VARIAN

Instrument Group/Walnut Creek Division
2700 Mitchell Drive, Walnut Creek, CA 94598/USA/Phone (925) 939-2400

BASIC HPLC

THEORY & TROUBLESHOOTING

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INTRODUCTION TO HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

Chromatography is a physical process used to separate components of a mixture of chemical compounds. The separation is accomplished by passing the mixture through a bed of particles. Some components of the mixture pass through the bed faster than other components, therefore causing separation to occur. When the mixture of compounds is carried through the bed by a liquid, the process is called "liquid chromatography". When very small particles are contained in a tube (column), the liquid is forced through the column by a pump and the components are detected as they come out of the bed by a sensitive detector, then the process is called High Performance Liquid Chromatography (HPLC).

Partitioning

Components are separated in HPLC by "partitioning" between the moving liquid phase (the mobile phase) and the surface of the particles (the stationary phase). Each component has a certain solubility in the mobile phase and a certain attraction to the stationary phase. "Partitioning" refers to the division of each component between the mobile phase and the stationary phase. When a component is "stuck" to the stationary phase, it is not moving through the column. When it is dissolved in the mobile phase, it is moving through the column as fast as the mobile phase is moving. Thus, the more time a component spends stuck in the stationary phase, the longer it will take to pass through the column; this is how separation of different compounds is achieved.

Injection of a mixture occurs when it is placed at the beginning (head) of a column. Partitioning occurs as the mixture passes through the column, and elution occurs as the sample components come out the end of the column:

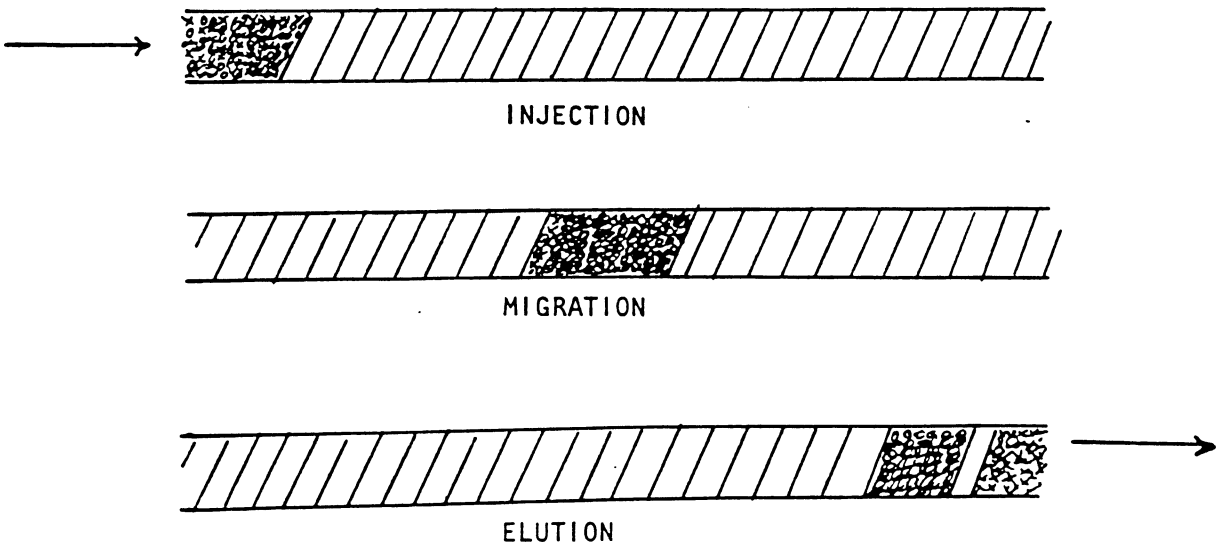


FIGURE 1

MECHANISMS OF SEPARATION

Column	Separation Type
Silica Gel	Adsorption
Bonded Phase - (Normal) Amino Cyano	Polar Interactions
Bonded Phase - (Reverse) Hydrocarbon	Non-Polar (Hydrophobic) Interactions
Ion Exchange	Ionic Interactions
Size Exclusion	Molecular Size

FIGURE 2

There are a number of mechanisms by which partitioning can be achieved in liquid chromatography. These include adsorption and so-called normal phase chromatography (polar-polar interactions), so-called reverse phase chromatography (non-polar interactions), size exclusion, and ion exchange chromatography. The choice of which mechanism to use for a particular mixture is based on the molecular weight of the components, their solubility and polarity, whether they are ionic or not, and what method was used previously for their separation (which, in many cases, can be found in published articles).

A Short History

The science of chromatography dates to the beginning of the 20th century. Michail Tswett is credited with the invention of chromatography, even though David T. Day was the first to achieve a separation of crude oil fractions on pulverized fuller's earth. Day did not understand the phenomenon, and called it capillary diffusion. Tswett demonstrated separation of chlorophyll pigments on a column packed with calcium carbonate (chalk). He named the technique after himself, calling it "Tswettography". As "tswett" means "color" in Russian, the term was later translated into "color writing", or chromatography.

Even though the roots of chromatography were founded in the technique of liquid chromatography, gas chromatography (where the mobile phase is a gas) became the most important instrumental technique through the 1950's and 60's, in large part due to the work of A. J. P. Martin, R. L. M. Synge, and A. T. James. Martin and Synge won the Nobel Prize in 1972 for their work on the theory of chromatography.

Liquid column chromatography metamorphosed into HPLC during the later 1960's and 1970's with the development of smaller particles, better column packing techniques, and precision pumping systems. More recently, HPLC research has focussed on very small particle packings, specific detectors, and small diameter columns (microbore). In addition to high performance liquid chromatography, HPLC has been referred to as high pressure LC, high speed LC, and high priced liquid chromatography.

