optimize the three levels of preparative runs (Fig. 11.1). Analytical preparative is concerned with isolation of up to microgram quantities of material and with obtaining enough material for spectrometric analysis; the most important factors are speed and resolution.

As we move to semipreparative separation, in the milligram range, we are usually purifying analytical standards of recovering impurities to do trace compound analysis. Resolution is still very important, but now load, not speed, is the trade-off. "True" preparative at the gram level can be run using a semipreparative column in an analytical system by making multiple sequential injections and collecting and combining similar fractions from each chromatogram. True preparative is usually run, however, on an HPLC system optimized for preparative runs with a much higher flow rate. Load is the major tradeoff; speed is secondary, with resolution the least important. Here we are gathering grams of material for biological testing and structural analysis, and as reaction intermediates.

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Table 11.1 A Guide to Preparative Scale-up

	Analytical	Semipreparative	Preparative
Size	4.6 mm × 25 cm	10 mm × 25 cm	25 mm × 25 cm
Packing (μm)	3, 5, 10	5	40
Max. load	150 μg	100 mg	5 g
Flow rate	1 mL/min	4.5 mL/min	30 mL/min
Resolution	1	0.8	0.3
User	Clinical Analytical	EPA/Process	Standards Organic Synth.

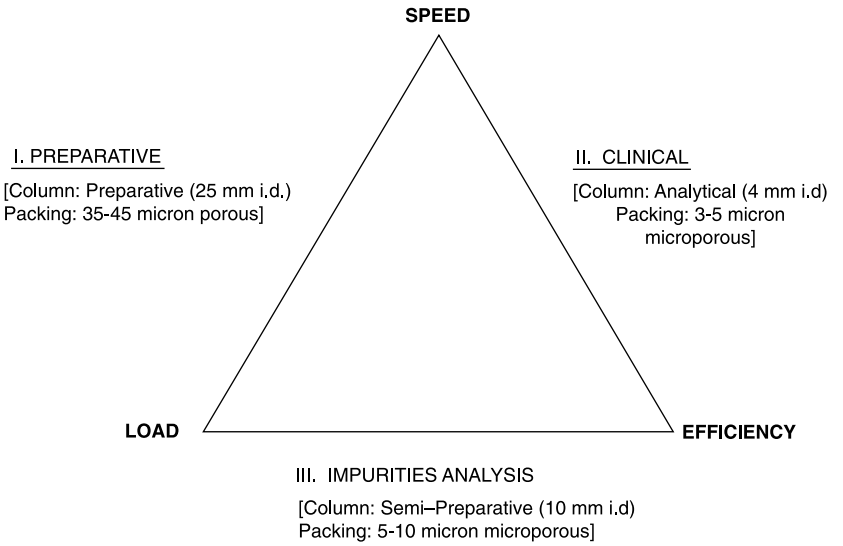

Figure 11.1 Speed–load–resolution preparative decision triangle.

11.1 ANALYTICAL PREPARATIVE

These separations are run at 1.0–2.0 mL/min on the same 4.5-mm × 25-cm column used for our analytical runs. Normally, in analysis we shoot from picogram to nanogram quantities. Most separations maintain their resolution until we reach an injection quantity of about 1 μg. The valleys between peaks begin to rise indicating some overlapping.

If we increase our first peak k' to 8–10, we can increase the interpeak gap allowing us to load to about 10 μg of compound/injection. As we increase the amount of sample, we need to go to lower detector sensitivity. We can increase flow rate to 2.0 mL/min, but we will lose some resolution by doing so. Generally, we have no problem increasing sample concentration and keeping the same injection loop size. If necessary, we can increase to the next size larger loop without affecting resolution.

If we have to increase load higher, say to obtain a 50-μg sample for NMR analysis, we can use the shave/recycle technique to be described in the "true" preparative section. These runs must be made isocratic and column overload occurs at 100–150 μg for most compounds. If this much material is needed, it is better to switch to a semipreparative column, which can easily produce milligram quantities in a single pass.

11.2 SEMIPREPARATIVE

Semipreparative separations are made on a 10-mm × 25-cm column packed with the same 5- or 10-μm packing used in the analytical separation. Simply replace the column and equilibrate with the analytical mobile phase used in analysis. A 1–5-mg sample can be injected with a flow rate, FR_2, calculated from the following formula:

$$FR_2 = FR_1 \times (D_2/D_1)^2$$

Where FR_1 is the analytical flow rate, D_2 is the semipreparative column diameter, and D_1 is the diameter of the equivalent analytical column and we use these to calculate the square of the column diameters differences. With our 10-cm column we would use a flow rate of 5 mL/min.

By using solvent polarity techniques to increase k' we can push the load to 20 mg. Going isocratic and using shave/recycle, the load can be increased to 100 mg with column overload occurring at 200–300 mg injections.

11.3 "TRUE" PREPARATIVE

Preparative separations in the grams per injection level are different. Separations are run isocratic in 1- to 3-in columns with large pore, fully porous packings (35–60 μm). An analytical, two-pump system can just barely reach the 20-mL/min flow rates needed to run a 1-in column. Special preparative HPLC systems deliver flow rates of 50–500 mL/min to handle the larger bore columns. A stream splitter is used to send part of the flow through a refractive index detector with a flow cell designed for concentrated solutions.

Injection samples need to be as concentrated as possible and this leads to problems. A column acts as a sample concentrator. If the solution starts out saturated, it will supersaturate on the column, precipitate, and plug the column. I have seen a column with a 3-cm-deep plug that had to be bored out with a drill bit and a spatula. A couple of injector loops full of the stronger solvent in a mixed mobile phase will clear this if there is still some flow, but the separation will have to be repeated. It is better to dissolve the compound, then add a half volume of additional solvent, ensuring that there will be no precipitation on injection.

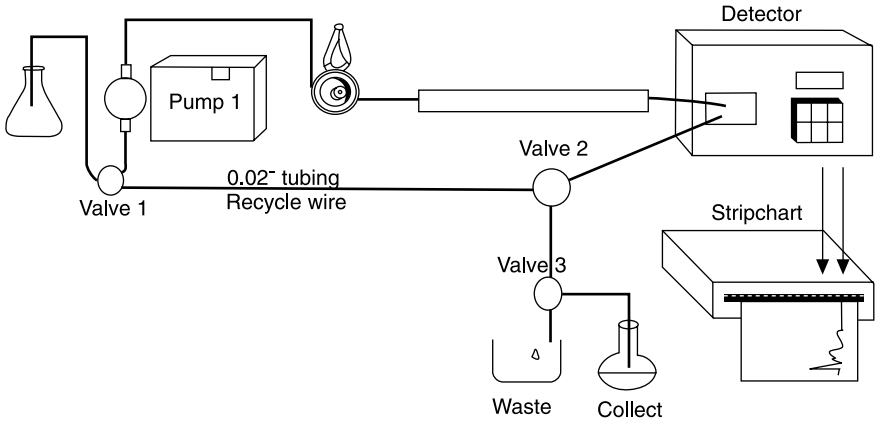

Figure 11.2 Recycle system.

A technique called *shave/recycle*, mentioned earlier, allows separtion of a pair of close resolving peaks. To use shave/recycle, it is necessary to plumb the HPLC system so that the output from the detector can be returned to the HPLC pumps through small diameter tubing and switching valves (Fig. 11.2). Twenty-thousandths tubing is used to connect the detector output to valve 2, the waste recycle valve; 0.02-in tubing connects from valve 2 to valve 1, the solvent select valve; and, finally, a third valve 3, the collect valve, can be placed in the waste line from valve 2.

The analytical separation is used as a guide to selecting load conditions for the preparative run. First, k' (Fig 11.3a) is increased until the first peak of the desired pair of peaks to be separated comes off with a $k' = 10$ (Fig 11.3 b). Load is scaled up until the valley between the two peaks is just visible (Fig. 11.3c). If there are peaks running later than our target pair, we will have to inject the sample and collect the fraction containing the compounds of interest for reinjection. If the only impurities come off before the target pair, the impurities can be discarded after making the shave/recycle injection. The preparative instrument can be a little intimidating to run because things happen so fast at maximum flow rate. With a top flow rate of 500 mL/min, a liter flask is filled in 2 min. The first time you run the analysis, I would suggest using the slowest flow rate possible to acclimate yourself.

To begin a run (Fig. 11.3d), a sample must first be injected with valve 1 turned to the reservoir and valves 2 and 3 to waste. You can use either a very large loop and valve injector or a stop flow injection in which the sample solution is pushed through the solvent line through an injection port, or you can pump the sample in using either the main HPLC pump or an analytical, loading pump plumbed in through a three-way valve 1. After injection (Fig. 11.3d), you will see the void volume peak followed by any early running impurities, which are all run to waste. If you are using a loop and valve injector,

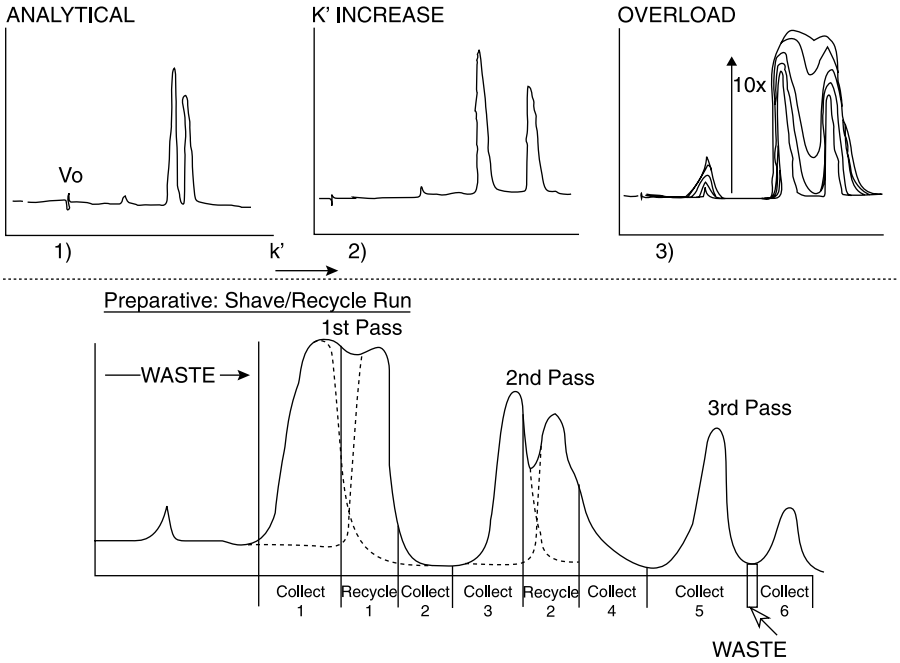

Figure 11.3 Shave recycle. (a) Analytical; (b) *k′* increase; (c) overload; (d) preparative shave/recycle.

wash through the loop with six-loop volumes of mobile phase, then *turn the handle back into the load position*. This is important because it removes a major source of dead volume from the recycle pathway.

As your first peak begins to elute, switch to collection of fraction 1 by turning valve 3 to collect. Continue to collect until you are over the maxima of peak A and one-third of the way down to the valley between the peaks. (Peak A tails badly into peak B, but there is little of B in peak A until we get well into the rear slope of A. Be brave, you can always reinject if your analytical system shows you were too late in your cut.)

At this point, switch to recycle by turning valve 1 and 2 at the same time; switch valve 3 to waste. We want to send the contaminated portion between peaks back through the pump head back to the column head for further separation. We continue to recycle until the detector shows we are well down on the backside of peak B. (Remember, A is tailing into B.) Change the collection flask while we are recycling to collect flask 2. Shut recycle valves 1 and 2 and switch valve 3 to collect in flask 2. Stop collecting 2 when we reach the baseline on the recorder and switch valve 3 back to waste and change to a clean collection flask 3.

Very quickly, peak A should begin emerging from its second pass through the column. Switch valve 3 and begin collecting faction 3 in its clean flask. We

will continue the cycle: 1) collect peak A, 2) recycle the middle by opening valves 1 and 2 and switching valve 3 to waste, and 3) close valves 1 and 2 and switch valve 3 from waste to collect the next fraction of B in a clean flask, until we have separated the peaks (three passes is usually enough to reach baseline). The slow flow rate is fine for the beginners, but you will find yourself quickly using only the maximum flow rate of the systems. Have plenty of volunteers on hand. People will be rushing around with flasks of collected sample trying to get to a flash evaporator before sample starts to crystallize.

Once all fractions are collected, we can take them to our analytical apparatus to ensure purity, then combine odd fractions—1,3,5, and so on—for recovery for peak A, and even fractions—2,4,6—for recovery of peak B. Volatile, organic solvents can be rotary evaporated for sample and solvent recovery.

Evaporation of large volumes of water mixture from samples eluted from reverse-phase columns can be very time consuming. Instead, you can use the preparative HPLC to recover pure compounds from aqueous solution. Dilute the combined fractions from peak A 5- to 10-fold with water and pump them back onto the column, either through the injector or through the pump. Dilution increases the compound's k', causing it to be retained strongly at the column head. Then, immediately elute and collect the compound with a strong solvent like methanol. Your sample can now be recovered rapidly by rotary evaporation from the relatively small volume of strong solvent needed for this elution. Each purified compound can be recovered in turn using the same technique.

A commercial customer had four compounds to recover from a synthesis mixture; they separated as two pairs of compounds. They injected and collected each pair together, then diluted each pair with water, reinjected onto the reverse-phase column, and ran shave/recycle. Using this technique they purified 50 gm of each compound in the two injections of the pair fractions on the 3-in column.

12

SAMPLE PREPARATION AND METHODS DEVELOPMENT

12.1 SAMPLE PREPARATION

Sample preparation is the key to getting the most out of an HPLC system. Unless you are working with purified standards or examining a compound in a very pure matrix, your chromatography becomes complicated with extraneous compounds.

A biological matrix such as serum provides a good example. Nature generally tends to make metabolites more polar than the original compound. These polars exist to aid in elimination and excretion as well as serving as building blocks and reaction components. At the same time, nonpolar molecules are present in transport and structural roles and end up in the circulating blood.

The effect on chromatography is to complicate the separation greatly. If we consider a reverse-phase separation, the first thing we notice is an almost irreversible binding of protein to the column. Even after protein removal, we find polar peaks, which overload the early part of the chromatogram and tail into the compounds of interest. The components that are more nonpolar than our compounds of interest adhere to the column and must be washed off before the next injection. To ensure polar elution before our target compounds and nonpolar removal afterwards, we are almost forced to run solvent gradients.

Sample preparation techniques are aimed at removal of as many of these extraneous materials as possible before injection onto the column. The expected result should be a dramatic reduction in run times, hopefully to a fast running isocratic separation instead of a gradient. A side benefit of much of this sample preparation is often trace enrichment, an increase in sample concentration with a corresponding increase in detectability.

HPLC: A Practical User's Guide, Second Edition, by Marvin C. McMaster

The first step in preparing a sample for injection is to ensure it is completely dissolved and to remove particulate matter. If we are working with blood samples, we need to get rid of blood cells, which is done by centrifugation. Be aware that in removing both particulates and red blood cells, there is a chance that you may remove some of your compound of interest. This needs to be checked by adding known amounts of your target compounds to a representative blood sample, removing the interfering materials, and then quantitatively checking your recovery of added standards.

12.1.1 Deproteination

The next general step is to remove charged molecules that interact with silica. Since silicic acid is a weak cationic ion exchanger, the compounds we are trying to remove will be positively charged. In serum, the most common of these are proteins, especially nonpolar proteins such as albumin. Proteins also can interact with bonded-phase columns through nonpolar partitioning. It is usually best to avoid putting them on the column since removal is difficult and time consuming.

As mentioned earlier, proteins can be removed by ultrafiltration through a very fine membrane filter. Ultracentrifugation at high speeds can also be used to separate proteins from smaller molecules based on size differences. The most commonly used protein removal techniques for HPLC involve protein denaturation. Heating denatures most proteins. If the compounds to be separated are temperature resistant, the crude mixture remaining can be boiled and then filtered or centrifuged. Particulates and denatured protein are removed together.

Chemical denaturation of proteins tends to be more efficient and less harmful to sensitive compounds. Acidification with trichloracetic acid (TCA; 5% in final solution), centrifugation to remove protein, and neutralization with sodium hydroxide remove better than 99% of the protein. A second reagent used for protein precipitation is perchloracetic acid. After protein precipitation occurs, excess perchloracetic acid is precipitated as $KClO_4$ by neutralization with potassium hydroxide. Both of these acid treatments, however, suffer from problems. TCA absorbs strongly below 230 nm, eliminating the use of low-wavelength detection. The perchloracetic acid treatment leaves large amount of salt in solution, which can precipitate with organic solvents or cause major early refractive index upsets of the UV baseline.

Probably the best chemical precipitant for use in HPLC is acetonitrile. Acetonitrile has the advantages of being a common solvent for HPLC and of being UV transparent to 190 nm. Mixing and centrifugation of an equal volume of plasma sample solution and acetonitrile will lead to precipitation of about 95% of the proteins; nonpolar proteins, such as the albumins, remain in the liquid phase. The supernatant can be injected directly if a guard column is used to remove the last 5% of the protein. The guard column will need to be repacked or inverted and washed out to a beaker periodically to prevent protein breakthrough to the main column.

12.1.2 Extraction and Concentration

The next step is to obtain the compounds of interest free of interferences. This has traditionally been done by extracting only interfering compounds from the solution, leaving all others behind, an ideal, but seldom realized goal.

Extraction of nonpolar compounds using equal volumes of sample and the Folsch mixture (2:1, chloroform/MeOH) gives a very broad polarity cut. Everything from steroids to triglycerides is pulled down into the bottom chloroform-rich layer. Extraction with methylene chloride from a sample acidified with sulfuric acid is more specific, pulling in steroids, fat-soluble vitamins, and free fatty acids. The triglyceride fraction can be extracted using i-PrOH/hexane (1:9) with little emulsification.

After extraction, these fractions should be dried to remove water. When dry, the extraction solvent is removed by evaporation and the sample is reconstituted with a solvent or mobile phase before injection. Care must be taken that these evaporated samples go completely back into solution. Sonicating the sample with your starting mobile phase is usually sufficient. However, at least the first time you perform an extraction, it is always good technique to sonicate the dry-down tube with a strong solvent and reinject this wash as a check that everything redissolved. For gradient work, the stronger of the two mobile phases is an excellent choice for this second sonication solvent.

It is always a good idea to make sure particulates are removed from these sonicates, or for that matter from any sample. Using centrifugation or filtration as a last step before injection protects the column filter from plugging and the system from pressure build-up.

12.1.3 SFE (Cartridge Column) Preparations

One of the most useful additions to the chromatographer's armament has been the off-line precolumn, sometimes referred to as SPE (sample preparation and extraction) columns. These disposable, low-pressure cartridge columns contain large particle packings with the same bonded-phase material as the HPLC column. The theory is that anything that will go through them will go through the HPLC column. Because they are silica based, protein sticks to them very tightly. The 50% acetonitrile solution prepared above can be passed down one of these cartridge columns to remove the last traces of protein.

Cartridge columns consist of 0.5–1 g of 40-μm particles sandwiched between 30-μm frits. The column's body may be a tube shrunk around a sandwich of bed support frits and packing. In another type, the frits and packing may be pushed into a small syringe barrel. The 0.5-g SFE has a sample capacity of about 25 mg and a void volume of about 1.5 mL.

The SFE can also be used for extractions. Reverse-phase cartridges are available that, after activation with methanol, can be used to remove nonpolar materials from solution. This is true chromatography, not filtration. The adhering material can be eluted in step-gradient fashion with increasing nonpolar solvent fractions. Although they do not have the efficiency of an HPLC

column, they can separate classes of materials. SFE cartridges are not limited to only C_{18}; you can buy C_8, phenyl, diol, and other intermediate polarity SFE cartridges from a number of companies.

For partition extraction, it is important that the compound is in its uncharged form. Extraction of organic acids from aqueous media is easier if you first acidicfy with sulfuric acid. Bases are easier to extract above pH 11. Most compounds are more volatile in their uncharged form, so be careful during removal of the extraction solvent. The C_2 to C_4 organic acids are often lost during evaporation of methylene chloride under a heated nitrogen needle dryer. If you plan to derivatize for increase sensitivity, add the coupling base to the methylene chloride solution before evaporation. The organic salt formed is much less volatile and will stay in the extraction tube.

A technique that works well in extraction of charged molecules is the use of ion-pairing reagents. These counterion compounds are used in the chromatography to make charged polar compounds appear more nonpolar so that they adhere to a C_{18} column. The same technique will work when extracting with organic solvents or with nonpolar cartridge columns. A word of warning, however, is needed: Unless you are planning to run ion-pair chromatography on the compounds in your main HPLC column, you may find the ion-pairing reagent difficult to separate from the target compound. Generally, if you must remove the ion-pairing reagent, pick the most nonpolar ion pairing reagent, extract, neutralize the target compounds charge, and back-extract with a polar solvent.

In a similar way, polar material that passes through a reverse phase cartridge will adhere to a hydrated silica cartridge column. Applying your sample directly to an untreated silica SFE cartridge and eluting with hexane will wash out very nonpolar compounds. A step gradient with increasing amounts of chloroform, then methanol, and finally water will remove almost everything from the cartridge. A notable exception is proteins, which adhere by ion-exchange as well as polar interactions. At neutral pH they seem to bind almost irreversibly. Passing an aqueous protein solution down a silica cartridge and washing with water is an excellent way to remove proteins if you do not exceed the column's capacity.

SFEs are available to use other separation modes to achieve size, ion-exchange, and affinity separations. If the compound to be separated contains a charged functional group or a group with an inducible charge, such as an amine, it can be removed from neutrals and compounds with the opposite charge. Charged molecules can also be removed with ion-exchange resins loosely packed in a Mohr pipette, but the SFE cartridge can be cleaned, regenerated, and reused. Apply the sample to the ion exchanger in a low salt buffer at a pH to maintain the target's charge. Wash out the breakthrough volume, then elute the compound of interest with either high salt or with a pH change sufficient to neutralize the charge on the target compound or the ion exchanger.

Although organic solvents may aid in making your compound soluble, be careful. Many polymeric ion exchangers will shrink in the presence of more

than 20% organic solvent. The bonded-phase silica ion exchangers will not tolerate pH below 2 or above 8, but can be used for short periods of time on SFE cartridges because they can be discarded. Apply the sample, wash quickly, and then elute the target. Silica SFE are resistant to organic solvents as long as the cartridge does not dissolve.

Size separations are seldom run on SFE columns because the column beds are not long enough to give an effective size cut. The only exception to this is the use of desalting SFE columns containing Sephadex G-25 for removing salt in a buffer solution from a protein fractionation.

Affinity SFE cartridges are coming on the market for antibody preparation (protein G and protein A columns), for hydrogenase preparations (blue, red, and green dye columns), and activated cartridges that can bind proteins and amino-containing compounds to prepare affinity columns for specific uses are available. These may go beyond the basic definition of the SFE, but they are just simply variations on the same theme.

12.1.4 Extracting Encapsulated Compounds

The final types of materials that may cause you problems in extraction are membrane-bound or encapsulated compounds. In the past, these have been removed by adding detergent to break the membrane, pulling everything in to solution and then extracting out the compound of interest. It is difficult to get clean separations because of the soap emulsification that is created. The detergent often contaminates the subsequent chromatography.

A better method is to first add an equal volume of dimethylsulfoxide (DMSO) or dimethylformamide (DMF) to the aqueous sample. This breaks both biological and encapsulation membranes and pulls polar and nonpolar material into solution. The second step is to dilute the sample with 10 volumes of water. At this point, nonpolars can be removed by solvent extraction or with a C_{18} SFE. Charged molecules can be recovered with pH-controlled extraction or with ion pairing reagents. The DMSO or DMF stays with the water layer. Customers have told me they can achieve almost complete recovery of both fat-soluble and water-soluble vitamins from polymer-encapsulated mixtures. Vitamins are encapsulated to protect potency from air-oxidation. Water-soluble vitamins have nonpolar encapsulation; fat-soluble vitamins have polar encapsulation. Either vitamin can be extracted by themselves, but they are difficult to extract under the same condition unless DMSO or DMF are used to break both capsules.