SIGNIFICANT EVENTS IN LIQUID CHROMATOGRAPHY

1903 - Michail Tswett - Plant Pigments on Chalk Column

1938 - Maria Shraiber, Nikolair Izmailov - First Paper on Thin Layer Chromatography

1947 - Martin and Synge - Theory of Chromatography Awarded the Nobel Prize in 1951

1958 - Stein and Moore - Automatic Amino Acid Analysis Awarded the Nobel Prize in 1972

1958 - Stahl - Published Handbook of TLC

1959 - Porath and Flodin - Size Exclusion with Cross Linked Dextrans

1969 - Journal of Gas Chromatography becomes Journal of Chromatography (Commercial "Liquid Chromatography" introduced)

1972 - Majors and Kirkland - Packing Microparticulate Columns

1977 - Microprocessor Controlled Pumps

1978 - 3 Micron Column Packings

1983 - Microbore Columns

The HPLC System

To achieve the best separations, very small particles are required for the stationary phase. To achieve reasonable flow rates through these beds of small particles, sophisticated pumping systems are required. In addition, sensitive detectors are required to measure minute quantities of components eluting from the column. A diagram of the components of a modern HPLC is shown below:

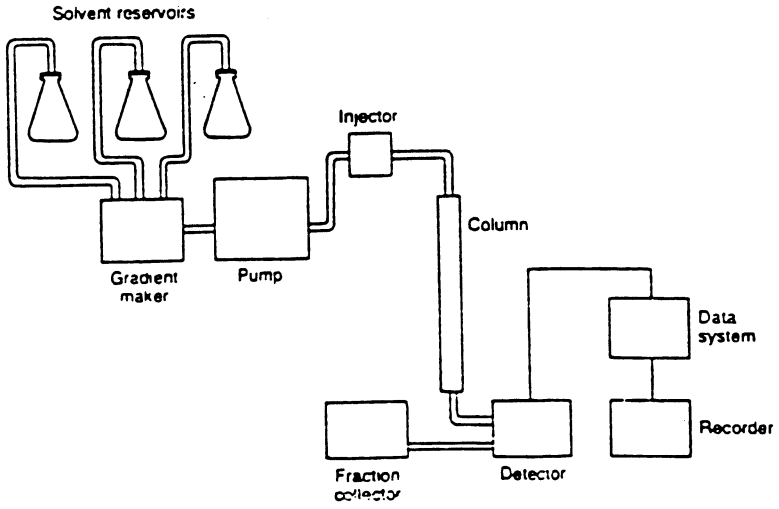


FIGURE 3

There are several solvent reservoirs so that different mixtures of solvents may be formed, usually with the aid of a microprocessor based programmer. If only one solvent composition is used throughout a separation (isocratic conditions), then only a single reservoir would be needed. However, many separations require the use of changing solvent compositions (gradient elution). Thus, the instrument must be capable of accurate mixing of the solvents in exactly the proportions required at any point during the separation.

The injector is used to introduce the sample into the system at the head of the column; it must be capable of doing this at relatively high pressures (1000 - 6000 pounds per square inch).

A number of different types of detectors are available for use in HPLC systems. These include detectors based on the principles of ultraviolet light absorbance, changes in refractive indices, and fluorescence. All detectors produce an electrical signal, which is then plotted on a strip chart recorder, or measured by a data system, or both.

Many HPLC's can also be adapted for completely automated separations of a number of samples.

ADVANTAGES OF HPLC OVER TRADITIONAL METHODS

Speed - Minutes

Resolution - Stereoisomers

Detection Limits - 10^{-9} - 10^{-12}G

Reproducibility - $\pm$ 1%

Easily Automated

LIMITATIONS OF HPLC

Cost - Instrument and Expendables

Complexity - Requires Training or Experience

Insensitive for Some Compounds

Sample Must Be Soluble in a Stable Form

ADVANTAGES OF LIQUID vs GAS CHROMATOGRAPHY

- Larger Number of Compounds can be Separated by LC
- Not Limited Due to Sample Volatility or Thermal Stability
- Suitable for Macro Molecules
- Easily Accomodates Ionic Species
- Employs Both Stationary and Mobile Phases
- Greater Variety of Phases

MAJOR AREAS OF APPLICATION OF LC

- Biomedical
- Agricultural Chemicals
- Polymers
- Pharmaceuticals
- Foods and Beverages
- Environmental Monitoring

THE THEORY OF LIQUID CHROMATOGRAPHY

One does not have to understand how a liquid chromatography column works to be able to use it. However, many problems can be avoided, and others diagnosed, by using your fundamental knowledge of what is happening inside the column. In addition, separations are more easily optimized, and manufacturers' claims more accurately evaluated by understanding these terms and relationships.

The primary purpose of this chapter is to impart a practical understanding of why separation occurs, and how a column works. This information becomes very important when one is trying to develop the best possible separation for a particular mixture. After all, you wouldn't try to repair a car without understanding how the internal combustion engine works, would you?

Separation

If you were to place a mixture of three components at the top of a liquid chromatographic column, and the components passed through the column with no column interaction, the LC recorder would show the plot in Figure 1A. In other words, the compounds would pass through the column in just the time it takes for a portion of liquid phase solvent to pass through the column. This time is called t_o. The plot of the concentration in Figure 1A is "square" because we have assumed that the mixture is unaffected by anything in its passage through the column, and thus elutes in the same concentration as when it was injected into the column: in a band.

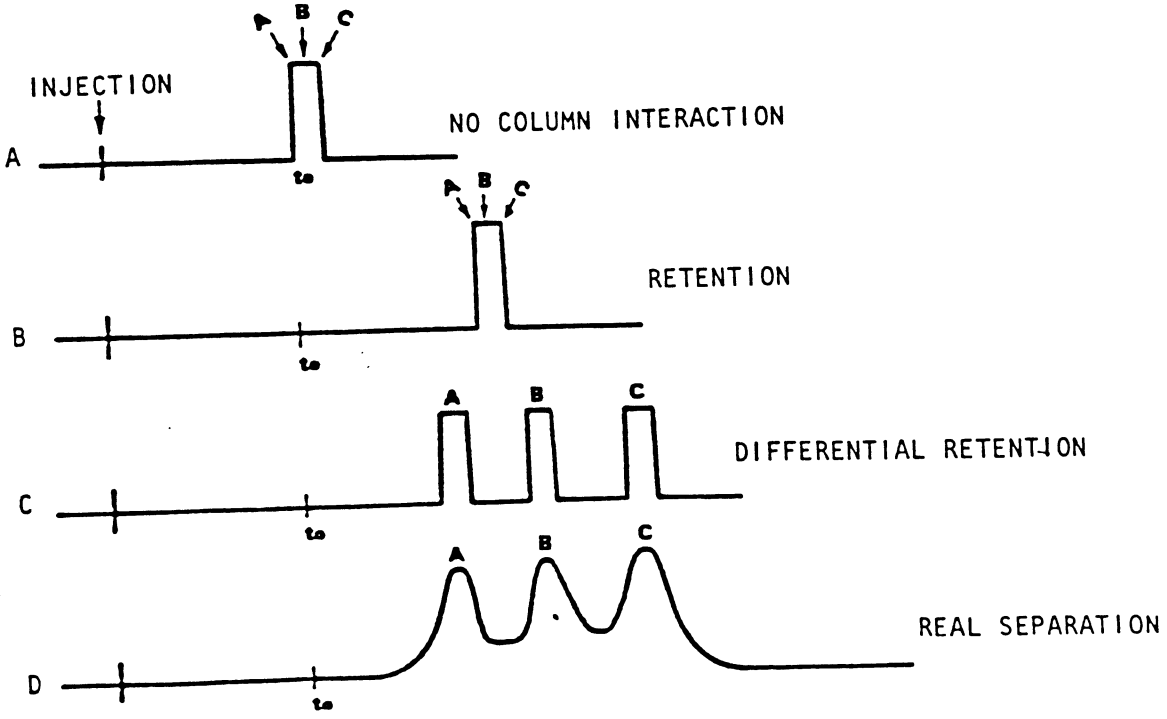


FIGURE 1

Figure 1B shows what the plot would look like if the three components are equally retarded in their passage through the column. **Retention,** but not separation, has occurred. To achieve separation, we need **differential retention,** as shown in Figure 1C. Here, the interaction of the three components with the column packing is unequal. Compound A shows the least interaction with the column and elutes first; C elutes last because it interacts strongly with the column packing. While all components spend the same amount of time in the mobile liquid phase, they separate because they spend different amounts of time adsorbed to or dissolved in the stationary phase.

Unfortunately, real separations do not show narrow bands representing the components upon elution. Instead, **band broadening** processes cause the concentration of the components in the solvent to change, which leads to a plot that looks like Figure 1D. This plot is called a chromatogram.

Resolution

When two compounds (represented by two peaks on the recorder chart paper) are completely separated, we say that they are completely resolved. The term, **resolution,** can be either descriptive or can be defined absolutely. The following figure shows the separation of two compounds on two different columns:

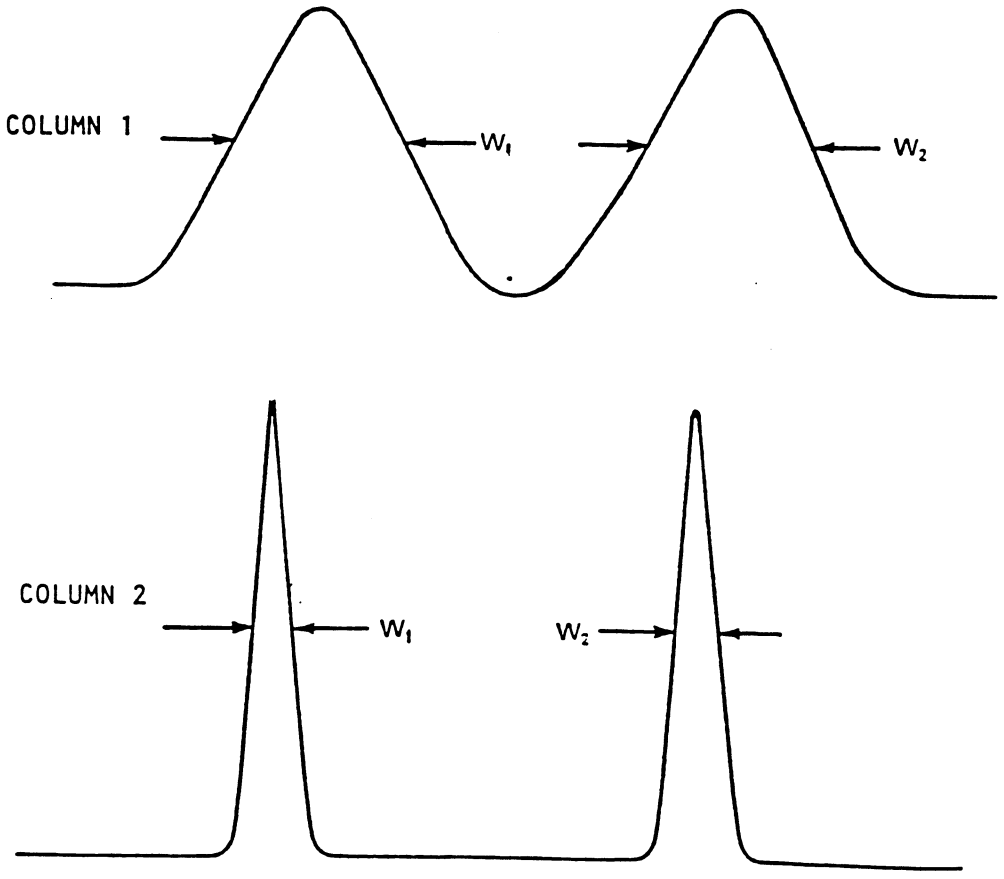


FIGURE 2

Even though the two compounds are completely resolved on both columns, we can describe the resolution (or amount of separation) as much greater on the second column. The resolution can be defined absolutely on either column using the formula:

$$R = \frac{1.18d}{W_1 + W_2}$$

W_1, W_2 = PEAK WIDTHS AT HALF HEIGHT

A calculated resolution of 1.0 means that the two peaks are 98% separated to the baseline, and a resolution of 1.5 means that the peaks are 99.7% separated to baseline. This means that if you divided the areas of the two peaks by drawing a perpendicular line from the valley to the baseline, 98% of the area of each peak would result from the primary component, while 2% would belong to the other peak:

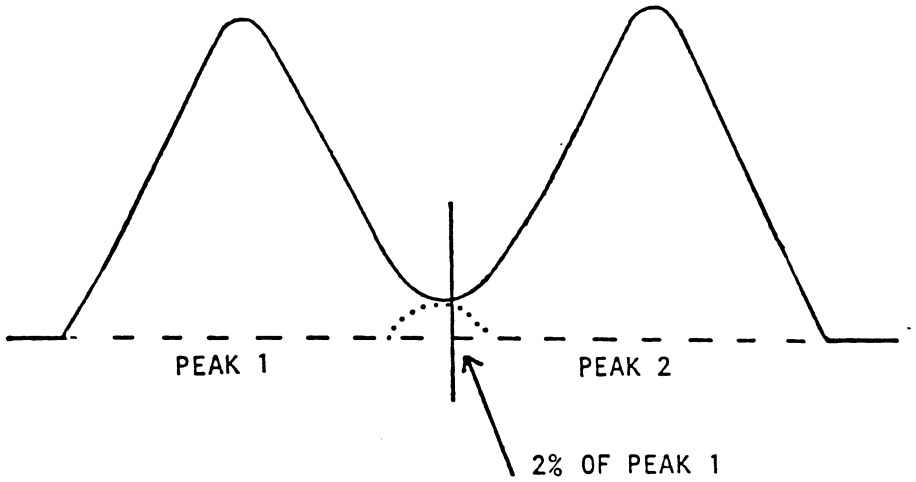


FIGURE 3

The first column in Figure 2 exhibits a resolution of approximately 1.5, but the second column shows a resolution of approximately 6.0.

Normally, a resolution of 1.0 is adequate for most separations, but if components vary greatly in concentration in your sample, you might need more resolution to achieve accurate quantitation of the smaller peak (see Figure 4).