It also should offer promise for cell extractions, which, after all, are lipid/protein encapsulated mixtures of polar and nonpolar compounds. It would be interesting to see the effect of DMSO or DMF on the extraction of proteins. My guess is that protein might denature in 50% DMSO and precipitate so they could be filtered off or might renature and refold on 10× dilution in water and stay in solution. This might make an interesting research problem for recovering membrane-bound proteins.

12.1.5 SFE Trace Enrichment and Windowing

To get the most out of the HPLC as a "time machine," we have to analyze the separation, then put to work the extraction techniques we have discussed to speed the separation. As an example, we will take a metabolite study carried out by one of my customers. His problem was to study the dispersion of a compound, XX, in the environment. He had to follow its fate and metabolites in samples of water, sludge, soil, and fish tissue.

In examining environmental run-off water he ran into a problem. His samples were very dilute, so the first step would be some form of concentration. Studies with pure material show that it is sufficiently nonpolar to stick easily to a C_{18} SFE cartridge from an aqueous solution.

He prepared the SPE by first wetting it with 2 mL of methanol and then with 2 mL of water. Next, the SPE was attached below a particulate filter in a line leading to a 1-L suction flask (Fig. 12.1a). The inlet of the SFE was fitted with a tube dipped into a 1-L flask of the runoff water containing our compound, XX, at 5 parts per trillion. (He was unable to detect this level of compound by direct injection into an HPLC system.)

The runoff water was pulled through the SPE by suction, leaving the sample adhering to the bonded phase support. He used a 2-mL water wash to remove adhering polar material and the sample was eluted with 2 mL of acetonitrile. This is a 500-fold concentration increase in going from 1 L to 2 mL. He nitrogen evaporated the eluant, dissolved the sample in starting mobile phase with sonication, injected the sample, and made a separation with a 45-min analytical gradient HPLC run obtaining a peak corresponding to the standard, XX, with correct peak height (Fig. 12.1b).

To check recovery of compound from the medium, he ran a labeling study. Radiolabeled sample was added to runoff water at 5 ppt, it was sonicated, extracted with a wetted SPE, eluted as before, and counted. He found a 97% recovery of radiolabel from the SFE.

The problem with the separation at that point was that he had increased concentration but not improved his run time. Even though he was interested in only a single component, he had to run a gradient to separate it from the rest of the mixture. He still needed to prevent early running, more polar compounds from running into the sample and washing out late runners before the next injection. His bottom limit on run time was about 45 min, with a 15-min reequilibration to achieve reproducible results.

An hour per run is a long time if you have many samples, as would be expected in a metabolite study. His next step was to simplify the chromatograph conditions by improving the sample preparation step.

At this point, I came into the picture. Together, we repeated the loading steps to the point where the sample was on the C_{18} SFE (Fig. 12.1a). Going back to the gradient run (Fig. 12.1b), we see that the sample peak came off at 50% acetonitrile. We decided to change the recovery step off the SFE cartridge. First, we washed the loaded cartridge with 2 mL of 30% acetonitrile

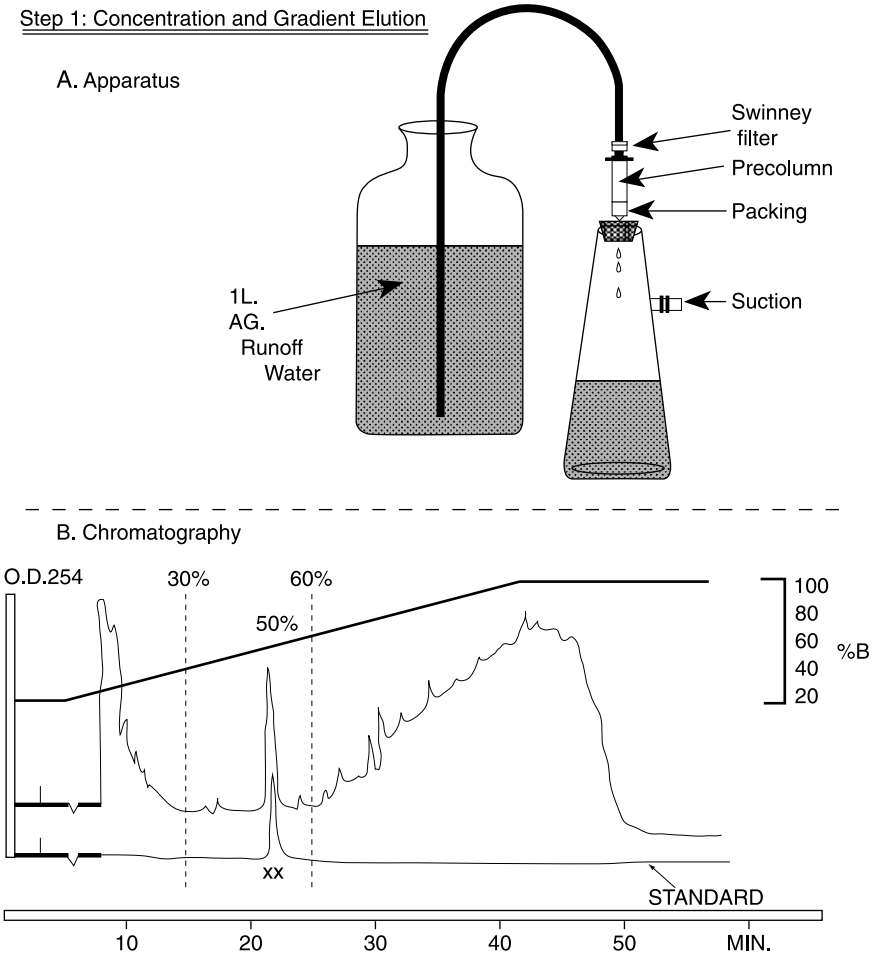

Figure 12.1 SFE concentration. (A) Apparatus; (B) gradient chromatography.

(Fig. 12.2a). Next, the cartridge was eluted with equal volumes of 60% and, finally, with 100% acetonitrile into fresh test tubes.

To check the recovered fractions, we equilibrated the HPLC with 60% acetonitrile. The standard, XX, and the first cartridge eluant were run under these conditions (Fig. 12.2b). The first cut chromatogram was examined for the presence of a peak with the same retention time as the standard. The remaining SFE elution fractions were each run at the same conditions and examined for peaks eluting with the standards retention times (Fig. 12.2, c and d).

We were trying to determine the location of the sample peak in the eluant cuts. Ideally, it should all fall in the "window" represented by the middle or second cut (Fig. 12.2c). If we find standard in the 30% eluant, then we have to move the window frame to exclude the sample from the 30% cut. We do this

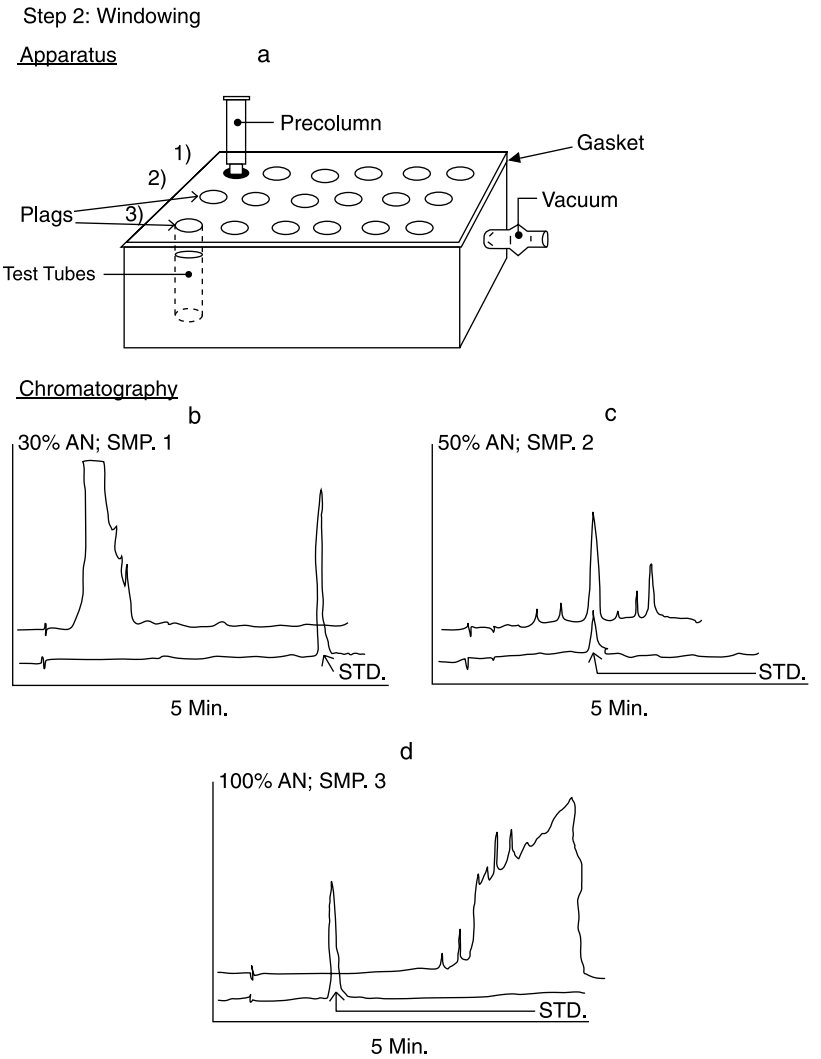

Figure 12.2 SFE windowing. (a) Apparatus; (b–d) isocratic chromatography: (b) fraction 1; (c) fraction 2; (d) fraction 3; AN = acetonitrile.

by repeating the concentration step, then using 25% instead of 30% acetonitrile for the first wash. If the XX had overlapped into the 100% eluant fraction (Fig. 12.2d), a similar window frame movement can be made by increasing the 60% (cut 2) fraction to 65%.

Once the window is optimized for the compound of interest, only the "window" eluant needs be run for each sample. Instead of a gradient, we can use a fast-running isocratic. We eventually got the separation down to a 5-min run time. The 30% "wash" and the 100% "stripping" eluants were run only to

optimize the "window" and to prove reproducible recovery. They were discarded for later runs.

To determine the effectiveness of the windowing procedure, the customer went back to his radiolabeled standard. Radioactive recovery studies showed better than 99% release from the cartridge and 94% activity recovery in the main "window" for the compound under study.

Using these techniques, we were able to increase concentration by 500×, while decreasing run time from the gradient to the isocratic chromatography by 12×. This is the type of simplification that can allow the full power of the HPLC to come into play.

12.1.6 Derivatives

Derivatives are used in HPLC only as a last resort. HPLC derives its usefulness from being able to run aqueous samples directly, with little if any work up. One place where derivatives are necessary is in preparing detectable forms of compounds that have poor UV absorption, such as carbohydrate, fatty acids, lipids, and amino acids. Useful derivatives for fatty acids and amino acids already exist, others should appear in the near future. Fatty acids derivatives are made with bromophenacylbromide in strong base; the resulting anhydrides can be detected at 50 ppt in a good UV detector. Amino acids can be removed from peptides in the Edmund degradation reaction as phenylthiohydantoins derviatives (PTH amino acids). Aqueous amino acids can be derivatized with *o*-phthaldehyde (OPA), and be detected with a fluorescence detector even in the presence of excess reagent. Much work has been done on post-column, in-line OPA amino acid derivatization with a reactor between the column and the detector.

The advent of the use of mass spectrometers as detectors and new mass detectors such as the charged aerosol detectors (CAD) and evaporative light scattering detectors (ELSD) should provide high-sensitivity detection of compounds that do not absorb UV light. The only problem with most of these is that they are expensive and, therefore, not readily available. When prices come down, they should finally eliminate the use of derivatives in HPLC analysis.

12.2 METHODS DEVELOPMENT

There are a number of methods for selection of conditions when approaching a new separation. You can rely only on published methods in the literature (sometimes an extremely dangerous step) or you can use published methods as a guide to selecting new conditions. If the compound has not been published, you can rely on methods published for compounds of similar polarity. You can even estimate conditions from consideration of the compound's structure.

All of these tend to be rather hit or miss. For unknown compounds or unusual mixtures, they generally are not very successful. It is usually better to approach a separation from a more systematic methods development based on scouting gradients (see Chapter 3).

When I demonstrate an HPLC system, it is often necessary to develop a separation of an unknown mixture of compounds in half a day to aid in obtaining an instrument sale. The techniques below arose from the need to speed such separations.

12.2.1 Standards Development

We are now going to walk through a typical methods development as you might carry it out in a clinical laboratory. Let us assume that you have just started your first day on the job. Your brand-new gradient HPLC is sitting on the laboratory bench with installation just finished. The chief pathologist for the hospital, your boss, walks in and hands you four vials marked A, B, C, and D. You are told that you will begin receiving patient blood samples containing any or all of these compounds at 10:00 A.M. tomorrow. Your boss wants to see the results before he leaves the hospital.

What are you going to do? You need to develop a rapid analysis of these compounds from plasma. The first step is to prepare 100× standard solutions of each compound for making dilutions. You make a carefully weighed 1-g/L solution of each compound in acetonitrile. A quick UV scan of each tells you that all compounds absorb well at 254 nm. Part of each stock solution is stored in the freezer; the rest is retained for development work. To make an injection solution, you add 1 mL of each 100× to a 100-mL volumetric flask and dilute with 25% acetonitrile/water, the starting scouting gradient mobile phase. You start the gradient system, equilibrating a guard column connected to a 25-cm C_{18} column at 2 mL/min in the same mobile phase. You inject a 7-μL sample and run a 20-min gradient. Your first peak comes off at 60% on the gradient trace. You get three partially resolved peaks (Fig. 12.3a).

Next, you drop back 10% from where the first peak came off and dial in 50% acetonitrile (AN)/water. You let the column equilibrate for 10 min and shoot the next sample. You get three well-resolved peaks, the last of which tails badly (Fig. 12.3b). There is no indication of a shoulder indicating an unresolved peak. Either one compound is very nonpolar or it is co-eluting with one of the other compounds. You need to make an alpha change.

You change the stronger solvent by switching to methanol (MeOH), while maintaining the same polarity. Since you used 50% AN/water, you switch to 65% MeOH/water, reequilibrate the column, and inject a new standards sample. You now have four resolved peaks, with the third tailing into the fourth (Fig. 12.3c). Guessing that the ionization problem is due to an organic acid, you make a pH change by add 1% acetic acid to the mobile phase and inject again (Fig. 12.3d). You get lucky and have four sharp, resolved peaks. They are a little farther apart than needed for the integrator, so you switch to 70%

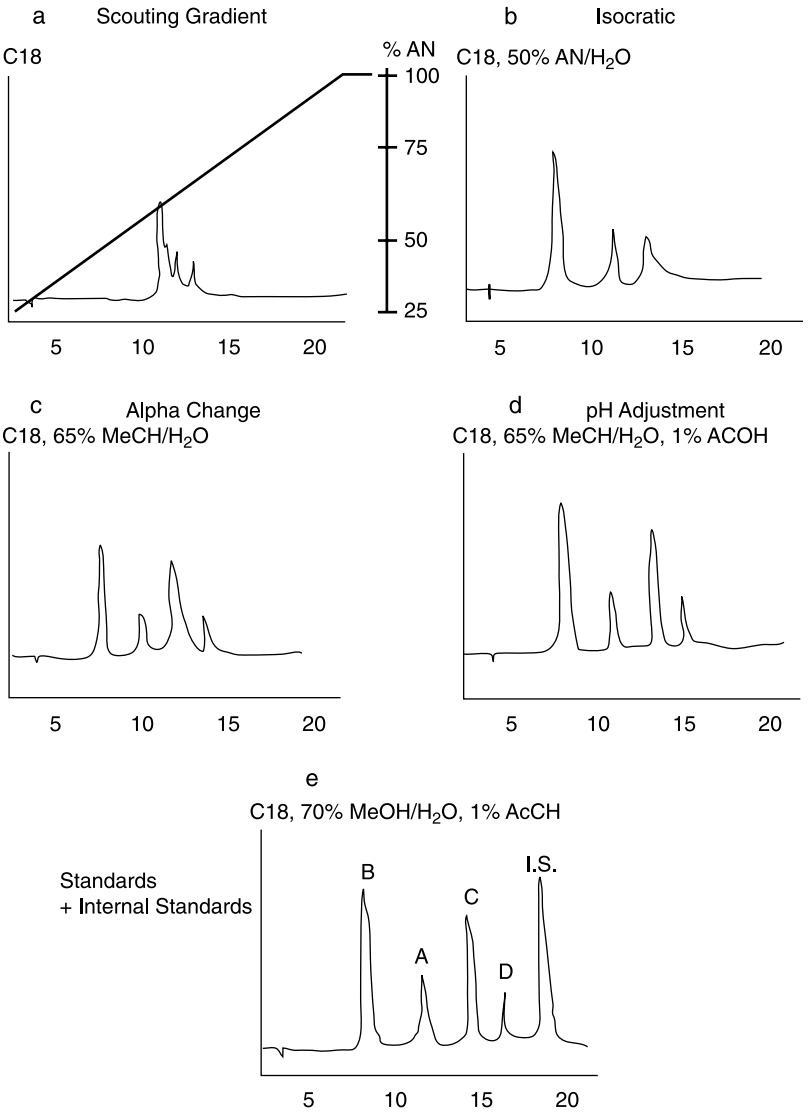

Figure 12.3 Systematic methods development: standards. (a) Scouting gradient; (b) isocratic; (c) alpha change; (d) pH adjustment; (e) standards plus internal standards.

MeOH/water with 1% acetic acid to draw them together and bring them off faster.

Note 1: Acids are easier to deal with in scouting than amines, so you lower the pH first with 1% acetic acid.

Note 2: Amine anions are more likely to be found in pharmaceutical compounds than acids. If acetic acid had not worked, you would add nonyl amine

to 5 mM to overcome interaction with silica surface cations and sharpen the peaks.

The next step is to identify each peak. You dilute and inject each compound separately and compare retention times to identify the standard mixture run. The elution order is B, A, C, and D. The last thing you need to find is an internal standard with a retention time just longer than compound D. You find compound IS, make a 1-g/L solution. You will add 1 mL to each standard mixture before dilution. You run a standard run with IS and calibrate each peak relative to the peak height of IS (Fig. 12.3e).

At this point, if you knew the identity of each component, you could rush out and publish the separation. Two or three years ago, many publications would have stopped here. However, the work is only about a third done. So far we have separated pure compounds under ideal conditions; we still have not entered the real world.

12.2.2 Samples Development

To finish the method, we need to add sample prep from plasma or serum and examine our standard separation under real conditions. Since you will be working on blood samples, you need to start looking at the standards in blood. You need fresh blood, uncontaminated with drugs or interfering compounds such as stabilizers and antioxidants, and representative of a wide population. The blood bank is out; they stabilize their blood for storage. You obtain volunteers, telling them how they can help push back the frontiers of ignorance and help society. Then you send them to an intern you know who needs practice drawing blood samples.

Once you get your blood samples back, you pool them, then split them into two parts. You refrigerate one part for later work with standards. You mix the other part with an equal volume of acetonitrile, homogenize, sonicate, place stoppered in a boiling water bath for 5 min, and then sediment proteins and particulates by spinning in a centrifuge. You place 2 mL of the supernatant in a volumetric flask and dilute with mobile phase. Running an 8-μL sample gives you a chromatogram of typical denatured blood plasma sample (Fig. 12.4a). You see a broad, early polar peak, which may overrun the positions of your first two standards, and a large compound lipid peak. The column may have to be washed with DMSO/MeOH to get the last of the nonpolar compounds off.

The next thing you want to see is the plasma blank sample with the standards and internal standard added to check for possible interferences. You add 2 mL of the treated blank and 1 mL each of the 100× solutions of A, B, C, D, and IS to a 100-mL volumetric flask, dilute, and inject. Sure enough, peak B is buried and A is on the shoulder of the polar breakthrough (Fig. 12.4b). You need to clean up the plasma blank with extraction or windowing techniques.

You decide to do windowing on a C_{18} SFE cartridge column. You take a mixture of the four original standards, dilute it 5-fold with water to increase the compound's k's, and pass it through the MeOH and water-wetted SPE

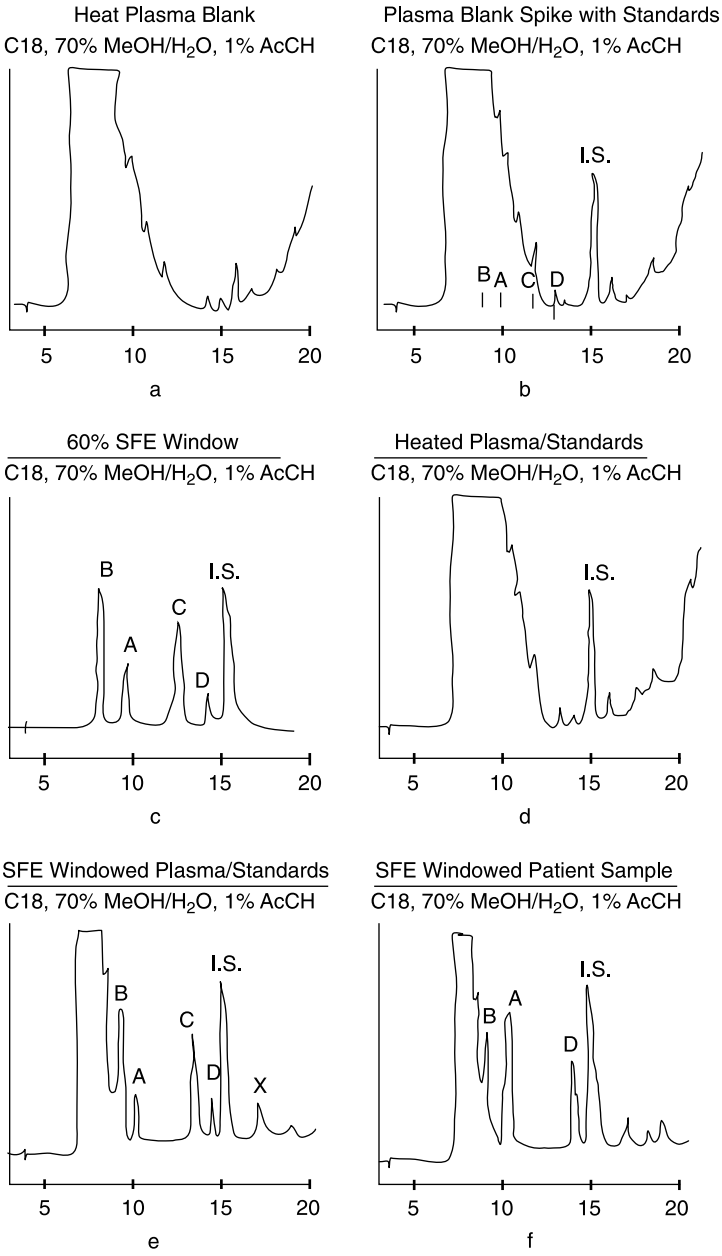

Figure 12.4 Systematic methods development: samples. (a) Heat plasma blank; (b) plasma blank spiked with standards; (c) 80% SFE window; (d) heated plasma/standards; (e) SFE windowed/plasma standards; (f) SFE windowed patient sample.

cartridge. You elute the cartridge with 2 mL each of 60%, 80%, and 100% MeOH, add 1 mL of IS to each cut, dilute 100×, and shoot each sample into the HPLC system. The 60% wash is contaminated with B and a trace of A. You repeat, moving the window frame to the left by eluting with 55%, 80%, and 100% MeOH. All your peaks are in the 80% window (Fig. 12.4c); none show up in either the 55% or 100% washes. Quantitization against the internal standard's peak height shows no loss of peaks B or D on the SPE cartridge.