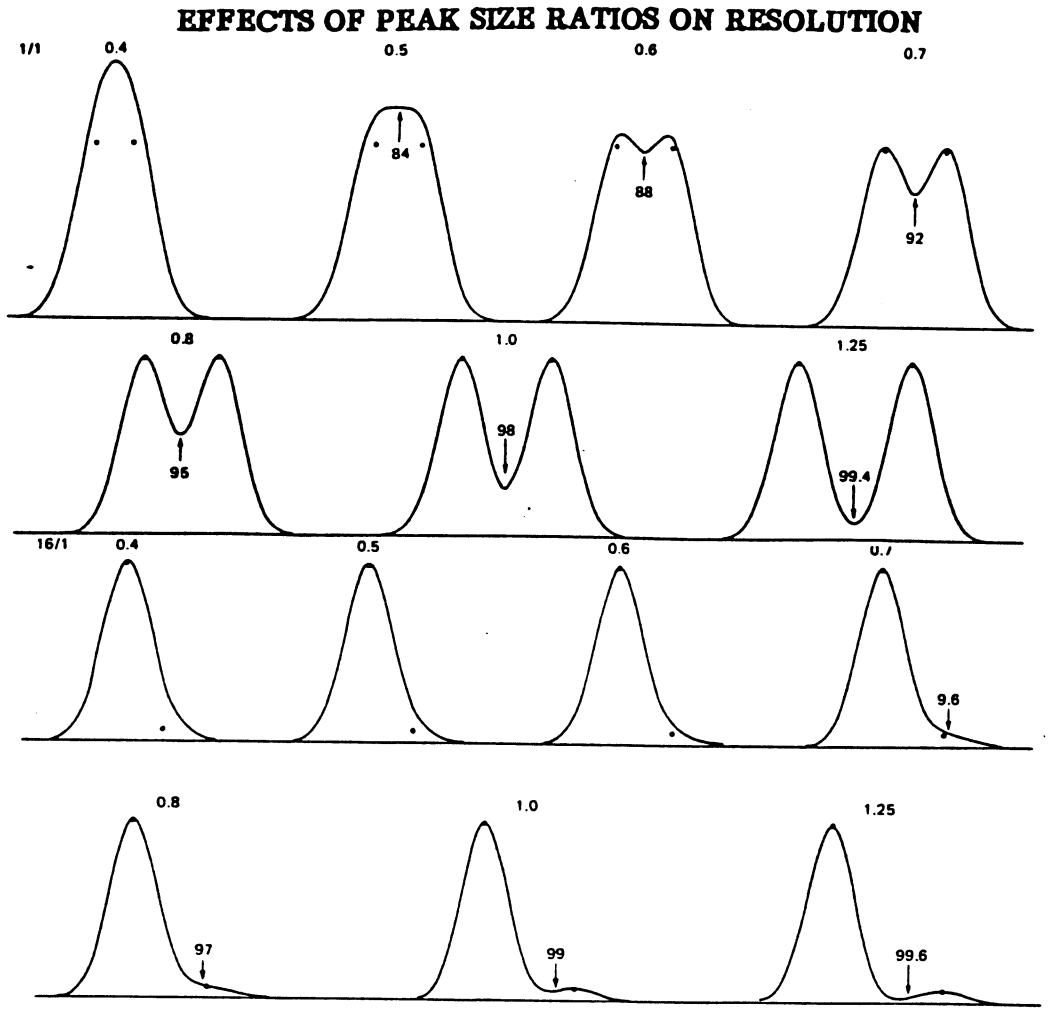


FIGURE 4

In addition, peak shapes will affect the amount of resolution required to separate two peaks completely. The "ideal" chromatographic peak shape is a Gaussian curve (the notorious bell-shaped curve of your college exam days):

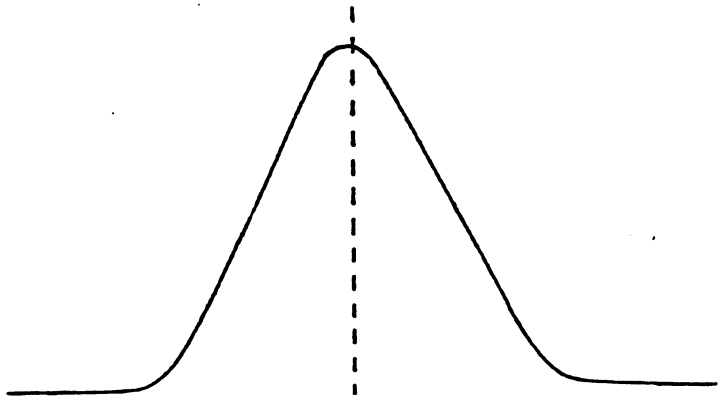


FIGURE 5

If you can imagine the molecules of the compound being injected into an LC column in a perfectly uniform, narrow band, then you should be able to imagine that band gradually taking on the Gaussian shape, since the molecules are always moving and the Gaussian curve represents the resulting statistical distribution of the molecules in the space that they occupy:

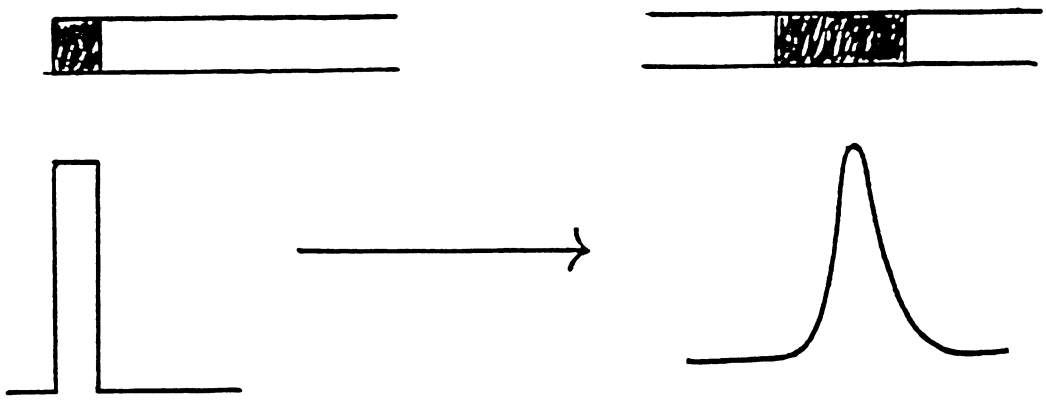


FIGURE 6

If anything happens within the column to affect the peak shapes, then separation will normally be degraded:

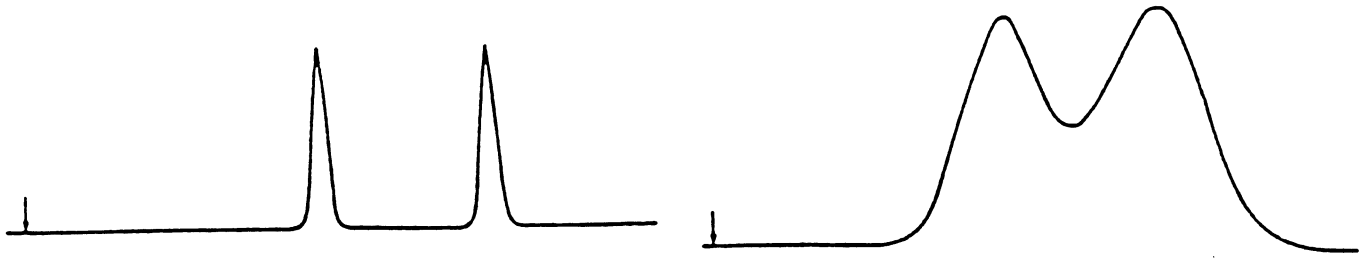


FIGURE 7

Thus, much care must be taken to ensure that peak shapes remain symmetrical in their passage through the column. The chapter on columns will discuss this point further.

How Do We Achieve Good Resolution?
The resolution between two peaks is a function of three other terms: **retention, selectivity,** and **column efficiency.** Retention is necessary to achieve any degree of separation. Retention and selectivity are functions of the chemical nature of the liquid phase and the stationary phase. In Figure 2 above, both columns show the same selectivity. In other words, the two peaks have the same retention times on both columns; the peak shapes are irrelevant.

The last factor, column efficiency, is a measure of how much the component bands "spread out" in their passage through the column. A more efficient column will give sharper peaks for the same retention time than a less efficient column. In Figure 2, the second column is more efficient than the first, thereby leading to the increased resolution.

Retention And The Capacity Factor
Retention can be measured and expressed in several ways. The **absolute retention time,** t_R, is the total time from injection of the sample into the column to the apex of the peak:

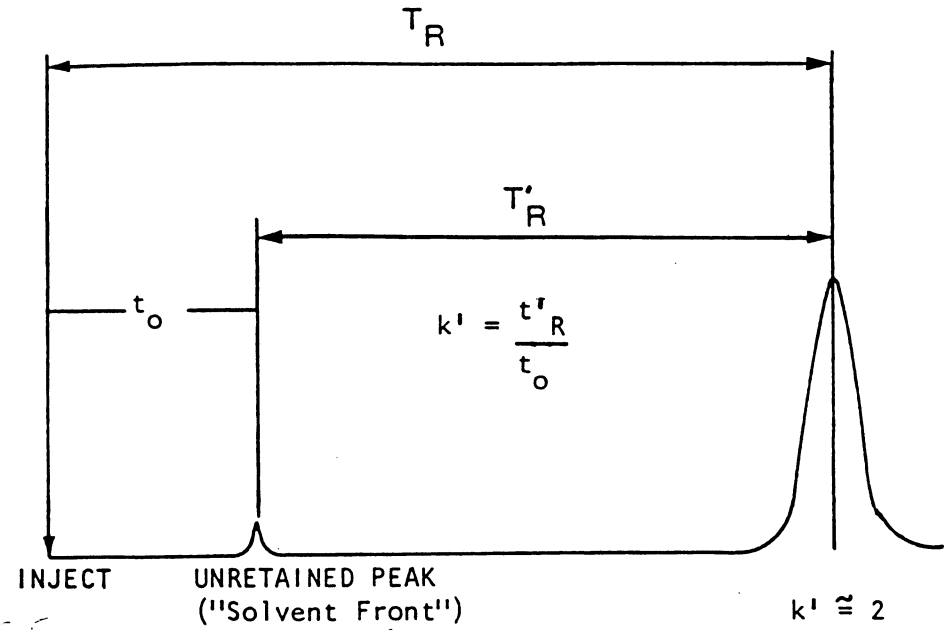


FIGURE 8

The **corrected,** or **adjusted retention time,** t_R', is the absolute retention time minus the unretained peak time: $t_R' = t_R - t_o$.

Finally, the capacity factor, k', is defined as the adjusted retention time of the peak divided by the unretained peak time. The capacity factor is a measure of the time that a sample component spends in the stationary phase. For example, a k' of 1 means that the solute has spent 50% of its time in the column in the liquid phase, and 50% of the time adsorbed or dissolved in the stationary phase; a k' of 2 corresponds to a 33.3%/66.6% division of time between the two phases.

The k' of a solute is also a measure of the partition ratio of that solute between two phases. For reasons which we will explain later, the k' for most solutes in an LC separation should be kept in the range of 1 to 10.

Selectivity

Selectivity, also referred to as **differential adsorption** or **differential solubility**, is a measure of the stationary phase's ability to distinguish between two compounds. If the two compounds are equally soluble in the stationary phase, then there can be no differentiation between them, and thus, no separation will occur. No matter how good the column efficiency of a particular column, if the stationary phase does not possess sufficient selectivity, separation cannot occur.

Mathematically selectivity is defined as the ratio of the adjusted retention times (t_R') of the two peaks. This ratio is named α:

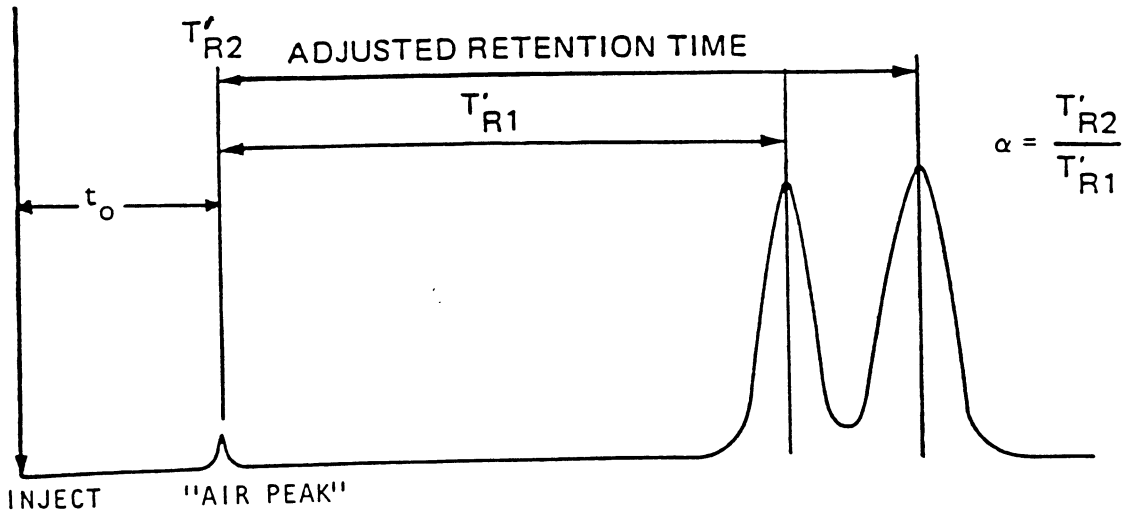


FIGURE 9

In gas chromatography, if the selectivity for a particular column is not adequate at any temperature, the only way to improve it is to change to a different liquid phase, using McReynolds numbers are guides. This is because the carrier gases used in GC are inert, and do not participate in the separation mechanism. However, the solvents in liquid chromatography are **not** inert, and play a very active role in the separation. Thus, selectivity in LC may often be adjusted by a different choice of mobile phase, without changing the column.