You remove the other half of the pooled blood sample from the refrigerator. You mix 4 mL of blood with 1 mL each of the four standards in acetonitrile, sonicate, heat in boiling water, and centrifuge. You place 2 mL of the super and 1 mL of IS 100× in a 100-mL volumetric flask, dilute with mobile phase, and inject into the HPLC. (*Note*: You are looking for loss of standards by adherence to precipitated protein.) Peaks A, C, and D are present; the last two standard peaks quantitate correctly (Fig. 12.4d). You take 2 mL of the remaining plasma plus standards supernatant, dilute it 5-fold with water, and place it on an activated SFE cartridge column. You elute with 55%, 80%, and 100% MeOH in water containing 1% acetic acid. The 80% fraction is mixed with IS, diluted and run. It shows a much narrower polar peak, compound B as a shoulder on the polar peak from the plasma peak, resolved peaks A, C, D, and IS, and a small amount of the latter running nonpolar peaks (Fig. 12.4e). All four standards give correct peak height response factor to the IS peak. We are ready to accept patient standards.

Two more comments are necessary. The internal standard is added to correct for injection variations. The way it was used in the last step, it was also checking for standard recovery from the protein precipitation step. It is mildly dangerous to use the same internal standard for two purposes. If the quantitization was not correct, it would have been necessary to repeat both the injections and the precipitation with another internal standard to find the problem. Also, you must check for possible interfering drugs (ones co-eluting with our standards) that might be given to patients taking these target drugs. I would use the plasma blank spiked with standards and IS to look for these interferences by changes in response factors of the standards. This study can be postponed for our work right now.

To run a patient sample, you will need to go through exactly the same deproteination, SFE cartridge extraction, IS addition, mobile phases dilution, and injection steps (Fig. 12.4f). From the peak heights relative to the IS height, we can now quantitate the amount of each drug in the patient's blood. To insure linearity, you may need to dilute our windowed plasma blank and spike it with different levels of each standard and plot calibration curves for each compound, but basically, our methods development is done.

12.3 GRADIENT DEVELOPMENT

It is sometimes not possible to develop an isocratic separation for complex mixtures of compounds. Binary gradient methods development starts with a

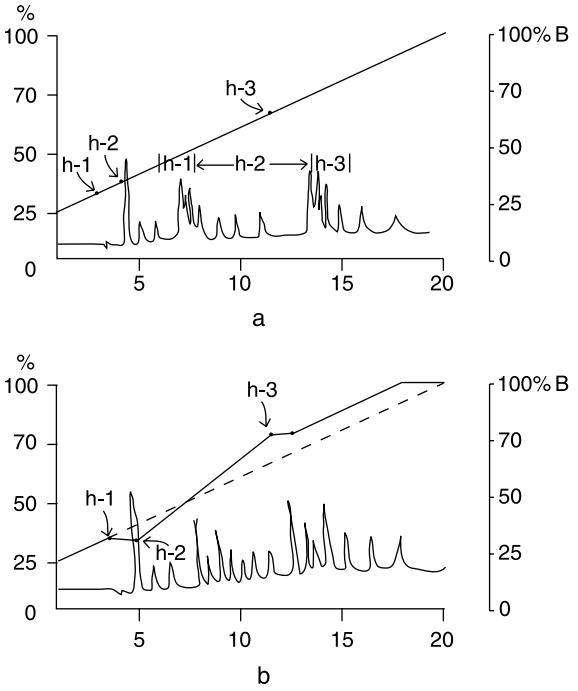

Figure 12.5 Gradient methods development. (a) Initial gradient; (b) hinge points adjustments.

linear gradient from 25 to 100% acetonitrile over 20 min (Fig. 12.5a), just as we did in scouting for an isocratic method. Next, inspect the gradient for areas in which peaks are jammed together (h-1, h-3) and areas in which they spread too far apart (h-2).

If the earliest peaks are jammed together at the void volume, we would want to drop the initial percentage of acetonitrile to 20% to allow these early peaks to interact with the column; if later peaks are taking too much time to come off, you would change the gradient slope so that we reached 100 acetonitrile faster to push the late peaks off earlier.

Assuming we get reasonable peak resolutions such as those in Figure 12.4a, imagine that there is a hinge point 10% before each of the compressed or expanded areas (h-1, h-3, and h-2) in the gradient trace. If the peaks are pushed too close together or unresolved (h-1 and h-3), place a hold in the gradient equal to the time in the original chromatogram for the last compressed peak to come off, then return to the original gradient slope. If peaks are too far apart (h-2), go back 10% to the hinge point and increase the gradient slope so that the last peak in the expanded area will be reached in half the time, then return to the original gradient slope (Fig. 12.5b). You may have to play with this slope change to get it to work out right and still resolve all the peaks in this set. Good scientific procedure would have you change one point at a time. I have been successful, however, in changing a number of points,

rerunning the chromatogram, checking for improvements, and then making new changes until I have the best separation I can achieve. I then program this hinge point gradient into my controller and run with it from then on. Remember, this is empirical development; don't get obsessed with finding the perfect separation.

13

APPLICATION LOGICS: SEPARATIONS OVERVIEW

At this point, I am going to try something a little different. Most HPLC texts include a series of figures showing you a separation, including the conditions, for various classes of compounds. I prefer to give you the tools to predict new separations. First, to give you an approximate set of conditions for making almost any type of separation, and, second, indicate why a particular column, mobile phase, and detector (or wavelength) was chosen for this separation—*the logics of the separation*.

To address the first objective, I've included my separation guide (Appendix A), designed as a quick reference to conditions that could be adapted to separate compounds similar in polarity, in size, in charge, or in absorption. Where possible, isocratic runs were chosen, rather than gradients. To handle the second objective, we will go through the various classes of materials exploring the chemical and physical differences that dictate certain HPLC conditions.

13.1 FAT-SOLUBLE VITAMINS, STEROID, AND LIPIDS

The first grouping is a mix of fat-soluble compounds that function as hormones, co-factors, and membrane components. *Fat-soluble vitamins* separate on a C_{18} column in 80% acetonitrile/water and are usually detected at UV, 280 nm, or with fluorescence. *Triglycerides* are slightly less nonpolar than fat-soluble vitamins and require 60% acetonitrile/water to run on C_{18}. They have poor extinction coefficients, and detection at UV, 220 nm, competes with refractive index detection in sensitivity. A phenyl column run in 50%

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acetonitrile/water gives some separation based on double-bonded side-chains. *Steroid hormones* require more polar conditions and separate on a C_{18} column in 60% MeOH/water. At UV, 230 nm, estrogenic steroids can be detected at 150 ng (the level in a pregnant woman's urine). *Adrenocorticosteroids* have higher extinction coefficients and can be seen at UV, 240 nm. *Prostaglandins* are hormonal, aliphatic diacids with double-bonds in their structures. They are separated on C_{18} in 35% AN/water containing phosphoric acid at pH 2.5. The phosphate is needed as a buffer, since detection is at UV, 192 nm, almost the bottom limit of the UV detector in air; even then not all of the prostaglandins absorb. These isocratic condition will separate most of the common therapeutic prostaglandins, but you will have to use a gradient to 100% AN to separate all of them, up to and including arachadonic acid, the precursor for the prostaglandins. This analysis might be a good candidate for using a charged aerosol detector (CAD).

The final type of "fatty" compounds in this group, *phospholipids*, are the hardest to separate and detect. They are naturally occurring "soaps" with long side-chains, alcohol, sugar or sugar alcohol bodies, and charged phosphate groups. They are soluble in nonpolar solvents when extracted from acidified media, but they differ in polar functional groups. They have very poor UV absorption and must be detected with "end absorption" at UV, 206 nm. The most successful separation has been on an acidified silica column eluting with 4% MeOH/AN containing 1% phosphoric or sulfuric acid. The CAD detector has been successfully used in detecting phospholipids.

13.2 WATER-SOLUBLE VITAMINS, CARBOHYDRATES, AND ACIDS

Water-soluble vitamins have a range of polarities. The vitamin B-complex, except for B_{12}, can be separated on a C_{18} column in 8% AN/water at 280 nm, using heptane sulfonate as an ion-pairing reagent. The ion pair slows thiamine and nicotinic acid so they will retain and run close to riboflavin. Vitamin C, an oxidizable organic acid, separates on C_{18} with 5% MeOH/water adjusted to pH 2.5, but has poor UV absorption and is better detected electrochemically for high sensitivity. All vitamins, except C and B_{12}, can be seen at UV, 254 nm. B_{12} may be a good candidate for high-sensitivity conductivity detection when it is available. It has a central cobalt atom that might be detectable at the right voltage with an electrochemical detector or with a CAD.

Free fatty acids can be separated on a C_{18} column based on carbon number using 50% MeOH/water pH 2.5 at UV, 280 nm; a fatty acid column (actually a phenyl column) will also separate them based on the number of double-bonds. Fatty acids have also been analyzed at UV, 210 nm, or by refractive index. For high-sensitivity work, they are derivatized with bromophenacylbromide and separated on C_{18} in a 15–80% AN/water gradient at 254 nm. Increase in early running C_2 and C_4 fatty acids measured by HPLC is used as an indicator of bacterial action. *Krebs cycle acids* are di- and tricarboxylic acids involved in metab-

olism of fats, sugars, and amino acids. They are separated by anionic ion exchange on an amino column using a pH 2.5 buffer gradient from 25 to 250 mm phosphate with detection by refractive index detector. If sensitivity is required, they could be derivatized post-column with bromophenacylbromide.

Monosaccharides can be separated on a polymeric cation-exchange column with a pair-bonded calcium or lead ion. The mobile phase is 80° water and detection is either by refractive index detector or UV, 195 nm. The elevated temperature speeds equilibration in the polymer column and reduces viscosity to protect the fragile polymer beads. Detection sensitivity is poor and numerous attempts had been made to prepare high-sensitivity derivatives, making this a good candidate for CAD and ELSD detection. This column can separate positional isomers, such as glucose and galactose, ring isomers, such as glucose and fructose, and all of these from polysaccharides and sugar alcohols.

Polysaccharides can also be separated on silica-based amino columns run in 75% AN/water and in polymeric "carbohydrate" size separation columns in 80° water with UV, 195 nm. The silica amino column separation can only go to about decasaccharides with 10 sugar groups and cannot distinguish ring or positional isomers. The size separators can go to molecular weights of about 6 million Da and offer separations of large polysaccharides that have only been separated previously by crystallization. A small amount of organic solvents will sharpen separations on either of the polymeric carbohydrate columns, but must be kept below 20% concentration to avoid damage to the column packing through swelling or shrinkage. Heating the water in the reservoir reduces column back-pressure by decreasing viscosity.

13.3 NUCLEOMICS

The *nucleic acids* family of compounds range from simple purine and pyrimidine bases to sugar- and phosphate-containing nucleosides, nucleotides, and poly-nucleotides, such as RNA and DNA. The nucleic acid ring structures all absorb well at UV, 254 nm. The free nucleic acids have been separated on a cation exchange column using high levels of ammonium acetate at pH 4.6. Most show pK_a's at 3–5 and might give sharper peaks at 2.5. The nucleic acids also should separated on C_{18} with hexanesulfonate at about 15% MeOH/water at pH 2.5.

Nucleosides, which have sugars connected to the bases, are separated on a C_{18} column in 8% MeOH/water pH 5.5 with phosphate. Adding the phosphate groups to form mono-, di-, and triphosphate *nucleotides* increases solubility, and they are separated with a quaternary amine ion-pairing reagent, tetrabutylammonium phosphate. A C_{18} column is run in 20% An/water pH 2.65 containing 10 mM TBA. Phosphate concentration is controlled at 30 mM; greater than this leads to loss of di- and tri-phosphate nucleoside resolution.

Polynucleotides pose a separation problem because of their large sizes and long, rigid shapes. *t*RNAs and some bacterial DNAs, which form ring

structures, can be separated on large-pore, TSK-type size separation columns. Mammalian m-RNAs and DNAs are double-helix molecules that form rigid rods with large Stokes radii. A size column with a 2 million Da molecular weight exclusion for proteins will exclude DNA restriction fragments larger than 100,000 Da. Added to this is the fact that nucleic acids are fragile and that pressure shearing on silica packings has been reported. Genetic engineering research has given this area considerable importance, and new rigid core packings are just now emerging for separating larger nucleic acid sections. Purification of cloned restriction fragments and removing contamination products from DNA amplifications reactors are increasingly important applications for HPLC systems.

13.4 PROTEOMICS

Separation of the family of compounds leading to enzymes, blood, and structural proteins has been an area of much recent research. *Amino acids* show "end absorption" below UV, 220 nm, but not high extinction coefficients. If a particular amino acid has a chromophore in its side-chain it may absorb well at higher wavelengths. Phe and Tyr groups absorb strongly at 254 nm and Trp at UV, 280 nm. The peptide bond between adjacent amino acids has good absorption at UV, 220 nm, in peptides and proteins.

Amino acids are derivatized two ways to increase sensitivity. *Free amino acids* in solution are reacted with *o*-phthaldehyde (OPA) to form a fluorescent derivative that excites at UV, 230 nm, and emits at FL, 418 nm. These OPA derivatives are separated on C_{18} in a complex mixture of An/MeOH/DMSO/water at pH 2.65. *PTH amino acids* are formed from the N-terminal end of peptides during Edman degradation for structure analysis of peptides and proteins. HPLC is used to identify which amino acids are released. PTH amino acids are separated at UV, 254 nm, on a C_{18} column with a gradient from 10% THF/water containing 5 mM acetic acid to 10% THF/AN. The separation with reequilibration takes 60 min. Work with short 3-μm columns has reduced this separation to a 10-min gradient.

Peptides (<99 amino acids) are separated at UV, 254 nm, on C_8 column in 30% n-BuOH/water containing 0.1% triflouroacetic acid (TFA). They can also be separated in acetonitrile/water gradients in which 0.1% TFA is added to both water and An. (Avoid going over 70% An in the gradient. TFA is reported to form aggregates in An concentrations greater than 70%, resulting in very large baseline shifts.) Peptides can also be separated at UV, 210 nm, on C_3 columns using An/water gradients buffered with phosphate ion at pH 5.5; these conditions are especially important if the peptides do not contain aromatic amino acids.

Enzyme *proteins* are separated with retention of activity in most cases at UV, 280 nm, on TSK-2000sw size separation columns in 100 mm Tris-phosphate buffered to pH 7.2 with added 100 mm NaCl with detection at UV, 280 nm.

Phosphate and sulfate will also work, but peaks are sharper with chloride. Protein stabilizers such as glycerol, EDTA, and dithioerythritol can be added if needed to the mobile phase. Enzymes can also be separated at pH 7.5 on TSK DEAE and CM ion exchange columns using salt gradients to 150 mM NaCl. DEAE is usually the first choice over carboxymethyl. Antibodies and larger proteins can be separated on TSK-3000sw columns. Proteins for structural studies can also be separated under denaturing, partition condition. A C_3 column can be used in 0.1% TFA gradient to 70% AN/0.1% TFA. Proteins with large nonpolar groups, such as albumins, tend to stick very tightly to this last column. Resolving power increases in the order size < ion exchange << partition. Load increase in the order partition < ion exchange << size.

13.5 CLINICAL AND FORENSIC DRUG MONITORING

Drug monitoring tends to be of two types: 1) assays for specific therapeutic drug levels, closely related analogs, and preparation enhancers and 2) rapid, broad screening for the presence and overdosage detection of drugs of abuse.

Theophylline, an asthma controller, has a very low safety/therapeutic ratio. One of the first clinical application for HPLC was to titrate theophylline levels in patient blood to avoid toxic overdoses. Blood levels can be controlled by assay at UV, 270 nm, on a C_{18} column in 7% An/water at pH 4.0 with phosphate buffer.

Catecholamines, nerve transmitters monitored in brain and heart patients, are separated on C_{18} using octane sulfonate ion pairing in 6% An/water (pH 3) with added EDTA and phosphate. Detection can be at UV, 270 nm, or by electrochemical detection at +0.72 V for maximum sensitivity. Other tyrosine and tryptophan metabolite neurotransmitters such as serotonin, VMA, and HMA can be analyzed with ion pairing and EC detection.

Anticonvulsants, used in controlling seizures, are analyzed on C_{18} columns at UV, 220 nm, eluting with 40% MeOH/water. They are also common drugs of abuse and are monitored for in toxicology laboratories.

Tricyclic antidepressants, major tranquillizers used in mental hospitals, are separated at UV, 254 nm, on C_{18} using 55% An/water at pH 5.5 with pentane sulfonate. Since these are very basic compounds, it is necessary to use hybrid or heavily end-capped columns and their separation benefits from organic modifiers, such as nonyl amine.

Basic drugs of abuse can be screened in a toxicology laboratory using a 20-min gradient from pH 3.0 phosphate buffer on a C_{18} column to 25% An/buffer at UV, 214 nm. This has recently been reduced to a 5-min quick check gradient on a 3-μm column. Similar screens can be set up for acidic drugs such as barbiturates. Designer drugs that are derivatives of acidic or basic drugs usually can be picked up in these screens, but a mass spectrometer may need to be used to confirm the identity of these anomalous peaks. Identity confirmation is very important in these labs to avoid false positives and for confirmation in a court of law.

13.6 PHARMACEUTICAL DRUG DEVELOPMENT

HPLC has played an important role for years in the drug discovery process in pharmaceutical laboratories. HPLC has proven a valuable asset in purifications of drug from de novo synthesis, from biological matrices, and from combinational synthesis. HPLC assay has moved from the discovery laboratory on through manufacturing, production, and metabolite monitoring.

LC/MS especially has been incorporated into building corporate-wide computer databases for tracking compounds throughout the process of candidate evaluation, approval, regulation, manufacturing, and environmental fate. This has lead to use of standardized LC/MS methods that are not optimized for each individual candidate, but allow computerized searching and comparison of compounds and structures.

13.7 ENVIRONMENTAL AND REACTION MONITORING

HPLC serves for some monitoring of air and water pollution. Air quality can be determined by pulling known volumes of air into an evacuated metal chamber and analyzing with a GC or into a pre-wetted C_{18} SFE cartridge column, then eluting under windowing conditions and analyzing on the HPLC. This technique has been used with belt monitors to analyze laboratory exposure in toxic or radioactive environments. Water pollution can be monitored in the same way. Instead of storing gallon bottles of water, the water can be pumped through an activated SFE cartridge column, placed in a plastic bag, and refrigerated or frozen for later assay.

Pesticides and *polynuclear aromatics* (PNAs) are the most commonly analyzed environmental contaminants. Analysis of PCBs, dioxans, and nitroorganics (explosives) is of growing importance. The major obstacles to adoption of environmental HPLC application are 1) awareness of the need, (i.e., environmental and drinking water contamination) and 2) the slow rate of development and acceptance of new AOAC and EPA-mandated HPLC and LC/MS methods.

Pesticides can be analyzed on a C_{18} column, the chlorinated hydrocarbon type (chlordane) at 80% An/water UV, 220 nm, the carbamate type (sevin) at 40% An/water UV, 254 nm, and the organic phospahate (malathion) at 50% An/water with UV, 192 nm or with a CAD. The organic phosphate types are hard to detect at low concentration and various phosphate analysis techniques have been evaluated. LC/MS, where available, is the technique of choice for analyzing all of these pesticides, but especially the organic phosphates, in a general gradient HPLC scheme.

PNAs are analyzed at UV, 254 nm, on C_{18} column in 80% An/water. PCBs can be analyzed with the same conditions. Dioxans require detection at UV, 220 nm, and 50% An/water on a C_{18} column.

Reaction monitoring is an important use for HPLC, both in the laboratory and in production. I have followed reactions in a 50-mL stirred, round bottom flask and in a stirred 6,000-gallon reactor. The HPLC becomes your window-on-the-reactor. It lets you analyze starting materials (QA or quality assurance), reaction intermediates, by-products, and final product (QC or quality control).

In one study, I followed temperature breakdown of final product by stirring it a three-necked flask while stripping solvent with live steam. I used an inverted pipette to pull samples over time, weighed each in a pre-weighed flask, threw in chloroform, shook it, pulled a sample of the chloroform layer, spun it to break the emulsion, and then injected a sample into the HPLC. I ran 5 min behind the reaction with my "reaction window." I could follow the disappearance of starting material and the appearance of product versus heating time. Using other sampling techniques, we could follow product from the reactor, into holding tanks, through steam strippers, through dryers, and on into product bags. It is simply a matter of scale; the analytical tool is the same.

13.8 APPLICATION TRENDS

What can we get out of all this that will help us the next time we run into a new separation?

First, *mobile phase and column*: we saw that most of the small molecule separations could be made on a C_{18} column in An/buffer water, with the exception of charged molecules and carbohydrates, which are too water soluble. We saw a range of polarity from fat-soluble vitamins, steroids, triglycerides, chlorinated pesticides eluting in 60–80% An/water, to carbamate, phosphate pesticides, anticonvulsants, antidepressants at 40–50% An/water, to nucleosides, nucleotides, aspirin, and water-soluble vitamins at 5–10% An/water. If you know something about the compound's structure or its solubility, you have a good clue as to what mobile phase can be used for its separation.

Second, *detection*: colored compounds absorb above UV, 300 nm; compounds with aromatic groups probably will absorb around UV, 254–280 nm; if it has an carbonyl group, it should absorb at UV, 220 nm. Any solvent or buffer with oxygen in it is almost useless below UV, 210 nm; water, acetonitrile, and phosphate are the choice for UV detection this low (<200 nm). Refractive index (RI) detection shows most compounds, but not at high sensitivity: it is good for preparative work, but can't be used for gradients. The charged aerosol detector (CAD) and mass spectrometer (MS) are both great mass detectors for general analysis, but both are fairly expensive and MS requires expertise beyond that normally required to run HPLC equipment. Florescence detectors (FL) give you high sensitivity, *if* your compound fluoresces and if you can find the wavelength combination for excitation and emission. Conductivity detectors (CD) require charged compounds for detection and a way of

removing interfering ions that does not remove the compound of interest. Electrochemical (EC) detectors give excellent sensitivity, but work only with oxidizable or reducible compounds and can be used only with <25% organics because of flow cell and reference electrode design.

It may not sound like much help, but with these as guides, and a little intelligent thought, you should be able to separate most of the compounds you will encounter. The rest are research projects and, therefore, publishable, so they are not a complete loss.

14

AUTOMATION

14.1 ANALOG-TO-DIGITAL INTERFACING

In the real world, changes are continuously *analog* variations with time. Temperatures rise and fall. Instrument parameters, such as signal output voltage, increase and decrease continuously. In the computer world, changes are discrete steps; computers process data using a two-value alphabet, 0 and 1. The next value may or may not be related to the one before it.

There are good examples of both analog and digital devices in electric light switches. The light switch you use to turn the room lights on and off is a digital device; it has two discrete settings. The dimmer switch you use to dim the lights to provide a romantic setting for a dinner party is an analog device; it yields a continuously changing current output.

For analog instruments to communicate with computers, the analog signal generated by a detector must first be converted to discrete digital steps (digitized). This is the purpose of the analog-to-digital (A/D) converter (Fig.14.1a). Most analog instruments put out a variable voltage data signal. Strip chart recorders work on an analog voltage signal that varies from 0 to 10 mV. As a detector measures a change in the flow cell absorbance due to a compound peak, the detector electronics output an equivalent signal change that increases then decreases in voltage. The strip chart faithfully traces this signal on paper to produce the peaks, valleys, and baseline of the chromatogram.