Column Efficiency

Whereas selectivities of two columns may be identical, there can be a very large difference in the **column efficiencies.** Qualitatively, more efficient columns give sharper peaks for the same retention times; less efficient columns give more peak broadening.

The term "column efficiency" comes from distillation technology. In fact, the theory of distillation, counter-current extraction, and chromatography is the same. In a distillation, the efficiency of the column in achieving the separation of two components is expressed in terms of how many vaporizations and re-condensations occur as the components pass through the column. The **bubble cap distillation column** exhibits very well defined vaporizations and re-condensations, as it is built with distinct plates where each vapor-liquid equilibration can occur:

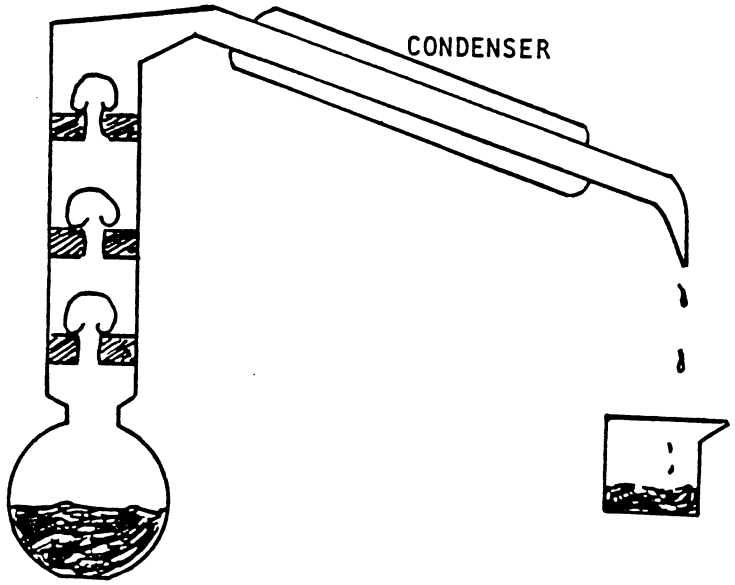


FIGURE 10

The above column, then, achieves three complete equilibrations between the vapor phase and the liquid phase. Any distillation column that could achieve exactly the same separation is said to have a column efficiency of three plates, or 3 **theoretical plates.** An average LC column has a column efficiency of around 10,000 theoretical plates, which should give you some idea of the separating power of LC. Even though the LC column does not have real "plates", a compound passing through it will equilibrate between the liquid and stationary phases approximately 10,000 times.

Quantitatively, the column efficiency is expressed as the number of theoretical plates calculated by the formula.

$$N = 5.54(t_R/W)^2$$ W = PEAK WIDTH AT HALF HEIGHT

where t_R is the total retention time.

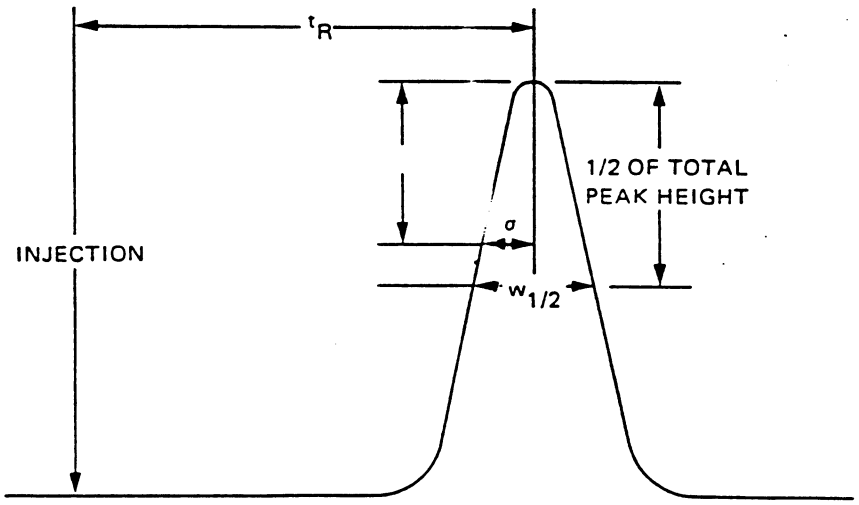


FIGURE 11

Column efficiency is primarily a function of the column length and the particle size and uniformity. In simple terms, the higher the column efficiency, N, the sharper the peaks. In other words, less peak spreading (band broadening) has occured as the compound passed through the column. There are three primary reasons (each with a mysterious name) for band broadening: eddy diffusion, longitudinal diffusion, and resistance to mass transfer.

Eddy Diffusion

Ideally, a sample is placed at the beginning of the column in the narrowest possible band. In a packed column, one of the factors that works to spread out the molecules in that band is **eddy diffusion**, which is also called the **multipath effect.** Because some molecules take longer paths through the particulate bed then other molecules, the overall effect is to spread out the peak:

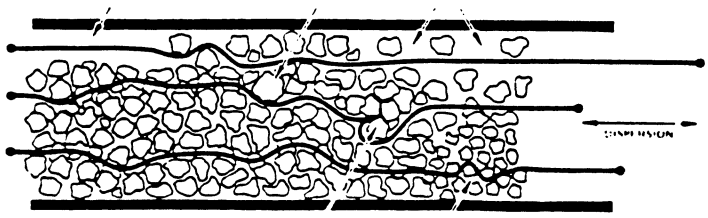


FIGURE 12

In a packed column, this effect is not affected by the flow rate, but is very dependent on the uniformity of the particle bed. Columns should be packed so that every possible pathway through the particles is nearly equivalent to every other pathway. This is best achieved when the particles are all the same size, and are packed carefully so that the packing density is maximized and uniform. There should exist no open channels through the column.