An integrator of a computer usually requires a stronger analog signal from the detector, either 0–1 or 0–10 V, but the output is of the same form. As the signal reaches the A/D board, it is sampled for a specific length of time and

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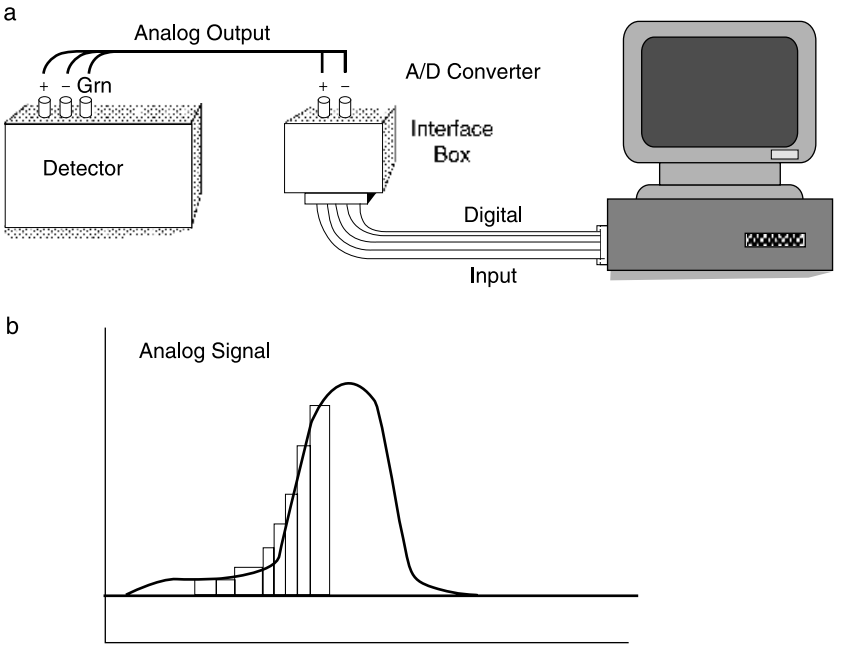

Figure 14.1 A/D conversion. (a) Analog output; (b) analog signal.

the average strength of the signal is converted to a digital number. This is done on some boards by charging up a capacitor, shorting, and then charging it again. The number of times the capacitor charges in a given time period is represented by a digital number. The slower it charges, the smaller the number; the faster it charges, the larger the final digital number. The stair steps in Figure 14.1b represent these digital values.

The frequency of this sampling is defined in samples per second. Most A/D boards can sample at about 30,000 points/sec, while an HPLC signal requires sampling at no more than 10 points/sec. The other controlling variable is the size of the output digital number. A typical A/D board can only process a number up to 65,000; to process the full range of detector outputs, at least a 12-bit board is required and many boards use a 16-bit data path that allows a word size that can handle numbers up to 1 million.

Once the signal is converted to a digital number, it can be transmitted to the computer for storage along with a number representing the sampling rate. Our signal now consists of a sample rate number followed by a series of digital numbers representing the voltage strength at each subsequent time point up to the end of the sampling time of the chromatogram.

This would be equivalent to taking a motion picture and breaking it into a series of individual film frames. Each frame represents a digitation of the motion picture at a given time point. Running the frames through the projector faster than 32-frames/sec gives the appearance of a continuous, analog,

real-world presentation. The digital-to-analog (D/A) converter board produces a similar effect with a digital signal. It takes the sampling rate and the series of digital numbers and converts them into an approximation of an analog signal with smoothing. This signal can then be sent to control a device that requires a continuously varying voltage signal. Using such a D/A converter, an HPLC controller can send a digital signal to change the motor speed on an analog HPLC pump.

14.2 DIGITAL INFORMATION EXCHANGE

The A/D board can reside either in the detector, an interface box, or in the computer. If the board is in the computer, it will have analog input terminals similar to a strip chart recorder or it will be connected to an interface box that will have this type of connector. If the A/D card is in the detector, the detector will have some type of digital interface port and a cable to connect it to the computer. In the personal computer world, once the signal is digitized outside the computer, it will be sent to the computer over one of three types of communication cables: parallel, serial, or GPIB.

Parallel cables transmit each bit of the digital word on a separate line within the cable. Serial cables code the digital words so they can be sent over a single line within the cable; they must be decoded on arrival at the other end of the cable. Other lines are used in both parallel and serial cables for communication housekeeping chores. Both cables are designed to send information only one direction at a given time or asynchronous communication. Parallel cables transmit data faster than serial cables, but are limited to less than 20 ft in length. Serial cables can carry signal up to 1,000 ft and longer with signal boosting.

The GPIB or IEE-488 cable is a bidirectional serial cable carrying module addressing information lines as well as signal and housekeeping lines. It can be used to send and collect information from different instruments on a cable bus. It is often used in instrumentation to automate a series of discrete instrument modules connected through a system controller.

14.3 HPLC SYSTEM CONTROL AND AUTOMATION

HPLC systems operate on a master-slave arrangement in setting up for automation. One module sets the timing and initiates the process, and the remaining modules accept a signal and follow the leader. In a gradient HPLC system, the master module can be a microprocessor-based controller, a computer software-based controller, an autosampler, or an integrator.

A system controller sends out pump speed control signals to set flow rates and to generate solvent gradients. It should also be able to send 0- to 5-mV contact closure signals to actuate external switches and equipment. It also may be able to send out a 0- to 30-V powered contact closure signal needed to run solenoid valves used to control air pressure and liquid flows. Finally, the

controller should be able to take in external 0- to 5-mV contact closure switching signals to control its internal functions (i.e., start a run, stop, pause, etc.). A smart controller may also have a serial or GPIB interface to allow upload/download of method control programs.

Other smart system modules that may be able to serve as system master are the autosampler or the integrator. In an isocratic system with no pump controller, an integrator might take a contact closure signal from a manual injector as a signal to start data collection and processing. A more complex system might be constructed using a master autosampler to initiate a series of washes, injections, and multiple injections from the same vial then send start/stop signals after every injection to the integrator to start data collection and processing. The integrator in turn can send back a hold signal to prevent further injections until it finishes printing out results. The autosampler might also send vial identification information from a bar code label to the integrator to be included in the printout. A third automation scenario might involve a master autosampler, a gradient controller, a smart detector, and an integrator interfaced to a data storage computer. Complete automation would have the autosampler making an injection and sending a signal to the system controller to start its gradient run and to the smart detector to change wavelengths or sensitivity during the gradient run, and the integrator would accept a vial I.D. and transmit it, along with its integration output, to the computer for data storage and further processing in other software modules to generate a final run report.

In general, in a system with an autosampler, this module usually initiates run start. The system controller usually controls external events occurring during a gradient run. The integrator may send a "hold further injections" signal until it finishes printing its processing report.

Life becomes even more complicated when a mass spectrometer is added as the detector in an LC/MS system. The system computer not only must run the pump gradient and control external events, but it also must control the mass-axis scanning program and lens setting as well as signal acquisition, digitation, processing, and annotated total ion current chromatogram presentation. In many laboratories, LC/MS systems are networked and the processing, chromatogram presentation, and other manipulations such as post-run spectral extraction and compound identification are moved off to a separate computer.

14.4 DATA COLLECTION AND INTERPRETATION

To quantitate data in either a computer or an integrator, you must first establish a baseline, then acquire data from an injection, detect peaks, integrate the peaks, and compare the peak integrations to response tables from known amounts of standards compounds.

14.4.1 Preinjection Baseline Setting

Before making an injection for a chromatogram that will be integrated, it is necessary to define an integration baseline. This can be done automatically in many integrators by pushing a button marked *Test* (or *Self-Test* or *Slope Test*). The integrator measures the chromatographic signal from the detector for a set period of time (<1 min). It then examines this data for the largest change in slope during the test time and sets a *minimum slope value*. Any slope change greater than this value will be defined as a peak start or a peak end. Next, it will average all deflections over the test period and use this average to set a *noise reject value*. Anything above this level will be treated as signal; anything below this level will be ignored. Finally, after the injection is made, the integrator will use a default *peak width* window and test to see whether the current peak falls in this window. If it determines that the peak is wider than the window, the peak width value will be automatically doubled for the next peak, and this value will continue to be tested during the chromatogram acquisition period. This corrects for the tendency of peaks to broaden the longer they stay on the column. In gradients, where peaks may sharpen as the gradient slope increases, the integrator can cut the peak width value in half if a peak fails the test.

I have been told that an integrator should be able to integrate a difficult separation four times more accurately than can be done with manual integration. This is often not happening if you visually examine repeated injections of a test sample rerun using machine-set parameters. The *Test* button is useful when running first-time or unknown samples and should be used when resetting detector sensitivities since these make a major change in baseline noise and slope values.

Be aware that when you press the *Test* button, you are allowing the computer to make a decision about minimum slope, noise, and peak doubling value for you. Once a separation has been run a few times, more precise data can usually be obtained by manually setting the slope, peak width, and noise values and by forcing peak doubling to occur at specific points in the chromatogram using time programs built into many integrators.

14.4.2 Peak Detection and Integration

Once data is in the computer, it must be processed to determine the identity and relative amounts of each material present. First, peaks must be defined. This is done by detecting peak starts and ends based on slope changes, determining *retention times* by detecting peak centers by inflection point changes from positive to negative slope at the peak maxima, and then calculating either peak heights or peak areas from the data. Next, relative areas or height are calculated by summing all detected areas or heights, then dividing each individual value by the total for the chromatogram. Then, this information, along with a chromatogram annotated with retention times, is printed as a table of retention times versus peak height and areas.

14.4.3 Quantitation: Internal/External Standards

Finally, these relative heights or areas are compared with equivalent values obtained from standards curves prepared from known amounts of target compounds to yield values for the amount of each target compound present in the chromatographic injection. Unknowns in the chromatogram can be identified with relative retention times, areas, and heights, but the amount of each present cannot be determined until they are identified, standard curves run, and response factors calculated for each compound.

The decision to use either peak height or area data is based on the nature of the mixture analyzed. Fairly simple mixtures, such as reaction mixtures or fairly clean extracts, are usually more accurately determined using peak areas. Very complex mixtures from biological sources, such as blood or urine, are best quantitized using peak heights, since target peaks are often detected on the shoulders of earlier, trailing peaks. This distinction is so common that I can usually separate a roomful of chromatographers into clinicians and researchers simply by asking who uses peak heights or who uses areas for quantitation.

Calibration standards can be of two types: external standards and internal standards. With external standards, multiple concentrations of the standards are injected, areas are measured, and a calibration curve is platted. Unknown samples are then injected, chromatograms run, and areas are calculated and compared with the calibration curves to determine amounts of each compound present. With internal standards, known amounts of an internal standard are added to each known concentration of standard compound and areas or peak height response factors relative to those of the internal standard are calculated. When unknowns are run, a known amount of internal standard is added to the unknown sample, response factors are calculated relative to the internal standards, and amounts of each unknown present are calculated from the standards calibration factors. Internal standards are usually used to correct for variations in injection size due to different operators and injection techniques. Internal standards can also be used to correct for extraction variation; in GC/MS target compound quantitation, this standard is referred to as a surrogate standard. Generally, an internal standard is used for one purpose or the other, not both at the same time.

14.5 AUTOMATED METHODS DEVELOPMENT

If you will pardon the pun, this is a developing field. There are no generally accepted approaches to methods development, but we will look at the underlying logic. Automated methods development is modeled after manual development of a standards separation. The instrument "looks" at a chromatogram, makes a change, and then "looks" at the new chromatogram to see if the separation has improved. Changes are made "systematically" until the "best" separation is reached.

A word of warning if you are planning to do automated methods development. The HPLC system you buy will generally dictate the type of methods development software that is available to you; this is not an add-on procedure that you can purchase at a later time, except from the manufacturer of your system computer. If you are contemplating a need for this technique, I would recommend strongly that you talk to other people who are already successfully doing this type of work and find out their HPLC system source and buy from the same place.

14.5.1 Automated Isocratic Development

Obviously, to make this happen we have to 1) have a way of measuring the completeness of a separation, 2) define what constitutes the best separation, and 3) define a systematic pattern for making changes.

When we look at a separation to judge whether two peaks are separated, we look at the centers of the peaks, but more importantly, we look at the valley separating the peaks. An ideal separation is one in which all peak pairs are baseline separated; the peak valleys all come down and touch the chromatographic baseline. If we were to draw a line connecting the two peak tops (Fig. 14.2, line 1–2), then drop a perpendicular line from the center of this connecting line to the baseline (A–B), the length of the resulting line would represent a standard of baseline separation for these two peaks.

If the valley did not touch the baseline, it would have a length (A–C) that would be less than the distance to the baseline. If we divide the length of A–C by the length of A–B, we have a mathematical measure of the baseline resolution for the two peaks. Summing these ratios of baseline resolution for every peak pair gives us a *resolution sum* for the whole chromatogram.

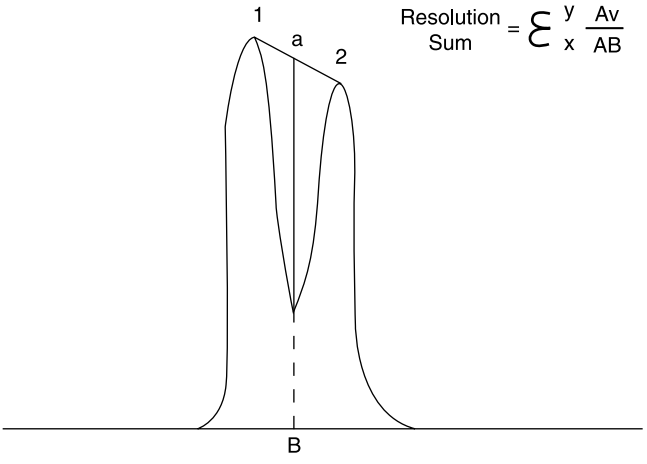

Figure 14.2 Resolution sum.

Once we can put a relative value on the degree of baseline resolution between two peaks, the rest is easy. The next step is to work to make the resolution sum number as large as possible, moving us toward a "better" separation. The computer changes one system variable, has the autoinjector make a new injection, and calculates a new resolution sum. Through repeated variable changes, new injections, and recalculation, it works to maximize this value.

Left to itself, the computer will carry this optimization to ridiculous extremes, optimizing for every baseline pair it finds no matter how long it takes. We would like the final chromatogram to run in a reasonable time, so we set limits on the length of the *expected run time*. We also need to specify the *expected number of peaks* in this run time. Now the computer will search until it has maximized the baseline resolution for the expected number of peaks in the preset run time.

The final information needed is a search pattern and a definition of the independent variable to be searched. Usual variables are flow rate, %B, and %C; %A is assumed to be a dependent variable (100%—the sum of the other solvent percentages). One commercial search pattern starts with all the variables at zero, then systematically changes one variable by a preset percentage and walks incrementally through all possible values, then repeats for the next variable. Once all injections and chromatograms have been run, each run is inspected and the best value is selected. I call this the "infinite monkey" theory of methods development; you will find it uses a lot of time, reagents, and paper.

A second search pattern makes injections with each variable in turn set at zero with all others at maximum. It then makes an injection with each pair of variables at half maximum and the remaining variable at zero. Finally, it makes an injection with all variables at half maximum and interpolates to predict the best separation. To visualize this "half monkey" technique, you would plot a triangle with sides defined as 0% to 100% of each variable. First, we would run compositional values at each corner, then the middle of each side, and, finally, at the center point of the triangle. This gets very complicated to visualize with more than two independent variables.

A more sophisticated method uses a random walk or simplex optimization search pattern, which was developed and is used to find downed aircraft or ships lost at sea. Variable limits are set, then three conditions within these limits are selected at random, injections are made, and chromatograms are run. The resolution sums for the injections are measured and calculated, the lowest value is discarded, and a new variable setting is selected directly opposite the discarded value and equidistant from the reject on a line connecting the two remaining values from the original triad (Fig. 14.3).

In Figure 14.3, our original triad would be made up of points 1, 2, and 3, if 1 gave the worst value it would be rejected and the new triad would be made up of 2, 3, and the reflection point 4. If the new point falls outside the limits, it too is discarded and a new point (4′) is selected halfway between the first triad discard (1) and the line between the two retained points 2 and 3. Now, a new run is made using the variable conditions of this new point (4′) and the

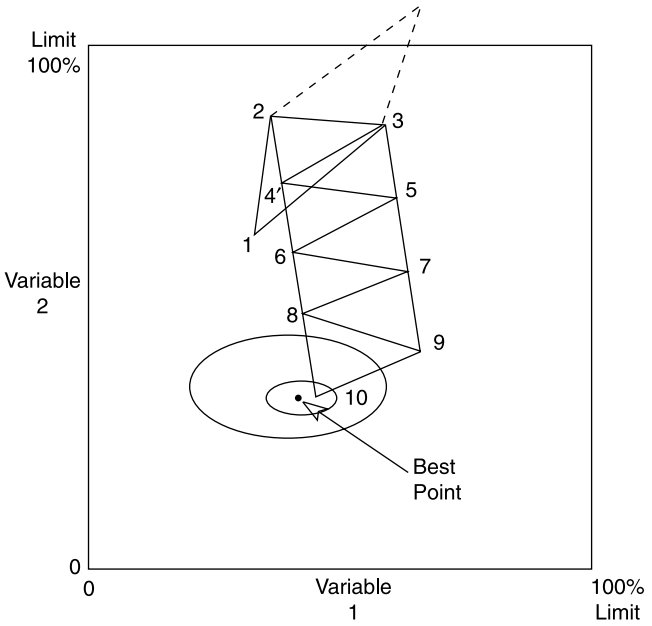

Figure 14.3 Random walk optimization.

resolution sum is calculated. This process continues with the lowest value of each new triad being discarded, reflection around the axis joining the best two points, and a new injection made at the new set of conditions. This technique will hunt and search until a point (10) is found that meets the search criteria. The search can be stopped at this point, but there is a danger that only a local "best" value has been found. If the overall best condition is desired, this final point can also be discarded, a new point selected at random, and the random walk can be continued. If the computer continues to return to the previous "best" point, then it probably represents the true best value within the limits. Obviously, a limiting maximum number of injections should be set to keep the computer from wandering around forever.

Although flow rate and solvent composition are the most commonly optimized variables, there is no reason why temperature and other mobile phase modifiers could not be used. Variables such as mobile phase pH, buffer concentration, ion pairing reagents, a chelator's concentration, or organic modifiers could all be optimized using resolution sums. If the computer can control the variable UV detector's wavelength, wavelength and detector sensitivity settings could both be included as independent variables to be searched and optimized.

To create a method, you would need a computer-controlled gradient HPLC system, with an autoinjector or autosampler capable of making repeated

injections from a large supply of the target solution, an HPLC column, and a detector. Data acquisition and processing can be done in an integrator and sent to the computer or can be handled using an A/D card and software running in the control computer. The system would be set up with sufficient mobile phase for an overnight run, limits set, and the system allowed to run unattended overnight. When you come in the next day, the system will be either still be running chromatograms or the report will be ready with the best chromatographic conditions on the final printout.

14.5.2 Hinge Point Gradient Development

The development system is usually designed to first try and optimize a fast-running, two-solvent isocratic separation (variables equal %B and flow rate). If this cannot be achieved within the run time and expected peak limits, a decision must be made by the operator as to the next type of development. If your peaks are nearly separated, you might try making an alpha change by selecting a different column and repeating the automated methods development. Unfortunately, there is no way of making column scouting an automated procedure.

If your system is a two-pump gradient system, the next step is probably development of a binary gradient. If you have a multisolvent gradient system, you usually try to create a binary solvent gradient method before trying to optimize a three-solvent or even a four-solvent isocratic method in the same fashion that we optimized a two-solvent isocratic separation. This decision may just be a case of linear thinking; it is much easier to visualize binary gradient development than multisolvent isocratic development.

To manually create a binary gradient, a linear gradient is run from 0 to 100%B, the resolution sum calculated, and then a hinge point development is begun, as discussed in Chapter 12. Automated gradient development works in a similar fashion; one hinge point at a time is selected and optimized to improve the separation of compacted peaks by introducing hold before this area. The hinge point can be entered after operator inspection or at random time intervals. After resolution is maximized for compacted areas, slope increases can be introduced at random hinge points to speed run time while maintaining resolution. Gradient software development is very much a research science at the moment.

If neither binary gradient nor three-solvent isocratics are successful, some systems will next try to perform a three-solvent gradient optimization. This development is very difficult to visualize. Assuming simultaneous optimization of %B, %C, and flow rate hinge points, it takes a long, computation-intensive time to carry out. It would be nearly impossible to carry out manually. The key is continually to use *the rule of one*: change only one variable at a time and to carefully select limits for evaluation.

These last changes are probably of academic interest only. Most separations can be achieved nicely with either a two-solvent isocratic or a binary gradient.

Most tertiary isocratics in the literature only use a constant level of the third mobile phase as a polisher. Amines that tend to tail under neutral pH complicate the development. Moving to an end-capped column of adding a fixed amount of organic modifier will usually fix the problem. Acids can be handled by going to a lower pH using a fixed amount of acid to buffer pH.

14.6 DATA EXPORTATION TO THE REAL WORLD

Raw data and reports can be stored in the computer's archival memory, but they must be transmitted to the real world to be of use. In the simplest case, they can be displayed on the computer's monitor in the form of chromatographic curves, tables of data, and reports, or they can be sent to the printer for printing. They can also be shared with other computers or with other software applications for further processing and extraction. To move data out of the resident software program, they generally have to be translated into some standard format recognized by other applications.

Laboratory Information Management Systems (LIMS) are computer software-based integrators for laboratory reports generation. They gather all the information on a particular sample, including history, source, supplier addressing, data reports from all wet and analytical instruments, and conclusions and results drawn from this analysis. They receive information from a variety of inputs, in a variety of formats, and must have inputs for data confirmation and checking.

14.6.1 Word Processors: .ASC, .DOC, .RTF, .WS, .WP Formats

The simplest of the formats used to transfer data into word processing applications is the ASCII (.ASC). ASCII is a standard set of 128 binary codes used by all computers to represent all the characters presented on the normal or shifted keyboard plus control codes, originally intended for use on teletypewriters. These code allow us to display lower case leters, capital letters, numbers, and punctuation marks, but formatting codes for underline, boldface, and italics are not included in ASCII, and are removed in converting formats. ASCII files have space-separated code and can be sent out over a modem or a serial cable to another computer and applications importing ASCII code.

Other word processing formats use higher-level coding in addition to the ASCII character codes to create proprietary coding specific to that manufacturer's software. Many of these can be recognized and translated by other writing applications, including the Word Star (.WS) file format and the Word Perfect (.WP) format. The most commonly used file formats today are the .DOC format used by Microsoft Word and Rich Text Formatting (.RTF), recognized by most word processing software and capable of retaining and transmitting formatting information along with the character coding.

14.6.2 Spread Sheets: .DIF, .WK, .XLS Formats

The next type of standard output is the spreadsheet. These file formats use comma-separated ASCII code, but also add calculation information and addressing information for the columns and rows they occupy. The simplest of these are .DIF files, which originated to allow information transfer between VisiCalc worksheets in the Apple II computer and have been retained as a standard format. .WK files are Lotus-1,2,3 formats and .XLS are Microsoft Excel formats that have become spreadsheet standards, allowing transfer of data, calculations, addresses, and macro programs.

14.6.3 Databases: .DB2 Format

To export data files into a database program, a database file format called .DB2 was developed in an early PC database, dBase II. Databases are made up of *files*, which could be compared to a Rolodex® file box full of cards, all containing the same type of information. The Rolodex® card would be equivalent to a database *record*. Each record has on it a series of entries, *fields*, in the same place on each card. To import data into a database record, all the entries in the report must be matched up with existing fields in the database's format. Most software that uses database formats has export/import subprograms that allow you to align fields between the two formats and allow you to select various ways of determining coding for end-of-file and end-of-record terminators.

14.6.4 Graphics: .PCX, .TIFF, .JPG Formats

Graphics, the fourth type of export from chromatographic data, is the most difficult. We can export copies of the monitor screen as bit maps in standard graphical formats such as .TIFF or .PCX files or in compressed .JPG files, but much of the fine detail and companion information will be lost. These bit map files can be manipulated, cleaned up, and labeled in "paint"-type applications, and then exported into word processing applications. However, the chromatogram can no longer be resized and data extraction and integration are no longer possible. In some graphical applications it is possible to write a printer format such as .EPS or .HTTP to a file similar to a postscript file, and these can be used by some applications to resize, rotate, and reprocess the graphical output.

14.6.5 Chromatographic Files: Metafiles and NetCDF

Chromatographic data file formats are very often in system- and manufacturer-specific metafiles. The formats that are used to store these files within an integrator or data processing unit are usually not designed for export, or they are designed for export only to other modules by the same manufacturer. They

may be in a proprietary format, in a compressed storage format, or even generated under a different computer operating system than in current usage. Many offer the capability of translating part of their contents to a standard computer format, but a great deal of information, especially graphical information, is lost in the process.

To overcome this problem, a standard chromatographic file format, NetCDF, was developed and approved by a committee of chromatographic companies in 1991. It languished for many years until the need to integrate information from across a laboratory lead to the appearance of LIMS to automate report generation. This would have been impossible with the babble of chromatographic information existing only a few years ago.

Every day, data systems are declared obsolete and no longer supported by their supplier, computer operating systems change and become obsolete, and hard drive and tape storage systems break down. It quickly becomes obvious to research laboratories how transient and fragile their archived data files really are. It is critically important to have access to file translation from these proprietary formats into a standard format running on modern computer systems.

15

RECENT ADVANCES IN LC/MS SEPARATIONS

Growth in HPLC systems sales had reached almost replacement level when adjusted for inflation until about five years ago. The rapid acceleration of the application of LC/MS systems to solving problems in pharmaceutical research reversed the trend and then gave it a new upward slope. The pharmaceutical industry has always been fruitful ground for developing HPLC uses and applications. LC/MS became the obvious, although expensive, answer for compound identification once atmospheric pressure ionization interfaces matured enough to provide a robust and reliable bridge between the workhorse HPLC and the definitive mass spectrometry detector. An additional spurt in systems sales occurred as proteomics discovered the advantage of using computer-assisted LC/MS/MS polypeptide fragmentation identification for protein characterization.

The mass spectrometer detectors place new demands on the HPLC system. The MS interface requires use of volatile buffers and reagents. Nanospray interfaces especially benefit from low-volume, high-resolution separations. The mass spectrometer is a fast response system and benefits from separation speeds higher than normally supplied by HPLC systems. All of these requirements have provided constraints on new development directions for HPLC systems.

15.1 A LC/MS PRIMER

One of the most important additions to the HPLC arsenal was the development of the evaporative ionization interface that allowed a mass

HPLC: A Practical User's Guide, Second Edition, by Marvin C. McMaster

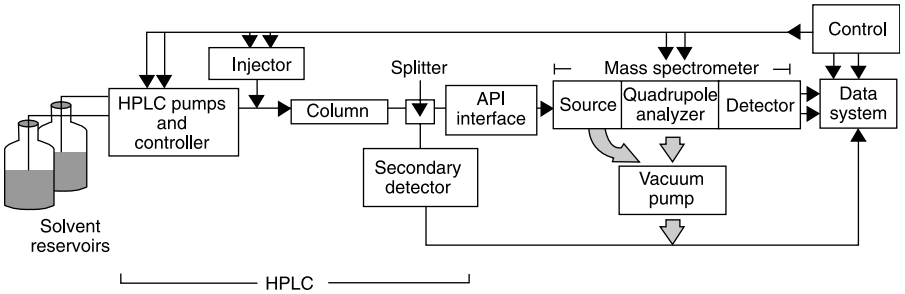

Figure 15.1 LC/MS system model.

spectrometer to be use as a detector. The basic LC/MS system (Fig. 15.1) consists of an HPLC pump or gradient components, an injector, and a column mated to a mass spectrometer through an evaporative/ionizing interface. The simplest chromatogram produced by this system is similar to a UV chromatogram, although possibly with peaks annotated with molecular weights.

The mass spectrometer has the advantage of not only being a universal mass detector, but also of providing a definitive identification of the compounds being analyzed. This advantage does not come without difficulties; mass spectral detectors are very expensive compared with other detectors, large computer data storage is required for the mass of information produced, and compound identification other than molecular weight requires more complex equipment and considerable interpretation skills. Although prices are coming down, mass spectral detectors are still primarily research systems costing in excess of $100,000, with interfaces costing $3–5,000. The high-vacuum pumps required to run the system have become much more reliable, more compact, and less expensive, but still require considerable maintenance. Fragmentation data needed to provide data for structure interpretation as provided by a GC/MS still requires use of LC/MS/MS systems costing $200,000.

But there are signs that simpler, less expensive LC/MS systems designed and priced for the general laboratory bench chemist, production facilities, and quality control laboratories may soon be possible. It remains to seen whether manufacturers will decide to produce these systems. Older MS systems have been purchased, attached to HPLC systems equipped with relatively inexpensive interfaces, and pressed into service for molecular weight determination as a $30,000 detector, indicating that the desire and need exists for general laboratory LC/MS systems. As prices continue to drop and technology advances work their way out of the research laboratories, the LC/MS will become a major tool for the forensic chemist whose separations must stand up in court, for the clinical chemist whose separations impact life and death, and for the food and environmental chemist whose efforts affect the food we eat, the water we drink, and the air we breathe.

With this in mind, let us take a look at the design of the LC/MS, its operation, and the way mass spectral data are manipulated to produce chromatographic information and compound identification. This will be simply an

overview; detailed information is available in *LC/MS: A Practical User's Guide*, listed in Appendix G. Mass spectrometry is a science in itself, but it is important for the chromatographer to have a working knowledge of its techniques.

15.1.1 Quadrupole MS and Mass Selection

The mass spectrometer has been around for a long time, with its major shift into the research laboratory occurring as an outgrowth of the Manhattan Project during World War II. In the 1960s, a useable GC/MS interface was developed, but the first commercial HPLC/MS interface did not appear until the 1970s. A useable atmospheric ionization interface was not developed until the 1990s because of the problem of seeing compounds in the presence of all that solvent.

Mass spectrometers work on the principle that a charged ion being propelled through a curved magnetic field will be deflected inversely proportional to its molecular mass and proportionally to its charge, allowing us to define an ion mass term corrected for its charge, m/z. The lighter the mass, the more deflection that will occur at a given charge. The higher the charge, the more deflection that will occur at a given mass.

The first research instruments were based on the ungainly magnetic sector mass spectrometers that used very large permanent magnets to establish the electromagnetic field and had very slow response times. The accelerated ions of different masses were detected at different impact points on the detector plate and mass ratios were measured (Fig. 15.2).

The first useful research instruments were based around the quadrupole mass spectrometer. Quadrupole mass spectrometers also employ an ion source, a lens to move the charged ions into the quadrupole mass analyzer rods, and a detector, all under high vacuum ($>10^{-5}$ mm Hg). Mass separation is accomplished in a direct current (dc) quadrupole electromagnetic field applied acrossed the mass analyzer rods and is modified by a radio frequency (RF) signal for mass separation and to select and focus the desired mass at the detector (Fig. 15.3). By sweeping the dc/RF field through a range of frequencies, the quadrupole can be made to focus a series of ions of increasing mass on the detector, allowing a continuous measurement of m/z through a selected AMU (atomic mass unit) range (SCAN mode). Alternatively, the quadrupole can be stepped to specific AMU values in a single ion-monitoring (SIM) mode. Scan mode is generally more useful when doing qualitative detection, mass scouting, and in fragmentation studies of unknowns. SIM mode is used for high-sensitivity detection and quantitation.

Another commonly used type of mass spectrometer is the tandem mass unit, also referred to as an MS/MS (Fig. 15.4) or a triple quad mass spectrometer. Originally, this was made up of two or three mass spectrometers used in series. One MS is used to separated ions, the middle unit is used as a collision chamber in which selected ions are allowed to impact heavy gas molecules and fragment, and the last MS is used to separate and measure the fragment ions. In one

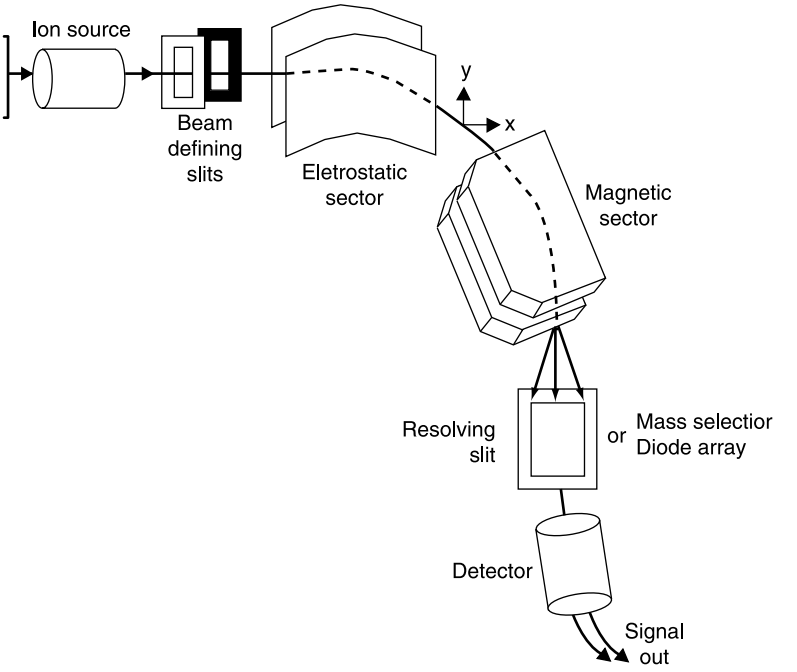

Figure 15.2 Magnetic sector mass spectrometer.

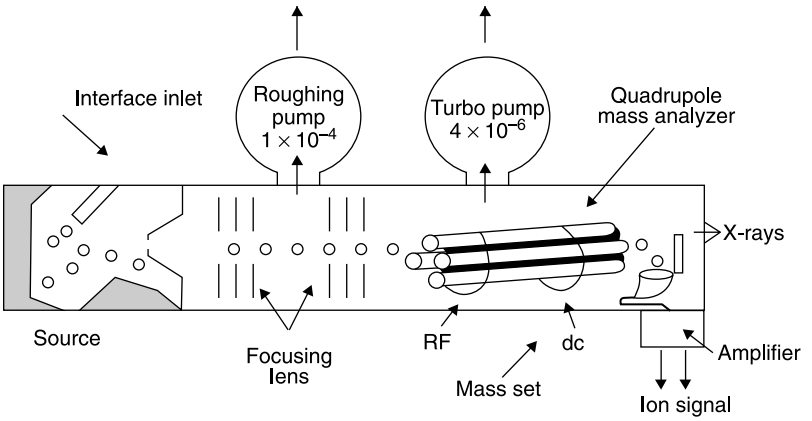

Figure 15.3 Quadrupole mass spectrometer.

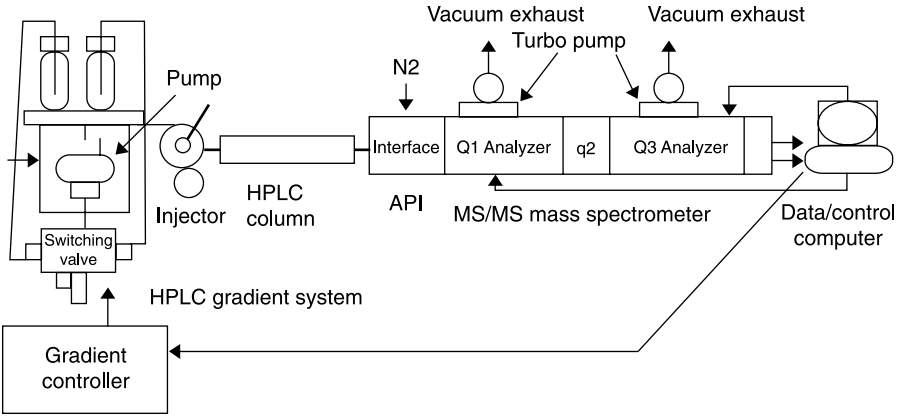

Figure 15.4 Quadruple LC/MS/MS system.

common MS/MS experiment, the first MS unit is used to separate out a specific molecular ion and the second MS is used to examine fragmentation daughter ions that can be used to determine the molecular structure of the original mass ion by comparison to know fragmentation patterns. Alternatively, the third quad can be used to scan the fragmentation ions looking for a specific mass ion to aid in confirming the molecular ion's identity.

15.1.2 Other Types of MS Analyzers for LC/MS

The quadrupole MS detector was the first, and is still the most common, detector used for LC/MS, but a number of other mass spectrometers have been adapted to this application. Both three-dimensional spherical (ITD) and linear (LIT) ion trap detectors offer tremendous potential for general, inexpensive LC/MS systems. They both offer the ability to be used as either a mass spectral detector or as a MS/MS detector. The 3D ITD (Fig. 15.5) allows ions to be trapped in the ion trap where they can be fragmented by heavy gas collision and the fragments released by scanning the dc/RF frequency of the trap.

The linear ion trap (Fig. 15.6) is essentially a quadrupole detector with an electrically controlled ion lens at either end. It can trap a much larger volume of ions in its trap, allowing much higher sensitivity in fragment ion detection for trace analysis as well as MS^n-type of experiments in which fragmentation ions can be trapped and further fragmented to aid in structure studies.

Time-of-flight (TOF) MS detectors (Fig. 15.7) are commonly used in proteomics studies of proteins and protein fragments because this type of detector can handle and analyze very large molecular and fragmentation ions. Fourier transform mass spectrometers (FTMS) are being incorporated into commercial LC/MS systems and offer the advantage of being nondestructive detectors that can trap and repeatedly analyze the same sample in order

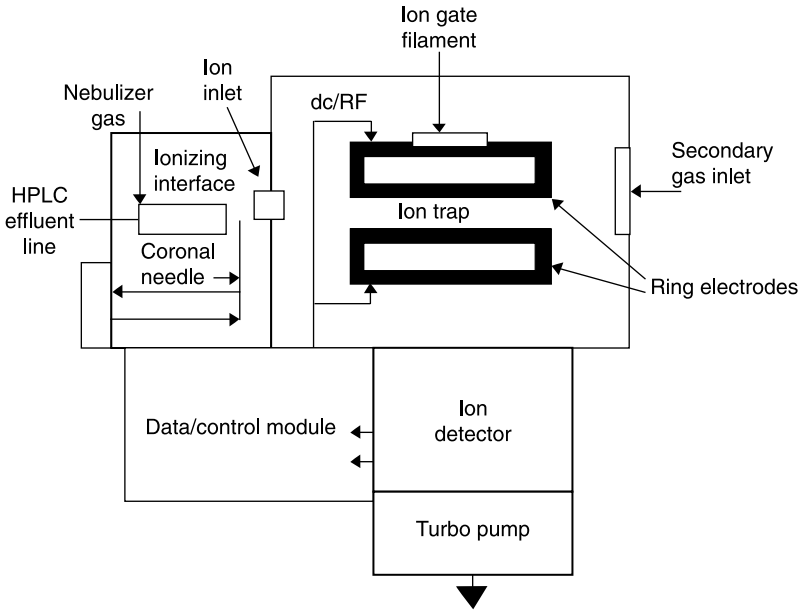

Figure 15.5 Ion trap detector.

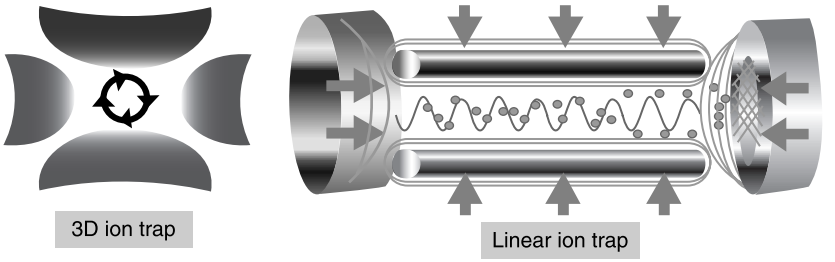

Figure 15.6 Linear ion trap MS analyzers. (Courtesy of Applied Biosystems.)

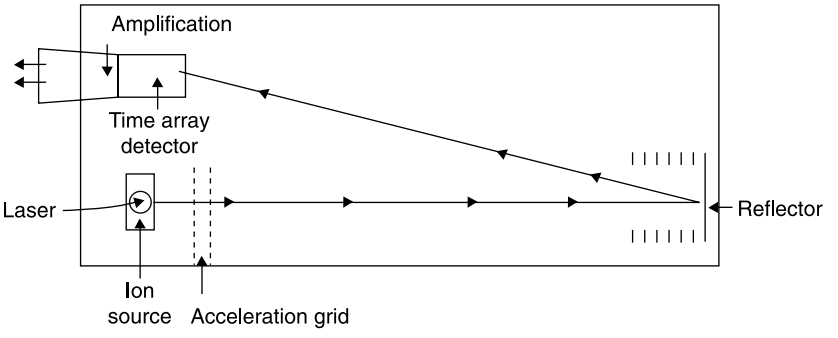

Figure 15.7 Time-of-flight MS analyzer.

to greatly increase analysis sensitivity for things like accurate mass determinations.

15.1.3 LC/MS Interfaces

The first modern LC/MS interface was a thermospray interface introduced in 1983 that allowed introduction of column effluent at 1.0–1.5 mL/min. The mobile phase had to be highly aqueous and contained large amounts of volatile buffer to induce chemical ionization. The mobile phase was forced through an electrically heated capillary and out through a fine orifice into the high vacuum source of the mass spectrometer. The entrance capillary often had to be heated to >200°C and the requirement for >100 mM volatile buffer often lead to sample decomposition and orifice plugging. Roughing pumps had to be added to the system to remove the large amounts of solvent and volatile buffer release in the ionization process.

Today, the two most common LC/MS interfaces are atmospheric pressure ionization interfaces, electrospray (ESI) and ion spray (ISI). Electrospray (Fig. 15.8) and its subtype, nanospray, are recommended for use with proteins and highly polar or ionized compounds. They are very soft ionization, concentration-dependent techniques that result in very little fragmentation and often produce multiply charged molecular ions.

Ionization is accomplished in the electrospray interface by passing the HPLC effluent down a heated metal capillary tube along which an electric charge differential is applied. The evaporating liquid sprays out of the tube end as charge droplets rapidly decreasing in size. A gas nebulizer often

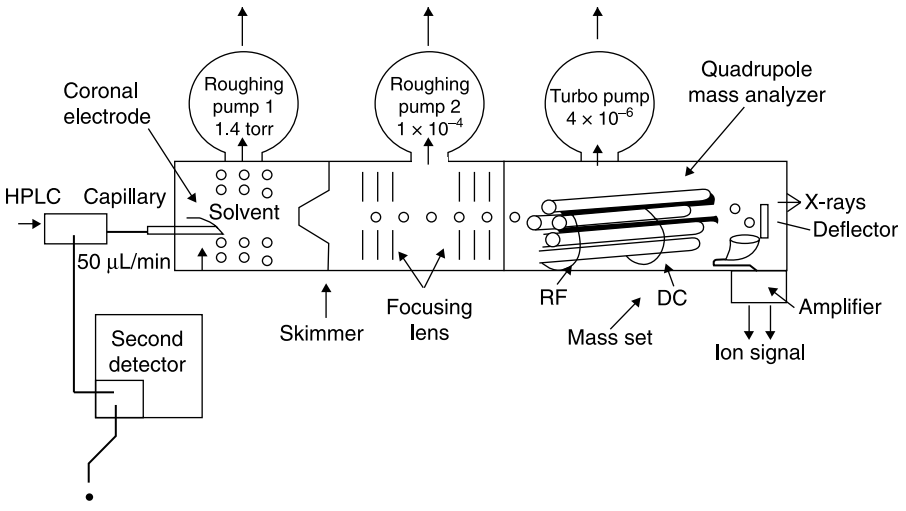

Figure 15.8 Electrospray interface.

encloses the capillary and aids in droplet evaporation. Electrospray is best done at reduced microliter/minute flow rates.

Proteins can acquire multiple positive charges at basic amino acids such as lysine to form multicharged molecular ions. Since the MS analyzer separates ions on the basis of m/z, or mass divided by charge, mass spectrometers with an operating range of 0–2,000 amu can still detect proteins with masses up to 100,000 amu if they have 10–50 charges per molecule. Deconvolution software can resolve this peak charge envelope developed by a single protein to allow calculation of the protein's molecular weight.

An ISI is used with less polar effluents and is the workhorse for standard HPLC systems since it can take flow rates up to 2 mL/min. It is most commonly used to produce intact molecular ions for molecular weight determinations, but it can be set up with an ion repeller to cause fragmentation that can provide preliminary compound identification and structural information.

The ISI interface (Fig. 15.9) also uses a nebulizing inert gas to entrain and break up the eluant stream into small droplets that are sprayed across a coronal discharge needle operated at about 25 KV to ionize the shrinking droplets. The impactor plate is equipped with a charge opposite to that applied to the coronal needle to draw the charged ion to the mass spectrometer entrance. Again, the nebulizer capillary may be heated to aid evaporation or an oppositely charged plate may draw the charged droplets into a heated tube before they enter the mass spectrometer inlet. The ISI is a mild ionization source and generally produces only a single, molecular ion per compound unless the molecular ion is very unstable. Fragmentation does not usually occur as it does in an ESI source of a GC/MS system. A MS/MS-capable system must be used to produce daughter ion fragmentation for structural studies.

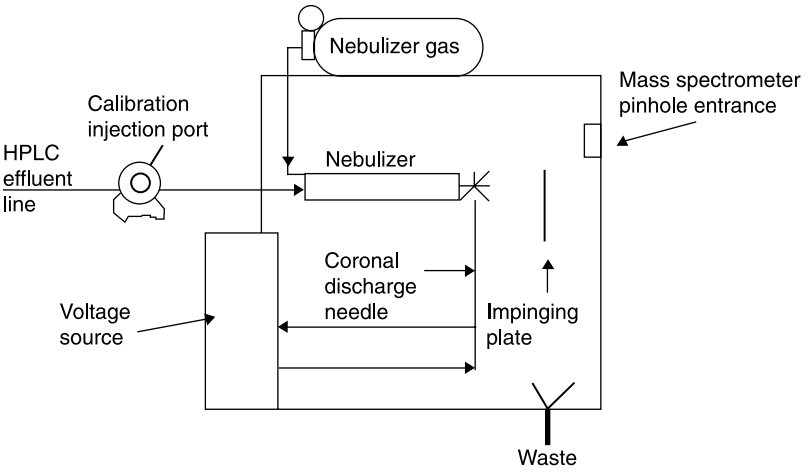

Figure 15.9 The ion spray interface.

Most modern LC/MS systems are equipped with replaceable ESI and ISI interfaces, depending on the types of compounds expected in the effluent. Agilent Technologies has recently released a multimode source capable of ionizing all compound types with only minor loss in sensitivity compared with dedicated sources. If this interface meets its initial claims and can be retrofitted to other LC/MS systems, it could rapidly replace either of the other two interfaces for general applications instruments.

Another interface commonly used for connecting HPLC to a mass spectrometer is not a true in-line interface. It is a robotically controlled spotter plate system for collecting samples from the HPLC to be injected into the MALDI time-of-flight laser ionization mass spectrometer for analyzing proteins and large peptides. The effluent sample dropped in the plate well is mixed with an ionization matrix already present, solvent and volatile reagents are evaporated, and the plate is then placed into the injector target and blasted with a pulsed laser to volatilize and ionize sample into the atmosphere of the interface where it can be drawn into the mass spectrometer.

15.1.4 LC/MS Computer Control and Data Processing

The mass spectrometer like the diode-array UV detectors produces a three-dimensional array of time-voltage-spectral information and requires considerable computer power to handle both MS scanning control and data processing. A simple general-purpose ISI-LC/MS is designed to separate compounds and present a chromatogram with peaks identified by retention times and molecular weights. The system computer must start/stop the system, scan the MS mass range, identify the molecular weight of the major molecular ion, and display the total ion chromatogram (TIC) annotated with the major peak molecular weight.

The MS detector is more sensitive and requires much less sample than UV detection. The LC/MS system is often designed either with an in-line secondary detector or with a splitter system before the interface and a second detector so that additional information can be obtained on the sample. Figure 15.10 compares the chromatographic signals from a variable UV detector and the full-scan total ion chromatogram (TIC) from a MS detector run in series for detection of an adhesive extract.

A more complicated task faces an ESI-LC/MS designed to separate and determine the molecular weights of proteins. The proteins have to be separated by the column, the multiply charged ion envelope must be measured, and deconvolution calculations made to determine the molecular weights of the separated protein(s). Often, there are partially resolved proteins mixtures and the overlapping peak envelopes must be resolved to determine molecular weight for both components present.

Working with a LC/MS/MS system adds further degrees of complication. The system computer must control both MS units either in a scanning or in a

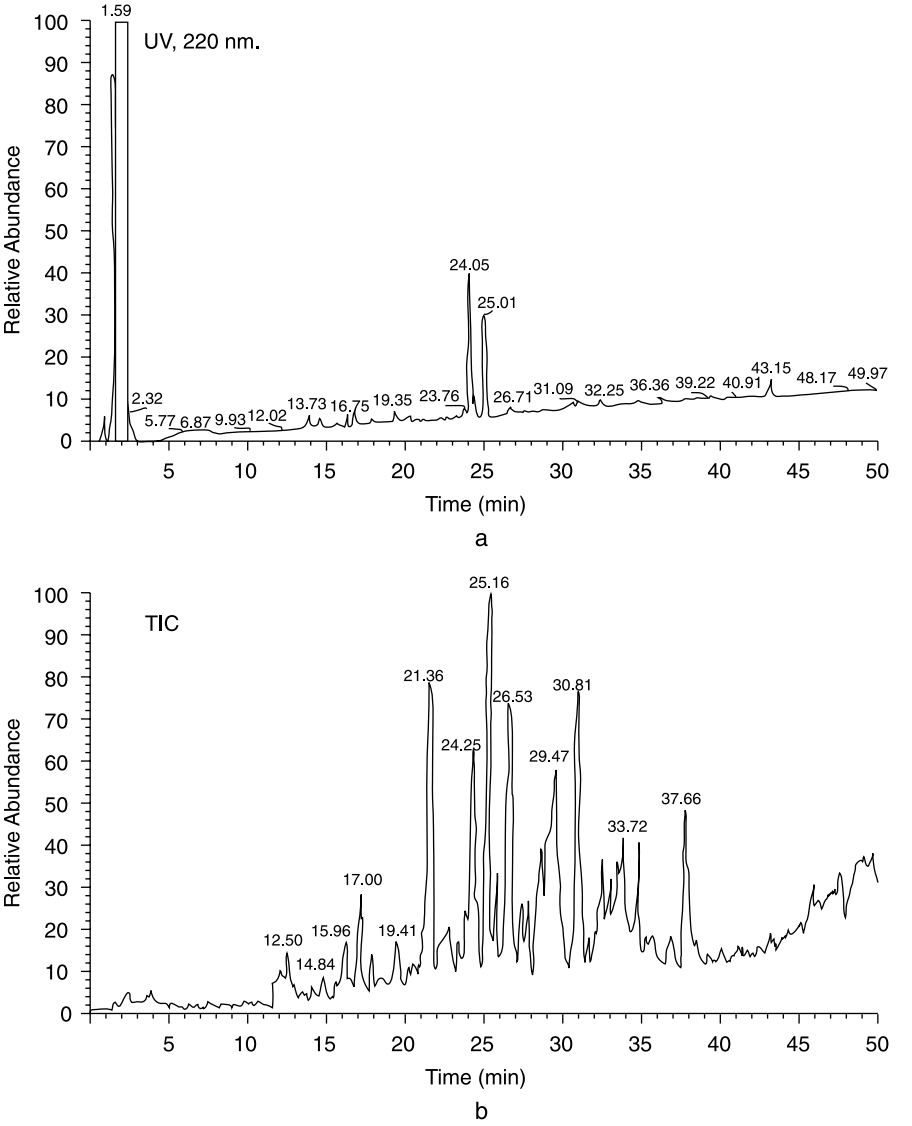

Figure 15.10 Comparison of (a) UV and (b) TIC full-scan MS chromatograms of adhesive extract. (From Tiller et al., Copyright © 1997 John Wiley & Sons, Limited. Reproduced with permission.)

single ion mode. The data array must be examined for fragmentation patterns for daughter ion identification, hopefully using a spectral library database. Anything but the simplest routine procedure becomes a mass spectrometry research project.

15.2 MICROFLOW CHROMATOGRAPHY

Two factors are driving the market for precise, very-low-flow HPLC pumping systems: extremely limited sample sizes in biotechnology and the electrospray and nanospray interfaces that are concentration and flow-rate dependent. It is very difficult to get precise flow and gradient formation from pumps that have a 5- to 10-μL plunger displacement, even using 3200-step stepper motor drives. This has forced manufacturers to resurrect a very old concept from the earliest days of HPLC, the syringe pump.

A syringe pump is a cylinder equipped with a tight-fitting piston and inlet and outlet check valves. Opening the inlet check valve and pulling back on the plunger fills the cylinder. Closing the inlet valve, opening the outlet valve and driving the plunger forward initiates flow. Syringe pumps generate smooth, pulse-free flow, but their delivery solvent volume is limited to the capacity of their heavy-walled cylinder. In practice, this translates to an upper volume limit of about 250 mL, which is insufficient for most standard HPLC separations. Beyond this volume, pressure-resistant cylinder wall armoring became cost prohibitive.

Micro- and nano-HPLC systems (Fig. 15.11) rely on small-diameter and capillary columns packed with high-efficiency packing materials and very slow flow rates to produce concentrated solutions and sharp chromatography peaks to feed electrospray interfaces for mass spectrometers.

Solvent volumes per run do not exceed the capacity of the syringe pump(s) because of the extremely low flow rates. Injection size is limited in these systems by incorporating a fixed-volume injector loop within the body of the

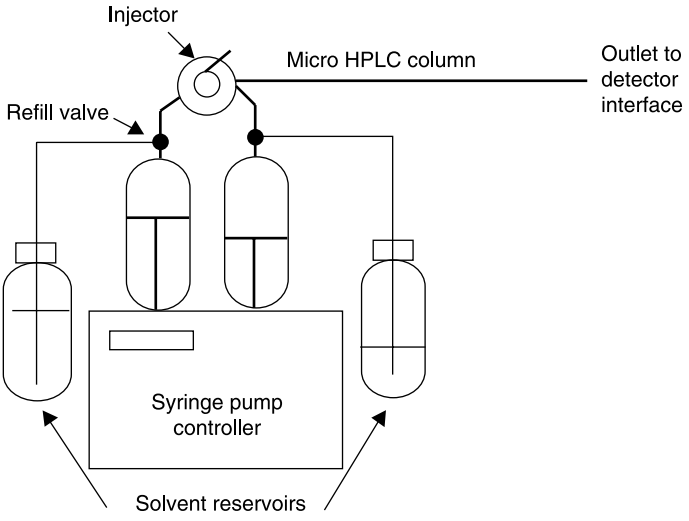

Figure 15.11 Syringe pump micro-HPLC.

injector. Decreased diameter and volume of tubing and connectors further increases the efficiency of these systems.

15.3 ULTRAFAST HPLC SYSTEMS

A different philosophy is taken with the ultrafast chromatography systems, such as the ACQUITY^{R} ultraperformance liquid chromatography (UPLC) system that dominated the pharmaceutical LC/MS market after its introduction in 2005. These systems are also designed to deliver highly concentrated effluent quickly to a mass spectrometer. But, the ultrafast systems use very small particle size HPLC-packed columns run at very high system pressures. Silica has always had the mechanical strength to handle pressures to about 15,000 psi. The problem has been in sealing the system to resist leakage.

When first introduced, the only column available for the ACQUITY^{R} system was a 1.7 μL C_{18} hybrid silica, but an expanded line of C_{18}, C_8, and phenyl bonded-phase columns are now offered for this system. As column diameters moved from 10 to 5 to 3 μ, it was found that the resolution loss at high mobile phase flow rate began to level off. Particles with 1–3 μ diameters show very little if any resolution loss at very high flow rates. Of course, very small diameter particles still show very high back-pressures at high flow rates, require very small pore frits to keep them in the column, and are very easily contaminated with particulate matter in the mobile phase. UPLC and nano-UPLC systems are now being sold that are optimized for LC/MS operation at 12,000 psi back-pressure and flow rates up to 10 mL/min flow rate with a corresponding decrease in separation times.

15.4 CHIP HPLC SYSTEMS

Another interesting HPLC technology is the miniaturized HPLC-on-a-chip system. An outgrowth of microfluidics technology used in inkjet printing and automated bioanalyzers, it consists of tiny closed channels etched onto a glass or plastic microchip. This system is designed to marry into a microfluidics nanospray interface on a second chip that will spray directly into a mass spectrometer. Used in an interface mounted on a mass spectrometer, they provide an easily replaced works-in-a-drawer solution to column and nozzle plugging while minimizing extracolumn problems normally associated with tubing and fittings.

Agilent Technologies currently offers commercial nanospray HPLC-Chip^{R} and HPLC-Chip Cube^{R} MS and MS/MS systems. Chips come with standard C_{18} packing or can be custom packed with standard HPLC column materials. They are aimed at proteomic labeling studies, and small molecule separations.

15.5 STANDARDIZED LC/MS IN DRUG DESIGN

Standard or generic LC/MS methodology was developed by pharmaceutical companies to provide rapid screening and a common informational database throughout a company's activities. Used from drug development screening through process monitoring and metabolite and degradation studies on compounds from almost any media, such as urine, plasma, or reaction mixtures, only the sample preparation will vary. No attempt is made to optimize the chromatographic separation for individual compounds or classes of compounds. A standard 5–20-min linear reverse-phase gradient from 5% organic in water to 95% organic at neutral pH is run on a specific C_{18} column type from a single manufacturer. The mass spectrometer is run in scan mode over a standard m/z range with a time delay to ignore the solvent spike and the retention time and molecular ion mass is determined for each compound.

The advantage of generic LC/MS run conditions is that it allows the preparation of an LC/MS separation database that can be referenced for compound mixtures from anywhere in the development and manufacturing process cycle. It trades off resolution for consistency, speed, and a decrease in methods development times. It permits creation of a computer-searchable database of information for all of the compounds being investigated in the company. The mass spectrometer provides sensitivity and resolution gain as well as information on retention times and molecular weights.

When I first saw the linking of combinatorial chemistry with generic LC/MS methods I was appalled to the depth of my traditional chemist's soul. It looked like they were using the power of the mass spectrometer resolution to try to fix bad chemistry and bad chromatography. But when I studied the technique I realized it used the same resolution, speed, and load triangle that we take advantage of in doing preparative chromatography. There we sacrifice resolution to gain load and speed. In generic chromatography, they trade off resolution for speed and analytical compatibility across the drug discovery and development process.

16

NEW DIRECTIONS IN HPLC

Separation speed and ease of use seem to be the primary factors driving changes in HPLC instrumentation. Resolution efficiency and stationary phase stability, especially at high pH, are the primary factors affecting current changes in column technology.

16.1 TEMPERATURE-CONTROLLED CHROMATOGRAPHY

Temperature has often been suggested as a useful control variable for HPLC to make α changes and to speed equilibrations leading to faster separations. The problem has been that both bonded-phase hydrolytic cleavage and solubility of silica in aqueous solvents are accelerated at elevated temperatures. Mobile phase boiling within the column can cause bubble formation and vapor locking if the critical point of the solvent is exceeded. Finally, thermal-labile compounds can suffer degradation at elevated temperatures.

Many of these problems disappear when a hybrid-silica column or a zirconium-based HPLC column is selected. Hybrid-silica packing material has an organic skin coating and protecting the silica surface. Advanced zirconium bonded-phases are chemically bound directly to the zirconium surface, which does not dissolve in aqueous solvents. ZirChrom has released a newsletter (vol. #5) 2001 (see Appendix G) claiming a 12-fold decrease in separation time without loss in resolution using an efficient column heater. The technique benefits from using narrower columns to avoid temperature gradients in the column and mobile phase preheating before entering the column. Selecting a

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relatively high boiling mobile phase such as aqueous acetonitrile allows separations at 120°C because the elevated pressure in the column raises the boiling point. The hybrid-silica columns show some of the pH stability of the chemically bound zirconium columns but probably would show decreased operating lifetimes due to increased packing material solubility.

16.2 ULTRAFAST CHROMATOGRAPHY

With large diameter HPLC packings, efficiency decreases rapidly at higher flow rate, as does back-pressure. Five- and 10-μm spherical packings show a maximum efficiency at 1–1.5 mL/min flow rate. However, when 3-μm packing appeared they showed little loss of efficiency at 3.0 mL/min; the back-pressure increases dictated the use of shorter columns, trading efficiency for shorter run times. Use of finer particles required the use of finer bed support filters and increased the danger of plugging by particulates in the injected sample.

The UPLC Acquity^{R} system mentioned in Chapter 15 takes this flow rate–resistant efficiency gain another step using a 1.7-μm hybrid-silica packing material run at back-pressures up to 12,000 psi to provide flow rate decreases of 3–5-fold. Silica has always had the capability to resist high back-pressures; many traditional HPLC columns are packed with 20,000 psi pumping systems. But, system components from pumps, injectors, and fittings all had to be redesigned to resist the extra pressure. Extra column volumes had to be reduced to avoid losing the increase column efficiency. A column heater is built into the system to take advantage of the increased hydrolytic stability of the hybrid-silica column in speeding flow rates. Obviously, a limit exists as to how much farther you can reduce the particle size of the packing material and still keep it with in a column and pump solvent through it at an acceptable back-pressure.

16.3 MONOLITH CAPILLARY COLUMNS

In the first edition of this book, I forecast that the ultimate HPLC column would be a "wall-bonded" capillary column that would avoid the voiding and back-pressure problems seen with packed columns. A new type of column, the monolith silica column, recently emerging from research laboratories very closely fits this description. A monolith column has a honeycomb foam of silica, which is bonded with an organic separating phase, completely filling the inside of the column.

One way of preparing the silica honeycomb is to mix tetramethoxysilane in a porogenic solution of polyethylene glycol and urea in the presence of acetic acid. This mixture is poured into a capillary tube treated with sodium hydroxide. The tube is first heated to hydrolyze the urea to form pores and then further heated to burn off organics leaving a continuous silica foam skeleton

with large through pores completely filling the tube. The monolith column formed is then treated with a silylation reagent to form a bonded phase within the silica pores. Commercial columns, such as the Chromolith SpeedRod™, run with efficiency of a 2–3-μm particulate column, but can be run at flow rates of 6–8 mL/min without exceeding approximately 2,500 psi back-pressure, greatly decreasing run times.

These columns offer the potential for creating a hybrid-silica monolith, which can be run on existing HPLC systems at high flow rates, that are temperature and pH resistant. By their very nature, these columns would be void free and the only column killers that they would suffer from would be particulates and bound organics. They probably could be reverse flushed for particulate wash out and bound materials could be washed off with strong solvents.

16.4 MICRO-PARALLEL HPLC SYSTEMS

A variation of the microfluidics HPLC-on-a chip mentioned in Chapter 15 is the polymeric Brio™ cartridge system. A replaceable 8- or 24-channel Brio™ cartridge contains side-by-side 30-cm × 0.5-mm i.d. column channels packed with standard HPLC stationary phase. The cartridge is run loaded into an autosampler-equipped Veloce™ gradient chromatography system. A Brio™ cartridge inserted into the Veloce™ system makes the same HPLC separations in all channels with simultaneous UV and/or FL detection. As a quality control or cost-per-test instrument, the 100-run cartridge allows accelerated assessment of compound purity, stability, and other physiochemical properties. Running this many 15 sec/sample separations on a single instrument can rapidly reduce a laboratory's sample load.

16.5 TWO-DIMENSIONAL HPLC SYSTEMS

Much of the pressure to develop automated sequential HPLC separations has come from the necessity to separate complex biological mixtures, especially protein mixtures. Traditionally, complex mixtures of proteins have been separated using two-dimensional gel electrophoresis (2D GEP). The first dimension gel separation is carried out with electrophoresis buffers, the gel plate is rotated 90° and the second SDS-PAGE separation is carried out under denaturing conditions, using sodium dilauryl sulfate. The separated spots are then visualized, scraped off the plate, and then extracted for further analysis. Protein analysis by MALDI time-of-flight mass spectrometry starts with this time- and labor-intensive 2D GEP separation mode.

In theory, combining two HPLC modes sequentially would provide an on-line LC/LC/MS/MS and speed the analytical procedure. Bands from the first separations could be detected and collected with an automated loop-and-valve injector, and then individual bands could be passed to the second LC for

separation using a different separation mode. A model first mode for LC_1 would be an affinity separation of antibodies followed by a partition separation in LC_2 of the purified effluent cuts passed from the affinity column, with the peaks from the bonded-phase column being analyzed by the MS unit. This sequence would benefit from removal of salt or small molecules used to displace the antibody proteins from the first column in the break through of the reverse phase column.

The main problem that has been encountered so far in the development of a LC/LC system is the large differences seen in the resolving power of the various HPLC modes. Partition separations are dramatically more efficient than either ion exchange or size separations, the other modes normally used for separating proteins. Attempts to do two-dimensional sequential partition separation using different types of bonded-phase columns have not provided significant improvements in separating power to justify the technique. Only affinity separations seem to provide a specific enough first separation to provide a useful feedstock for the partition dimension purification.

16.6 THE PORTABLE LC/MS

Advances in the art of chromatography and micro-miniaturization of hardware components and electronics are rapidly approaching the point where an HPLC in a suitcase is a real possibility. Components such as high-charge density batteries, nano-flow syringe pumps, the chip HPLC, monolith cartridge HPLC columns, and tiny portable computers leave only a compact RI or UV detector as the missing element. Add to this a chip LC interface and a miniaturized quadrupole or ion trap MS detector and the picture is complete. Already, high vacuum fist-size turbo pumps and 5-in long quadrupole analyzers are available.

The system would be put together in a vented case with the fluidics on the bottom. A syringe pump, pre-loaded with solvent before leaving the laboratory, the battery, and a waste vessel would be in the first layer. Stacked above it would be a nebulizer helium lecture bottle, chip ISI interface, the internal loop injector, the column, and the mass spectrometer. The MS unit would also be evacuated with a roughing pump before leaving the lab and the turbo-pump spun up and left running on the internal battery. The final layer would be the touch-pad flat screen portable to control the MS unit and display MW annotated TIC chromatograms. A portable thermal dye sublimation printer would be sold as an option, but chromatography reports could be printed on return to a central laboratory. The whole unit could be powered by its internal battery, but most likely would draw most of its power from an automobile hot point charger.

The demand for such an LC/MS luggable would come from the field environmental chemist, from the arson investigator, and obviously from your local forensic CSI and drug enforcement teams. It would avoid the problem of

sample aging and delays in compound analysis by providing an immediate answer on-the-spot, based on tables of retention times and molecular weights for suspect compounds.

I hasten to add that this system does not exist at the moment. You probably do not want to include it in your equipment proposal for this year. But, it is rapidly becoming a viable option for development. And, if successful, would the portable linear ion trap (LIT) based LC/MS/MS, for definitive compound identification by searching a MS database, be far behind?

APPENDICES

I have enclosed seven items as parts of the Appendix. The first, a separations guide, points out starting points for chromatographic separations and also suggests trends in usage of columns, mobile phase, and detectors.

The second item is a list of frequently asked questions (FAQs) that I have encountered while serving as an in-house trouble-shooting resource for customers and as an instructor for extension courses. I taught 30 two-day HPLC course in seven states before I began teaching at the University of Missouri–St. Louis. Many of these answers arose as responses to problems raised by students in my classes.

In the third appendix, I have added tables of solvents and volatile buffers important for use in LC/MS. Nonvolatile buffers cause problems when you remove solvent and ionize effluent for injection into the mass spectrometer and need to be avoided when this detector has been selected.

The fourth item is a glossary of HPLC terms. I have tried to include much of the terminology and buzzwords used in the field.

The fifth part is a trouble-shooting quick reference. It is not intended to replace the systematic trouble-shooting discussion in Chapter 10. When things go wrong, however, you may find it helpful. I have arranged it in the way things flow through the system: from pumps to the integrator or data acquisition computer.

The sixth item is a series of three HPLC laboratory experiments. The first familiarizes the student with getting a system up and running and calibrating a column with standards. The second experiment shows how to clean a column and pacify a system. The last is a first, quick look at methods development.

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These are three areas where I feel new users tend to get hung up or ignore when first approaching an HPLC system.

The last appendix is a selected reference list. It is not intended to be exhaustive, but simply to give you a point to enter the literature in the field. To stay current you probably want to subscribe to *American Laboratory*, *LC/GC Magazine*, the *Journal of Liquid Chromatography*, and *Chemical Abstracts—HPLC Selects*. *Chemical Abstracts* are also on-line as part of Dialog's computer database. I have found Google to be a very usefully search engine when I am trying to learn about a new instrument or obtain background information about a new technique. HPLC literature is extensive, published in many and surprising places, and of variable quality and reproducibility.

APPENDIX A

PERSONAL SEPARATIONS GUIDE

Application	Column	Detector	Conditions [a,b]
1. Vitamins (water soluble)	C_{18}	UV (254 nm)	8% AN/H_2O, C_7SO_3
2. Vitamins (fat soluble)	C_{18}	UV (280 nm)	80% AN/H_2O
3. Steroids	C_{18}	UV (230 nm)	60% MeOH/H_2O
4. Triglycerides	C_8	UV (220 nm)	60% AN/H_2O
5. Phospholipids	Si	UV (206 nm)	130/5/1.5AN/MeOH/ 85% H_3PO_4
6. Prostaglandins	C_{18}	UV (192 nm)	35% AN/H_2O, PO_4. pH 2.5
7. Bromphenacyl acids	C18	UV (254 nm)	15–80% AN/H_2O
8. Krebs cycle acids	RNH_2	UV (210), RI, CAD	25–250 mM PO_4, pH 2.5
9. Monosaccharides	CX-Ca	UV (195 nm), RI, CAD	H_2O (80°C)
10. Polysaccharides	TSKpw	UV (195 nm), RI, CAD	H_2O (<20% AN)
11. Nucleic acids	CX-Na	UV (254 nm)	0.4 M NH_4HCO_2, pH 4.6
12. Nucleosides	C_{18}	UV (254 nm)	8% MeOH/H_2O, PO_4, pH 5.5
13. Nucleotides	C_{18}	UV (254 nm)	20% AN/H_2O, TBA, PO_4, pH 2.6
14. PTH amino acids	C_{18}	UV (254 nm)	10% THF/5 mM AcOH →10% THF/AN
15. OPA amino acids	C_{18}	Fl (230/418)	8% AN/PO_4, pH1.6→ 3/25/30/40–DMSO/ MeOH/AN/H_2O

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Application	Column	Detector	Conditions [a,b]
16. Peptides (<99 amino acids)	C_8	UV (254 nm)	→30% n-BuOH/0.1% TFA, H_2O
17. Peptides	C_3	UV (210 nm)	40–70% AN/H_2O, PO4, pH 5.5
18. Proteins (enzymes)	TSKsw	UV (254,280)	0.1 M Tris, PO_4, pH 7.0
19. Proteins (enzymes)	TSK_{DEAE}	UV (280 nm)	50 mM PO_4, pH 7.5→ +150 mM NaCl
20. Proteins (structure)	C_3	UV (280 nm)	0.1% TFA→75% AN, 0.1% TFA
21. Catecholamines	C_{18}	UV (270 nm)	6% MeOH/H_2O, C_8SO_3, EDTA, PO_4, pH 4.
22. Theophylline	C_{18}	UV (270 nm)	7% AN/H_2O, PO_4, pH 4.0
23. Anticonvulsants	C_{18}	UV (220 nm)	40% MeOH/H_2O
24. Tricyclic antidepressants	C_{18}	UV (254 nm)	55% AN/H_2O, C_5SO_3, pH 5.5
25. Aspirin, acetominophen	C_{18}	UV (254 nm)	10% AN/H_2O, AcOH, pH 2.5
26. Aflatoxins	Si	UV (235), Fl, CAD	6% MeOH/hexane
27. PNA	C_{18}	UV (254 nm)	80% AN/H_2O
28. Pesticides (carbamate)	C_{18}	UV (192), RI, CAD	50% MeOH/H_2O
29. Pesticides (PO_4)	C_{18}	UV (192), CAD	50% MeOH/H_2O
30. Pesticides (chlorinated)	C_{18}	UV (220 nm) CAD	80% AN/H_2O

[a] These separations are intended as a guide. They are not intended as recommended or standard procedures for *in vivo* diagnosis. Conditions will vary from compound to compound and from column to column.

[b] Abbreviations: AN, acetonitrile; AcOH, acetic acid; CAD, charged aerosol detector; DMSO, dimethyl sulfoxide; PO_4, pH 2.6, phosphate buffer, pH 2.6; RI, refractive index detector; TFA, trifluoroacetic acid; C_7SO_3, heptance sulfonate; TBA, teriary butylamine; UV, ultraviolet detector.

APPENDIX B

FAQs FOR HPLC SYSTEMS AND COLUMNS

I've made a list of common HPLC questions I hear from students and customers and the answers that I found. This list is not exhaustive. I have tried to leave out some of the more inane question that I have gotten. One of the most common questions that I did not include was, "Why won't my system start up?" I would ask the person on the phone if the system were plugged in. After the explosion on the other end settled down, I would say, "Sir, sometimes the janitors unplug lines so they can plug in their polishers. Would you please check to see if it is plugged in?" Usually after about a minute or so I would hear a quiet, embarrassed click as the phone was hung up.

HPLC SYSTEM FAQs

Why do I need to use helium gas on a liquid chromatography?

Helium might be used for two reasons. Low pressure mixing valve gradient systems suffer from bubbles being pulled out of solution and stalling the pump head unless air is flushed out of the solvents by helium purging. Sometime, the solvent reservoirs are pressurized with helium gas to aid in smooth solvent flow. Helium or nitrogen also may be used as the nebulizer gas in an atmospheric pressure ionization interface to remove solvent, volatile buffers, and aid in ionizing compounds in the LC effluent.

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Do I need a gradient system? And if so, why?

Gradient systems let you control flow rate and solvent/buffer changes to improve chromatographic separations. They can be used to sharpen separations and to speed column re-equilibration. A four-solvent gradient system is useful for methods development when equipped with methanol, acetonitrile, ammonium acetate buffer, and formic acid solution. But, many quality control laboratories prefer to use inexpensive isocratic systems that run a constant-composition premixed mobile phase for rapid separations.

Do I need an autosampler?

Autosamplers and robotic workstations provide reproducible injections and allow automation of the chromatographic separation, but add significant cost to the system. Many university laboratories prefer to substitute graduate students to do the same job.

Why does my LC system keep shutting itself off?

HPLC pumps are equipped with over-pressure settings to protect fragile columns. Perhaps your settings are set too low or your column frits may be plugged, providing too much back-pressure. If the pressure settings are correct, you may have to clean the line from the injector to the column or the column frit. The most common cause of plugs in lines or frits is material not filtered from samples or mobile phase. Buffers precipitate when you switch between incompatible solvents; washing buffers out with water before moving to a new organic mobile phase will help prevent this problem in the future.

Why would I need an inert HPLC system?

Inert systems are used for two reasons. Purification of proteins can be contaminated and enzymes can be deactivated with metals ions extracted out of stainless steel. Also, inert systems are resistant to concentrated salt solutions. Some protein purifications require in excess of 150 mM salt.

What kind of sample preparation do I need to do before I inject?

That depends on what you are analyzing. Interfering compounds need to be removed as much as possible; proteins precipitated, lipids extracted, cells and particulates filtered or removed. Some samples need to be concentrated to aid in detecting trace amounts in dilute samples. Check the literature for your particular compound, use traditional procedures for compound purifications, and look into the possibility of using SPE columns for pre-column purification and concentration (see Chapter 12).

Do I need HPLC-grade solvents and water? Do I need to filter samples?

The answer to both of these is Yes! HPLC water is the most important ingredient. Triple-distilled water used for HPLC has ruined many a chromatography run because of co-distilling nonpolar organic contaminates. Use HPLC-grade solvents and columns from a reliable supplier. Unfiltered samples lead to injector and column frit plugging. Filtering mobile phase after pre-mixing them will cut wear and down time on pump check valves and lines. If you are going to invest money in systems and operators, don't cheapen out the system by trying to save money on supplies. Also, pick a column manufacturer and stay with them to get the best reproducibility from column to column. Otherwise, you will continuously be wasting your lab's time doing methods development to adjust your chromatography to the new column.

What are all these special detectors I keep hearing about?

Some compounds are transparent and undetected by UV and FL detectors. They may be present in tiny enough quantities to be undetected by RI detectors. Evaporative light scattering detectors (ELSD) and charged aerosol detectors (CAD) can see almost any compound with good sensitivity. Mass spectrometric detectors (MSD) also can see almost any compound at high sensitivity and can also determine its molecular weight.

What is pacification and how can it help protect my LC?

Pacification is a technique for removing organics and buffers from HPLC metal and Teflon surfaces and protecting them from salt corrosion with 6 N nitric acid (see Chapter 4). First, remove the HPLC column and replace it with a column bridge. Do not flush this wash into the mass spectrometer. Wash the system with water. Remove the column and replace it with a column blank. Flush with 6 N nitric acid for at least 30 min, then overnight with water. Ensure the effluent pH is back to that of lab water. Replace the column and flush with mobile phase. This should be done at least once a month to clean check valves, line, and injectors. Under no circumstances should this wash be done with an HPLC column in place or into the mass spectrometer!

What is a fast chromatography system and do I need it for my laboratory?

You are probably referring to the new UPLC system that Waters introduced last year. It is a system that was designed to significantly cut run times and interface with mass spectrometric detectors. It is designed from the ground up to run 1.7-μm bonded-phase packings at high flow rates and very high system back-pressure (see Chapter 16). Another fast system is created to run

zirconium columns at elevated temperature with a special column heater. If you simply want to speed your chromatography on your existing system, try going to a short 3-μm column and see if that does not give your separations at boost. Make sure you filter your sample through 0.22-μm filters, because these columns plug up easily.

What kind of detector do I need to do carbohydrates and phospholipids?

A refractive index detector can see both of these types of compounds. A variable UV detector at 206 nm or 195 nm will work for both at reasonable concentrations. I would recommend either a CAD (see Chapter 5) or a mass spectrometer (see Chapter 15) if you need to do gradients or high-sensitivity detection. Both are expensive but give good results. The MS will give you molecular weight data for your separated peaks as well.

What do I need to upgrade my HPLC to do mass spectrometry?

The best answer I have heard for this question is deep pockets. You will need an ionizing interface and a mass spectrometer. The least expensive conversion that I know of was a customer who bought a slightly used GC/MS unit from a hospital lab for $50,000, pulled off the GC, bought an ion spray interface for $4,000, hooked it up to his HPLC, and got the instrument up and running. Normally, you would expect to pay at least twice that for a basic LC/MS, but prices show signs of coming down, so check around.

HPLC COLUMN FAQs

Which is the best column: reverse phase, ion exchange, or size separation?

Each column type has its own place of use. Column variety is what gives HPLC its versatility. It really depends on your compound and application. Approximately 80% of all separations are done on 5–10-μm reverse phase C_{18} silica columns. Much of this is tradition. Reverse phase columns offer high-resolution separations for a wide variety of compounds and can be run in aqueous mobile phases. Ion exchange separations require salt solutions for separations, and these are not compatible with mass spectrometers. Size separations have lower resolving power and longer run times, but may be the only way to separate proteins solutions that will irreversibly stick to reverse phase columns. Use small pore size separation columns to remove salt from effluent from other chromatography separations. Zirconium and polymeric column are newer and offer possibilities for unique separations.

Do I have to use buffers to achieve separation? What is ion pairing?

Buffer and ion pairing reagents are used to sharpen and control separations. Buffers adjust the pH of the mobile phase and the compounds it contains. Compounds such as organic acids, phenol, and amines are partially ionized at their pK_a. They exist as two species that the column tries to separate, leading to broad, tailing peaks. By using buffer to control pH, we force the compound into one form or the other. For LC/MS, choose volatile buffer, such as ammonium acetate, instead of nonvolatiles such as sodium phosphate. Ion pairing reagents form nonbonded complexes with ionized compounds to control where they elute in a separation.

How long will my HPLC columns last?

There is no specific life span of an HPLC column. A number of things can "kill" columns, including bonded phase loss, voiding or channeling, dissolving support, and irreversible binding of material from injections. Most can be either prevented or treated. Many cost-per-test commercial laboratories set a goal of 1,000 injections for a column. Silica columns should be kept as much as possible at a pH between 2.5 and 7.5 and pressure shock or sudden changes of solvents should be avoided. Zirconium and hybrid-silica columns are more forgiving of pH extremes. Polymeric columns can be used from pH of 1 to 11, but should be protected from bed collapse under high pressure. For more information on column killers and column healing see Chapter 6.

How long does it take to change columns?

Column changes are a viable methods development tool in HPLC. Column can be switched at any time. Attach the new column with a slow flow of solvent from the injector outlet line to fill the inlet fitting of your column so you don't leave air in the column entrance. Figure on flushing a column with at least six column volumes of a new solvent to re-equilibrate the column before injecting a sample. Plan on ignoring the first injection. Usually, the second, third, and subsequent injections will be reproducible.

Why does my new column give such bad looking peaks?

You either used the wrong diameter of tubing for the line from the injector to the column or you used tubing from a previous column connection. Manufacturers use different depth in column fittings. The wrong diameter tubing or a line made for a different column can create extra column volumes that can ruin your chromatography. Use fine internal diameter tubing and always make the fitting in the column inlet in which it will be used; 0.009-in tubing for 10-μm packing and smaller diameter tubing for 5- and 3-μm packing. I once saw a brand-new 5-μm column give terribly broad, tailing peaks in a laboratory. The inlet line had come from a mixed tubing drawer and I found it to be

0.02-in i.d. The column gave perfect needle-sharp peaks when we replaced the line with 0.009-in tubing and a fitting made in the inlet hole.

How do I go about cleaning columns?

Column cleaning is an art and extensively covered in some detail in Chapter 6. Most things that adhere to a column will stick on a guard column of the same packing. Guard columns are cheaper to replace than analytical columns. Remove bound material from a C_{18} partition column by washing with a solvent most like the column in polarity. First, wash the column out with water if you are using a buffer in the mobile phase before doing the organic wash out.

My chromatography has changed. How can I fix it?

Chromatography changes because the column is either degrading or something is sticking on the column and needs to be washed out. Controlling pH and pressure shock on a silica column will help prevent degradation. Washing with solvents of the same polarity as the column will remove bound organics. High-concentration salt washout will remove most things from ion exchange columns.

We have a reverse phase zirconium column, but it doesn't run anything like my waters C_{18}. How do we fix it?

You may not be able to fix it. Zirconium columns have a different chemical structure than silica. They are more pH resistant and can be used at elevated temperatures, but they run differently than a silica C_{18}. First, find out if it has a chemically bonded tetraphosphonic acid chelator. If not, you may have to add a chelator to the mobile phase. If you are not getting sharp peaks, make sure you have made new inlet tubing specifically for this column. Old tubing can ruin the performance of a new column. Run a standard mix on both columns and see how they differ. This column can give you the ability to separate mixture that you can't separate on a reverse-phase silica column.

I have heard that monolith columns are the wave of the future. What are they?

Silica monoliths are a new class of bonded-phase column in which the inside of the column is completely filled with silica foam that has had a bonded phase attached to it. It should run like a high-efficiency reverse-phase column, but with much lower back-pressure. Realize that they are a new type of column and you may have to adjust your chromatography to get them to run in the same way as your other silica-based columns. Look at it as an adventure into the future.

APPENDIX C

TABLES OF SOLVENTS AND VOLATILE BUFFERS

Table C.1 HPLC solvents

Solvent	Formula	Molecular weight (Daltons)	Boiling pt. (°C)	UV cutoff (nm)
Acetonitrile	CH_3CN	41.05	81.6	190
Chloroform	$CHCl_3$	119.38	61.7	245
Dichloromethane	CH_2Cl_2	84.93	40.0	235
Ethanol	CH_3CH_2OH	46.08	78.5	210
Ethyl acetate	$CH_3CO_2CH_2CH_3$	88.12	77.1	260
Diethyl ether	$(CH_3CH_2)_2O$	74.12	34.5	220
Heptane	$CH_3(CH_2)_5CH_3$	100.21	98.4	200
Hexane	$CH_3(CH_2)_4CH_3$	86.18	69	200
Methanol	CH_3OH	32.04	65	205
n-Propanol	$CH_3CH_2CH_2OH$	60.11	97.4	210
Isopropanol	$CH_3CH(OH)CH_3$	60.11	82.4	210
Tetrahydrofuran	C_4H_8O	72.12	66	215
Toluene	$C_6H_5(CH_3)$	92.15	110.6	285
Water	H_2O	18.02	100	None

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Table C.2 HPLC volatile buffers

Volatile buffer	Structure	pKa	Buffer range
Triflouroacetic acid	CF_3CO_2H	0.5	3.8–5.8
Formic acid	HCO_2H	3.8	—
Ammonium formate	HCO_2NH_4	3.8	2.8–4.8
Acetic acid	CH_3CO_2H	4.8	—
Ammonium acetate	$CH_3CO_2NH_4$	4.8	3.8–5.8
4-Methylmorpholine	$OC_4H_8N(CH_3)$	8.4	7.4–9.4
Ammonium bicarbonate	NH_4CO_3H	6.3/9.2/10.3	6.8–11.3
Ammonium acetate	$CH_3CO_2NH_4$	9.2	8.2–10.2
Ammonium formate	HCO_2NH_4	9.2	8.2–10.2
1-Methylpiperidine	$C_5H_{10}N(CH_3)$	10.1	10.0–12.0
Triethylammonium acetate	$CH_3CO_2NH(CH_3)_3$	11.0	10.0–12.0
Pyrrolidine	C_4H_8NH	11.3	10.3–12.3

Note: Usually, 1–10 mM buffer concentration is recommended for LC/MS. TFA is known to quench ionization in electrospray LC/MS, leading to lower sensitivity and should be avoided.

APPENDIX D

GLOSSARY OF HPLC TERMS

Adsorption Chromatography—Separation mode resulting from compounds that have different adhesion rates for the packing surface. (See Normal-Phase Chromatography.)

Alpha (α)—(Separation or chemistry factor). A measure of separation between two peak maxima. Ratio of their k' values.

Attenuation—Measure of detector sensitivity. A larger value means less sensitivity.

Autoinjector—An injection device for automated methods development in which the sample loop is repeatedly filled from a large sample reservoir rather than a sample vial carousel.

Autosampler—A multiple sample injector, usually with a rack or carousel to hold sample vials or a sample well plate, designed for unattended programmed operation in which a sample is loaded by either pushing or pulling sample into the loop injection loop with air or hydraulic pressure.

Autozero—Detector, integrator, or computer function capable of setting detector signal value (baseline?) to zero.

Band—The disk of resolved compound moving down the column. Band spreading cause by diffusion tends to remix already separated bands.

Baseline—Detector signal versus time if no peaks are present. Good indicator of pulsing, air bubles, electrical noisse, or impurities.

Baseline Resolution—Chromatographic goal of methods development in which all valleys between adjacent peaks touch the baseline, indicating complete resolution of peaks.

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Buffer—Mobile phase modifier used to control pH. Usually salts of weak acids or bases, most effective at their pK_a, where concentrations of ionized and unionized form are equal.

C_8 (Octyl)—Nonpolar column or packing with 8 carbon hydrophobic hydrocarbon chain bound to silica.

C_{18} (Octyldecyl)—Nonpolar column or packing with an 18 carbon hydrophilic hydrocarbon chain bound to silica. Used for reversed phase separations. (See ODS.)

Cartridge Column—Disposable off-line tube packed with >1 g of packing used for sample and solvent preparation. (*See* SFE and Windowing.)

CAD (Charged Aerosol Detector)—A universal, mass detector that evaporates column effluent using a gas nebulizer in the presence of a caronal discharge needle that ionizes compound droplets so they can be detected on an electrometer.

Check Valve—Mechanism in the pump head inlet and outlet to ensure one-way solvent flow; usually a sapphire ball in a stainless steel cone. Major point of buffer precipitation and pump pressure loss.

Chip HPLC—Nano-flow, micro-sample HPLC system in which the packed column resides within the body of the injector. Originally touted as the HPLC of the future for nano-level LC/MS, but its potential has been slow to materialize.

Chromatography—A separation technique producing a qualitative record of the relative amounts of components, a chromatogram. HPLC modes include partition and adsorption (polarity), GPC or SEC (size), ion exchange (charge), or affinity (compound-specific retention).

Column—A metal tube in which the HPLC separation occurs, packed with porous packing held in place at each end by a fritted filter in an end-cap. End-caps are secured to the column with ferrules and can be opened for frit cleaning.

Column Blank—A length of tubing, fitted with compression fittings simulating column ends, used to replaced the column for system cleaning and diagnosis.

Column Heaters—Heaters designed to allow elevated temperature operation by jacketing the column, injector, and tubing lines. Especially useful for shortening run times and inducing α changing when using temperature-resistant zirconium columns. Best systems use fast response Peltier healing/cooling.

Compression Fitting—A device for connecting tubing to other system parts. Usually made up of a ferrule and a threaded screw or cap, which slide over the tubing. Tightening the screw cap forces the ferrule into a conical hole, squeezing (swaging) it permanently onto the tubing.

Dead Volume—Unnecessary volume in a system that can remix separated bands of compounds, usually in tubing or fittings, especially from the injector to the column and from the column to the detector.

Deoxygenation—Removing oxygen from a solvent by vacuum replacement with nitrogen or helium gas to prevent oxidation of sensitive compounds or columns (such as the amino columns).

Efficiency (*N*)—A measure of the narrowness of elution bands, the sharpness of peaks, and the performance of a column. Results are in theoretical plates. The Huber equation calculates efficiency versus flow rate, which is plotted on as a Van Deampter plot, which compares column efficiency with flow rate.

ELSD (Evaporative Light Scattering Detector)—A universal, mass evaporated detector that measures the amount of compounds present by the amount their droplets deflect an incident light beam.

Elution—Washing bands of separated bands out of the column with mobile phase. The liquid output of a column is the eluant; the amount of solvent needed to reach a peak's maximum is its elution volume.

Elutotropic Series—Solvents ranked in order of polarity or eluting strength. The strongest solvent is the one most like the packing material in polarity.

End Absorption—UV absorption, from 210 nm down, going nonlinear at 180 nm due to dissolved oxygen. Most carbon-oxygen containing compounds absorb in this area.

End-capping—After silylation, reaction of bonded-phase packing with a reactive small molecule to tie up unreacted silanols on the silica surface. Sharpens peaks from basic compounds.

Exclusion Volume—In size-exclusion chromatography, *Vo*, the volume of solvent necessary to washout unretarded compounds too large to penetrate the pores of a size-separation column. The inclusion volume, 2*Vo*, is the elution volume needed to elute all compounds small enough to fully penetrate the pores.

Fines—Small particles of packing material in a column that tend to migrate and plug the outlet frit, raising column back-pressure. Commonly seem with irregular packings that have microfractures that break off small pieces of packing material under pressure changes.

Flow Cell—Low volume (8–20 μL) detector cell designed to accept eluant output from an HPLC or an ion chromatography.

Frits—Porous stainless steel filters at either end of the column that serve as bed supports and filter the sample coming in from the injector.

GPC (Gel Permeation Chromatography)—Separation mode based on the molecular sizes of the compounds. [See SEC (size exclusion chromatography).]

Gradient—A reproducible change in a separation parameter that can be used to speed a separation. In a binary solvent gradient, % solvent B increases while %A decreases, causing late-eluting peaks to come off faster and sharper.

Guard Columns—Short, protective columns placed in-line between the injector and the main column.

Helium Sparging—A solvent degassing technique in which helium gas is bubbled through solvents to displace dissolved gases before solvent mixing, compression, and pumping.

Interface—An ionizing, evaporative device designed to take effluent from the HPLC and prepare it for injection directly into the source of a mass spectrometer.

Ion Displacement—Use of strong salt solutions to displace compounds bound to ion-exchange columns.

Ion Exchange Chromatography—Separation mode for ionized compounds on charged columns. Anion-exchange columns attract and separate anions; cation-exchange columns separate cations.

Isocratic—Constant mobile phase composition. The opposite is a gradient in which the mobile phase composition is altered during the run. Isocratic conditions are not restricted to single solvents or solvent mixtures, but can include multiple components in the mobile phase.

***k′* (Retention Factor)**—A measure of the relative solvent volume needed to wash a compound off a column at a given solvent polarity. Normalized with the void volume of the column to make it independent of column length.

Lamps—Light source for a detector. A deuterium lamp is fully variable from 190 nm to 400 nm; a tungsten lamp from 370 to 700 nm. Other lamps show discrete bands; mercury, 254 and 436 nm; cadmium, 228 nm; zinc, 214 nm.

LC/MS (Liquid Chromatography/Mass Spectrometry)—Chromatography system in which an HPLC is married to a mass spectrometric detector through an evaporated, ionizing interface. A variety of mass spectrometers are used to produce various LC/MS and LC/MS/MS configurations. MS detectors are universal, mass detectors that provide molecular weight information and can give a definitive identification of separated compounds.

Loop and Valve Injector—Device for placing sample onto the column head. Modern design consists of a loop, partially or overfilled at atmospheric pressure, that is rotated into the flowing stream from pump to column. Sample is back-filled from the end of the loop closest to the column, described as "last in, first out" filling.

Microporous Packing—Modern, fully porous, high-resolution separations packing with average particle diameters of 3–10 μm.

Mobile Phase—The solvent mixture pumped through the column carrying the injected sample; the liquid phase of the solid-liquid equilibration.

Monolith Column—Porous silica column prepared in situ to completely fill the column tube with a fully porous silica foam skelton. After the organic polymer support is heated off, the silica surface is silylated in place to product bonded-phase surface. Column is high resolution and can be used at high flow rates with relatively low back-pressure (see Chapter 16).

Multichannel HPLC—HPLC system designed to run parallel HPLC columns into a multi-flow cell UV or fluorescent detector. Designed for production laboratories to speed QA/QC monitoring (see Chapter 16).

Nanoflow HPLC—HPLC system with accurately controlled reciprocating and syringe pumps designed to use capillary and small diameter, high-resolution columns as front ends for electrospray and nanospray mass spectrometer interfaces.

Needle Port Seal—Teflon^{R} throat seal in injector needle port that prevents flow back of injected sample solution.

Normal Phase Chromatography—Separations mode run on nonbonded, anhydrous porous silica using a nonpolar mobile phase. (*See* Adsorption Chromatography.)

ODS—Octadecylsilyl bonded phase material or column in which the material bound to silica is an 18-carbon saturated hydrocarbon chain. (*See* C_{18}.)

Pacification—Treatment of a column-bridged HPLC system with 20% (6 N) nitric acid to remove buffer and organic deposits and protect metal surfaces from corrosion. The column must be removed before acid treatment. Overnight water wash is needed to remove the last traces of acid.

Peak Areas versus Peak Heights—Integration and quantitization can be based on either the height or area of the peak. With well-resolved peaks seen in research labs, areas give more accurate results; with less well-resolved peaks or shoulders seen in clinical or biomatrix separations, peak heights give best results.

Pellicular Packing—First analytical packing; it had a solid core and a crust of porous silica. Now used primarily for packing guard columns and columns for separating very large molecular weight compounds (i.e., DNA, RNA).

Plate Count—A measure of column efficiency derived by comparing peak width to retention time. A higher number indicates a more efficient separation. Theoretical plates are an arbitrary unit assigned to the efficiency value, in analogy to efficiency units in open column distillations.

Plunger—A piston, usually of sapphire, driven by the pump motor into the pumping chamber to pressurize and displace solvent through the outlet check valve. The rear of the chamber is sealed by the plunger seal, made of hardened Teflon^{R} that fits tightly around the plunger.

Polarity—A measure of a solvent's, column's, or compound's ability to attract similar molecules. Polar compounds have large dipole moments, large dielectric constants, and usually form hydrogen bonds (e.g., water). Nonpolar compounds such as hexane are on the opposite end of the polarity scale. (See Elutotropic Series.)

Pulse Dampeners—Device used to control pump pulsing. Usually a tight coil of metal tubing in a metal container that acts as a baffle and counters pulsing by a spring recoil effect.

Reciprocating Pumps—Single- and dual-headed pumps that use a piston and check valves to pump solvent from a reservoir into the system.

Resolution (R)—A measure of the completeness of a separation. Influenced by k' (solvent polarity), N (column efficiency), and α (system chemistry).

Retention Time—The time or mobile phase volume need to elute and detect a component of the mixture in a detector.

Reverse-Phase Chromatography—Separation mode on bonded phase columns in which the solvent/column polarities are the opposite of normal-phase separations. Polar compounds elute before nonpolar compounds, Nonpolar columns require polar solvents.

RP_{18}—Reverse phase, bonded packing with 18-carbon side chain. (*See* C_{18}, ODS.)

Rotor Seal—Teflon^{R} surface that seals the injector and separates the flowing mobile phase from the sample loop until an injection is completed.

Sample Clarification—Removal of particulates from the injection sample by either filtration or centrifugation.

Saturation Column—Sacrificial column placed before the injector to protect the main column from pH degradation.

Seal—Wear surface that both lubricates and separates moving parts in the HPLC. (*See* Plunger Seal, Rotor Seal, and Needle Port Seal.)

SEC (Size Exclusion Chromatography)—A separation mode employing control pore size packing to achieve resolution of molecules based on size and shape. (See GPC.)

Separation Factor (α)—A measure of peak separation between peaks. Product of dividing one peak k' by the other. Also called the *chemistry factor* because it is controlled by changes in the chemistry of the column, mobile phase, and the sample.

SFE—Separation and filtration cartridge column. Also referred to as a SPE (solid phase extraction column). (*See* windowing in Chapter 12.)

Silica—Particles or spheres of crystalline silicic acid used in chromatography. Its surface is polar, acidic, and tends to attract water of hydration and polar compounds.

Silylation—The first step in forming bonded-phase packings from dried silica and chlorodialkylchlorosilanes.

Stationary Phase—A term used to describe the column packing, indicating that it is part of a two-phase equilibrium with the mobile phase or column solvent.

Syringe Pump—A pulseless pump made up of a motor-driven piston or plunger in a solvent-filled cylinder. Useful only when small solvent volumes are to be pumped; often used in micro-flow or nano-flow HPLC systems.

Tailing—Unsymmetrical peak formation in which the side of the peak away from the injection returns very slowly to the baseline. Usually due to an unresolved equilibration and incomplete separation.

Ultra-fast HPLC—An HPLC system designed to use <2-μm spherical packing at high flow rate and pressure (~12,000 psi) to produce very rapid, high-

resolution separates. This system is designed for interfacing into an LC/MS system or to increase separation speed.

Voids—Spaces or openings in the column bed leading to poor chromatography. End voids are directly under the inlet frit. Center voids are channels through the center of the packing bed.

Void Volume—The solvent volume inside the packed column. It usually can be measured as an early refractive index baseline upset when injecting a sample dissolved in a solvent even slightly different from mobile phase.

Windowing—A technique using cartridge columns (SFE) to speed chromatography by first removing polar and nonpolar impurities, leaving only a solvent fraction containing the compounds of interest.

Zero Dead Volume—Fittings designed to leave no extra column volumes that might cause band spreading or remixing of peaks.

APPENDIX E

HPLC TROUBLESHOOTING QUICK REFERENCE

This section is designed to assist in troubleshooting system problems. It is not a systematic approach as described in Chapter 10, which always yields better results. Keeping this in mind, I have listed a series of commonly seen problems, possible causes, and suggested treatments.

Problem 1: No power. (Display does not light up on module or system.)

Cause a: Not plugged in.

Treatment: Check the plug at the socket and at the module. It may have worked loose or been unplugged accidentally.

Cause b: No fuse, blown fuse, or incorrect fuse.

Treatment: On a new system, make sure fuse(s) were installed. If variable voltage, make sure the selection card is correctly positioned. New systems are often shipped with fuses in a bag. If fuse is broken, replace it and contact service.

Cause c: Not switched on.

Treatment: Turn on the switch (from the front, try the upper right-hand side of the back plate). Someone may have been helping you to conserve energy. I always set my system up on a common surge protector with an on/off switch. That way I can turn everything on with one switch and protect against line surges and ground loops at the same time.

Problem 2: Leaking frits, puddles on desk top, fountains of solvent.

Cause a: Compression fittings not tight enough.

Treatment: Tighten leaking fitting another 1/4 turn or until leak stops. (Leaks will be at the back of the fitting around the tubing.)

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Cause b: Incorrectly made fitting or wrong ferrule.
Treatment: Stop pump. Loosen fitting with a wrench. Examine for correct preparation. Cut off and replace if necessary. (Leak is usually around the base of the connected fitting.)
Cause c: Fitting scored by silica packing left in threads.
Treatment 1: Wrap fitting with Teflon^{R} tape and reseat.
Treatment 2: Cut off ferrule and replace fitting.

Problem 3: Inconsistent or too slow pump flow rate.
Cause a: Air bubble in pump head.
Treatment 1: Open a purge valve. Prime the pump with degassed solvent.
Treatment 2: Open the compression fitting at the top of the outlet check valve with a slow pump flow until solvent leaks around the fitting. Tap the side of the check valve with a small wrench until small air bubbles come out with the liquid. Reseat the compression fitting.
Treatment 3: Pacify the pump with 20% nitric acid after first removing the column and replacing it with a column bridge. Wash repeatedly with water.
Cause b: Plugged solvent sinker in reservoir.
Treatment: Replace filter (sinker is plugged, pump is starving). Try sonicating stainless steel frits in 20% HNO_3, then sonicate in fresh water.
Cause c: Outlet check valve is sticking open.
Treatment: Pacify pump with 20% HNO_3 (*see* 3b).
Cause d: Sticky inlet check valve. Solvent flows back into reservoir.
Treatment 1: Replace check valve.
Treatment 2: Pacify system (*see* 3b).

Problem 4: Pump pressure shuts down pumping.
Cause a: Pump overpressure setting set too low.
Treatment: Reset overpressure setting to a higher value, if the column can tolerate it.
Cause b: Under-pressure setting too low. No solvent.
Treatment: Reset under-pressure setting higher. Check solvent reservoir and add more solvent if necessary.
Cause c: Column or system plugged.
Treatment 1: If pressure exceeds 4000 psi, find the plug and clear it. Remove the column and run pump(s). If pressure persists, trace pressure source back toward the pumps until the pressure drops. Reverse the line and use pump pressure to blow plug out.
Treatment 2: If pressure leaves with the column, replace the inlet frit (See Chapters 6 and 10).

Problem 5: No peaks detected after injection.
Cause a: No sample pulled up into injection syringe.
Treatment: After drawing up sample, pull back on syringe barrel so you can see the meniscus. Tap out bubbles. Push syringe back to injection size mark. Wipe off excess liquid with tissue. Inject.
Cause b: Injector loop plugged.

Treatment: Make sure solvent flows from injector waste line when loading loop. If not, disconnect injection loop at point closest to column and place injector in inject position with pump flow on. Wash loop into beaker and reconnect.

Cause c: Wrong elution solvent used. Gradient run not started.

Treatment: Use correct elution conditions. Make sure you start gradient run.

Cause d: Column has bound impurities.

Treatment: Wash column into a beaker with strong solvent. Reequilibrate with mobile phase. Inject new sample.

Cause e: Detector setting too high. Wrong wavelength selected.

Treatment: Check detector settings. Increase sensitivity. Inject again.

Cause f: Sample cannot be seen by detector.

Treatment 1: Select a wavelength at which the sample can be seen. Scan standards in spectrophotometer to find a useable wavelength.

Treatment 2: Select a universal detector that can see sample, if one is available. RI, MS, CAD, and ELSD detectors can see most samples.

Problem 6: Injector leaks around body or around needle when in port.

Cause a: Injector body leaks because of torn injector seal.

Treatment: Rebuild injector by replacing injector seal.

Cause b: Needle seal has been scored by sharpened injection needle.

Treatment: Tighten sleeve around needle seal or replace the seal.

Problem 7: Ghosting peaks occur when injecting only solvent.

Cause a: Dirty sample loop.

Treatment: Clean sample loop by washing with strong solvent.

Cause b: Small rotor seal tear is trapping sample.

Treatment: Replace the rotor seal.

Problem 8: Increasing column pressure.

Cause a: Column inlet frit is plugging.

Treatment 1: Filter samples before injecting them.

Treatment 2: Wash column with water before switching from buffer to a stronger organic solvent. Wash with water before return to buffer.

Treatment 3: Remove frit and replace it. Wash old frit by sonicating with 20% nitric acid and then water (*see* 3b).

Cause b: Column bed is plugged.

Treatment: Avoid injecting a saturated sample solution. Column will over concentrate and precipitate sample. Wash out at best flow rate with a strong solvent without triggering over-pressure setting.

Cause c: Outlet frit has become plugged with packing material fines.

Treatment: Replace outlet column frit. Sonicate old frit with 10% NaOH, water, and again with water (*see* 3b).

Problem 9: Column retention time and plate count changing.

Cause: Bound material on column or column voiding.

Treatments: See Chapter 6.

Problem 10: Loss of detector sensitivity and dynamic range.

Cause a: Old detector lamp is failing.

Treatment: Replace detector lamp. Record new intensity value, if it can be measured, for later reference.

Cause b: Dirty flow cell window.

Treatment: Clean windows. First, try cleaning in situ. Disconnect detector from system, wash with strong organic solvent from syringe and tube connected to flow cell inlet. If this does not solve problem, wash with water. Check manual to see if flow cell can be pacified with nitric acid. Push 20% nitric acid in from the waste line and trap in flow cell. Leave for 15 min. Flush out copiously with water. If necessary, disassemble flow cell and clean window with acetonitrile, chloroform, and then hexane, wiping with tissue. Dry and reassemble.

Problem 11: Baseline increases with no solvent flow and detector lamp on.

Cause: Decomposing coating on flow cell windows.

Treatment: Wash windows or pacify with 20% nitric acids (*see* 10b).

Problem 12: Rising and falling chromatographic baseline.

Cause a: Late running peaks are still eluting off column.

Treatment: Wash column with stronger solvent. Equilibrate with fresh mobile phase.

Cause b: Detector warm up is not complete.

Treatment: Go have another cup of coffee before shooting another sample. If warm-up time becomes excessive, consider replacing the lamp or calling a serviceperson.

Problem 13: Noisy baseline or baseline spikes.

Cause a: Bubbles in flow cell.

Treatment 1: Add 40–70 psi back-pressure device to detector outlet line.

Treatment 2: Disconnect detector. If acid-resistant, pacify by pushing 20% nitric acid into flow cell and trapping it, followed by water wash out (*see* 10b).

Cause b: Electronic noise coming from the power line.

Treatment 1: Get a line noise filter.

Treatment 2: Make sure the detector signal line is properly shielded. Only one end of the line should be connected to ground.

Problem 14: Chromatographic peaks have plateaus or unexpected shoulders.

Cause: Strip chart or printer slide wire or bar is dirty and sticking.

Treatment: Pen or printer head is sticking. Wipe slide wire or bar with a lint-free tissue. Spray WD40 on tissue and wipe slide wire or slide bar if problem reoccurs.

Problem 15: Retention times vary in both directions on rerunning sample.

Cause: Stretched tension spring on strip chart drive.

Treatment 1: Check strip chart bed speed with a stopwatch. Reposition spring attachment to increase tension.

Treatment 2: Get an integrator or a computer-based data system. Put the strip chart back on the GC or trash it.

Problem 16: Continuous retention time printing is occurring.

Cause: Noise level is set too low. Each baseline deflection is seen as a peak.

Treatment: Increase noise setting level until only retention times of peaks of interest are printed with retention times.

Problem 17: Integration start/stop marks occur too early or late.

Cause: Peak width is too small. Slope reject is too high.

Treatment: Use auto zero or test button before making injection. Recalculate using higher noise value, lower slope value, or a wider initial peak width until chromatogram is correctly displayed. (see Chapters 9 and 14)

APPENDIX F

HPLC LABORATORY EXPERIMENTS

The following laboratory experiments have been designed to let you try out the tools you need to run an HPLC system and its columns on a daily basis. In Laboratory 1, you will practice starting up an HPLC, recovering a dry column, and quality controlling a new column. In Laboratory 2, you will run a scouting gradient to select an isocratic condition. An SFE cartridge column will be used to window out peaks in the chromatogram. In Laboratory 3, we will look at the effect of changing the stronger solvent and changing column types on our standards separation. Finally, we will remove the column and pacify the system with 20% nitric acid followed by a water washout. Using these tools on a regular basis should keep your columns and systems up and running and provide procedures when you have to develop new separations.

LABORATORY 1—SYSTEM START-UP AND COLUMN QUALITY CONTROL

Purpose

1. To start up an HPLC protecting seals, plungers, and a "dry" column.
2. To run column standards.
3. To calculate plate counts/retention times.

Equipment and Reagents

1. Isocratic HPLC system
2. C_{18} column (5 μm, 15–25 cm)

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3. 25-μL injection syringe
4. HPLC-grade methanol and water
5. Column Standards (P.J. Colbert Ct. No. 962202)
6. Column blank (5 ft of 0.010-in tubing, fittings, and unions)
7. 70 psi back-pressure device on the detector outlet

Protocol

1. Prepare 200 mL of 50% methanol/water and 100 mL of 80% methanol/water. Vacuum filter through 0.54-μm filters.
2. Remove the C_{18} column, cap, and set it aside. Set up the HPLC system with a column bridge in place of the column. Prime the pump(s) with 50% methanol/water. Set the over pressure setting on the pump to 4000 psi. Stat flow at 0.1 mL/min and slowly ramp to 1 mL/min. Watch the pump pressure indicator for fluctuations (air bubbles? dirty check valves?). (*Lab Note:* Air bubbles can be cleared by opening the compression fitting on the outlet check valve with a wrench until solvent bubbles out, then tapping the valve housing lightly to release bubbles. Retighten the compression fitting and move to the next check valve fitting until you have checked them all or the problem has disappeared.) Stair-stepping pressure problems may indicate a dirty check valve, which should be replaced or pacified (see Laboratory 3).
3. When the pressure is steady, turn the injector handle to inject (or load if it was already in the inject position) and watch the pressure. If the pressure does not jump up, the loop is not blocked. Cycle the injector handle to the inject position.
4. Watch the recorder or computer baseline. When it is stable, slow the pump flow to 0.1 mL/min, remove the column blank, and connect the $C1_8$ column to the injector. *Do not* connect the column to the detector yet. Wash the column solvent into a beaker (start a slow flow ramp up from 0.1 to 1.0 mL/min) for six column volumes (12–18 mL). Pressure should slowly increase to around 2000 psi at 1 mL/min due to column back-pressure. (*Lab note:* Always hook up a column with solvent running to prevent introducing air from the column head into the column.)
5. When the pressure is stable, *record column back-pressure* from the pump pressure gauge in a logbook. Connect the column to the detector inlet fitting. Turn on the detector (select 245 nm, 1.0 AUFS) and the recorder at 0.5 cm/min chart speed. Observe the baseline. Drifting indicates that the detector is still warming up or something is washing off the column. (*Lab note:* The pump pressure gauge should be monitored periodically when making changes to a system. A sudden pressure increase indicates a blockage problem. Adjusting the pump over-pressure setting should prevent problems, but shut off the flow yourself and figure out what is causing the extra pressure to be sure.)

6. When the baseline is stable, inject 15 μL of column standards. (*Lab note:* Inject by overfilling the syringe, point the needle up, pull the barrel back until you can see the meniscus, tap out visible bubbles in the liquid, push the plunger to the 15-μL mark, wipe outside the barrel with a lab wipe with a pulling motion. Insert into injector. Load the injector loop slowly, and leave the needle in place.) Turn the injection handle quickly. Remove the injection needle, and flush three times with solvent.
7. On the chromatogram paper, *mark the inject point. Record date, time, operator name(s), flow rate, mobile phase, sample type, number, injection amount, column, detector wavelength, attenuation, and the chart speed* so you could duplicate this run. Record chromatogram until the baseline is reached after the four peaks.
8. Repeat standards run. Increase recorder speed to 2 cm/min. Inject standards solution. Record the four-peak chromatogram.

Results

9. Using the chromatogram recorded at 2 cm/min, measure V_0, the exclusion volume of the column, V_x for each peak (the solvent volume at the peak center), and W (the 5σ width) for peaks 1 and 4.
10. *Calculate k′ (peaks 1 and 4), α (peaks1,2), and N (1 and 4).* (*Lab note:* Remember $k'(1) = V_1 - V_0/V_0$, $\alpha(1,2) = k'_2/k'_1$, $N_1 = 16(V_1/W_1)^2$. Also remember that W_1 is measured by projecting lines parallel to the sides of the peak to where they intersect the baseline. W_1 is the distance between the intersection points.) (see Ch. 4)

LABORATORY 2—SAMPLE PREPARATION AND METHODS DEVELOPMENT

Purpose

1. Run a scouting gradient.
2. Select SFE cartridge column windowing conditions from the gradient.
3. Run SFE window cuts in selected dial-a-mix conditions.

Equipment and Reagents

1. Gradient HPLC system
2. C_{18} HPLC column (5 μm, 15–25 cm)
3. C_{18} SFE cartridge columns (Whatman Part No. 6804-0405)
4. 5-mL B-D disposable syringes
5. Seven-components test mixture (P.J. Colbert Cat. No. 962201)
6. HPLC-grade methanol and water
7. 10-mL test tubes

Protocol

1. Purge pump A with water and pump B with methanol. Dial-a-mix 20% B and equilibrate the column at 1.5 mL/min (six column volumes or a stable baseline at 254 nm, about 10 min). (*Lab note:* If the gradient system is a low-pressure mixing system, solvents must be degassed by purging or running under helium.)
2. Inject 15 μL of the seven-component standard. Run a 15-min gradient to 100% methanol, hold at 100% for 5 min. Watch the chromatogram during the run and *record %B of the first and last peaks*.
3. For a 25-cm column, deduct 10% from the first peak's %B and equilibrate the column with this dial-a-mix mobile phase (i.e., if the first peak came off at 80% B, dial-a-mix 70% B). For a 15-cm column, deduct 7% from the first peak %B to find your dial-a-mix isocratic. Equilibrate the column with this mobile phase.
4. Inject standards and run the chromatogram.
5. Pretreat a C_{18} cartridge column with 2 mL MeOH, then 2 mL, of water. (*Lab note:* Remove plunger from a 10-mL syringe. Put the cartridge column on the luer end of the syringe tip. Put 2 mL of MeOH in the syringe barrel. Put solvent and air through the cartridge with the plunger and collect eluant in waste test tube. *Do not pull back on the syringe barrel while cartridge is attached.* Remove cartridge. Pull out plunger. Replace cartridge on tip. Go to next wash solvent.
6. Dilute 1 mL of standard solution 3-fold with water. Put sample in syringe barrel with cartridge in place. Insert the barrel and push the sample into the cartridge. Collect the effluent as collect tube 1. Based on the scouting gradient, select three washes to window off standards. Select a window that should leave the three middle compounds in the second cut (*Lab note:* In windowing from a scouting gradient, start at the injection mark and move to the last peak you want to wash off in the first cut. Find its equivalent %B on the gradient trace. Deduct 7–10% B to find its isocratic equivalent for your first wash condition. Wash the cartridge with 1 mL of this eluant. Collect it as collect tube 2. On the chromatogram, move to the last peak you want in your second window fraction and determine its equivalent wash off %B. Wash the cartridge with 1 mL of this %B. Collect it as collect tube 3. Finally, wash the remaining peaks off the cartridge with 1 mL of 100% methanol. Collect this as collect tube 4.
7. Run 15 μL of each collect tube in the isocratic dial-a-mix mobile phase chosen in step 4.

Results

8. Examine the four chromatograms of the breakthrough and windowing cuts (collect tubes 1–4). *Measure retention times of the peaks in each*

collect tube and compare with the final methods development chromatogram. If you found more or less than three peaks in collect 3, or if some of the cut 3 peaks are found in cuts 2 or 4, indicate how you would adjust the %Bs of the window frame to improve cut 3. Do not repeat the windowing experiment to prove your point! (*See* Section 12.1.3–5 if you have questions on windowing.)

LABORATORY 3—COLUMN AND SOLVENT SWITCHING AND PACIFICATION

Purpose

1. Study the effect of changing the stronger solvent or the column.
2. Do a column washout and QC check.
3. Do a system pacification using a column blank.

Equipment and Reagents

1. Gradient HPLC system
2. Back-pressure device on the detector outlet
3. C_{18} column (5 μm, 15–25 cm)
4. C_8 column (5 μm, 15–25 cm)
5. Column blank (5 ft of 0.01-in tubing, fittings, and unions)
6. Four-component column standards (P.J. Colbert Cat. No. 962202)
7. Seven-components test mixture (P.J. Colbert Cat. No. 962201)
8. HPLC-grade acetonitrile, methanol, and water
9. Concentrated nitric acid

Protocol

1. Purge line A with water and line B with MeOH. Dial-a-mix 70% MeOH and equilibrate the C_{18} column at 1.0 mL/min. When stable, inject 15 μL of the seven-component test mixture and annotate the chromatogram's start. Run an isocratic chromatogram.
2. Reduce the flow to 0.1 mL/min, remove the C_{18} column, and replace it with the C_8 column (do not connect to the detector). Increase the flow to 1.2 mL/min and wash with six column volumes of mobile phase. Connect the column to the detector inlet and run until the baseline is flat.
3. Inject 15 μL of the seven-component standards test mixture. Annotate and run an isocratic chromatogram.
4. Dial-a-mix 60% methanol/water. Equilibrate the C_8 column. Inject 15 μL of the seven-component standards mixture. Annotate and run an isocratic chromatogram.

5. Put acetonitrile in the B reservoir. Purge the pump inlet line with acetonitrile. Dial-a-mix 60% acetonitrile/water. Reconnect the C_{18} column at 0.1 mL/min. Increase the flow to 1.0 mL/min and equilibrate the column. Inject 15 μL of the seven-component test mixture. Annotate and run the chromatogram.
6. Make up 100 mL of 20% nitric acid (1 part acid added to 4 parts water).
7. *Very Important Lab Note: Remove the column!* Replace the column with the column blank. Put end fittings on the column for storage. Put water in the reservoir and wash the system at 2 mL/min with water.
8. *Check your HPLC system manual to make sure the system is compatible with nitric acid washing. Make sure the reservoir sinker is made of stainless steel and not monel metal.* Replace the water in the solvent reservoir with 20% nitric acid. Stop! *Note: Make sure the column has been replaced with the column bridge. Do not pump nitric acid through a bonded-phase column.* Wash the system for 15 min at 2 mL/min with 6 N (20%) nitric acid. Discard the wash carefully.
9. Wash the system with water (2 mL/min) (set UV detector at 230 nm, 2.0 AUFS) and monitor baseline for disappearance of nitric acid. When baseline is flat or your class time has elapsed, collect effluent and check pH against laboratory water.

Results

10. Examine chromatograms of standards run on the C_8 and C_{18} columns for peak shifting. Examine chromatograms run in methanol/water and acetonitrile/water for peak shifting. *Measure last peak retention times in all three chromatograms. Look for peak switching by looking at peak heights and positions.*
11. Pacification of a columnless HPLC with nitric acid: *Observe the length of time necessary to wash all the nitric acid out of the HPLC system.*

APPENDIX G

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