Longitudinal Diffusion

This term refers to the fact that, as time passes, the band will tend to diffuse toward both the detector and injector ends of the column. The longer the peak is in the column, the more longitudinal diffusion will occur. In fact, if carrier flow is stopped completely for a long period of time, the component will spread out evenly throughout the entire column. Thus, the magnitude of this term is inversely proportional to the flow rate.

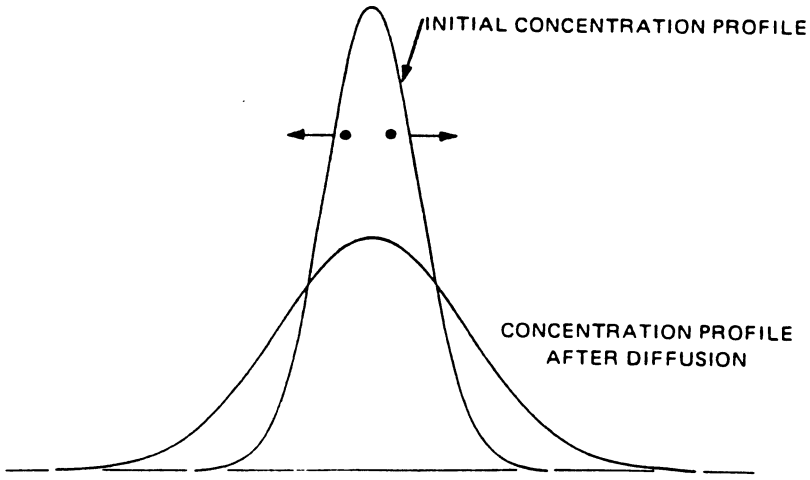


FIGURE 13
Longitudinal Diffusion

In gas chromatography, longitudinal diffusion is an important contributor to peak spreading. In liquids, though, diffusion rates are roughly 10,000 times less than they are in gases. Thus, longitudinal diffusion is of little importance in liquid chromatography. In fact, you can stop the flow through a column in mid-separation, leave it overnight, and start the flow the next day. The peaks trapped in the column will elute and look much the same as if they had eluted the day before.

Resistance to Mass Transfer
This formidable-sounding term simply refers to how readily sample molecules move from the liquid phase to the stationary phase and back again:

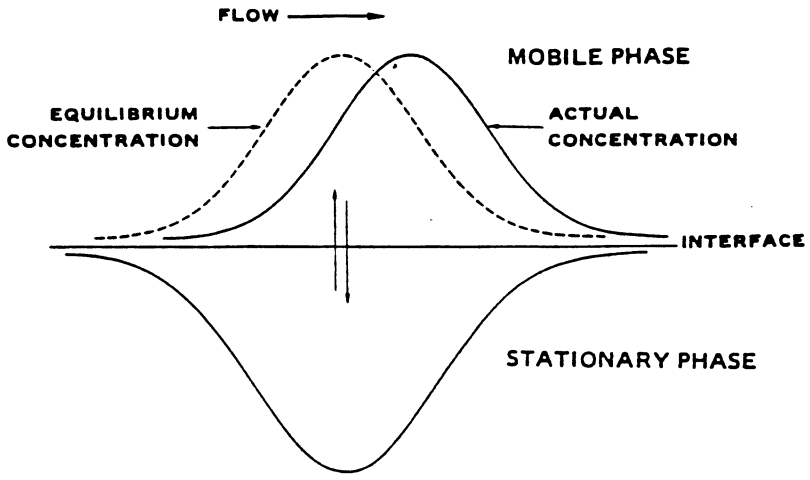


FIGURE 14

The ideal situation would be at very low liquid phase flow rates, where the sample molecules would have plenty of time to come to equilibrium between the stationary phase and the liquid phase. As higher liquid phase flow rates are used, the molecules dissolved in or adsorbed to the stationary phase will begin to "lag behind" those in the mobile phase, and band broadening will occur. Thus, the magnitude of this effect is directly proportional to the flow rate.

Other factors that affect this term are the particle size (the smaller, the better, since you don't want your molecules to get "lost" inside a large particle), and stationary phase film thickness (the thinner, the better, again to facilitiate rapid equilibration between the stationary phase and the liquid phase). In addition, the viscosity of the solvent will affect mass transfer. Acetonitrile, a "thin" solvent, will give approximately 40% better column efficiency than methanol, a "thick" solvent, because the rate of molecule transfer between phases is faster with the less viscous solvent.

The Van Deemter Equation

The sum of the above three contributions to band broadening is called the van Deemter equation:

$$HETP = A + \frac{B}{\mu} + C\mu$$

where the A term is due to eddy diffusion, the B term is due to molecular (longitudinal) diffusion, and the C term is due to resistance to mass transfer. The factor in the last two terms, μ, is the abbreviation for mobile phase linear velocity. HETP is the abbreviation for Height Equivalent to a Theoretical Plate, which is just the length of the column divided by the column efficiency:

$$HETP = \frac{Length}{N}$$

The lower the HETP, the higher the column efficiency, and the sharper the peaks.

The B term and the C term are both affected by the mobile phase linear velocity, μ, but in opposite directions. This means that there exists an optimum linear velocity at which the HETP will have its lowest value. This is most easily seen by looking at the plot of each term in the van Deemter equation separately, and then the sum of all three plots:

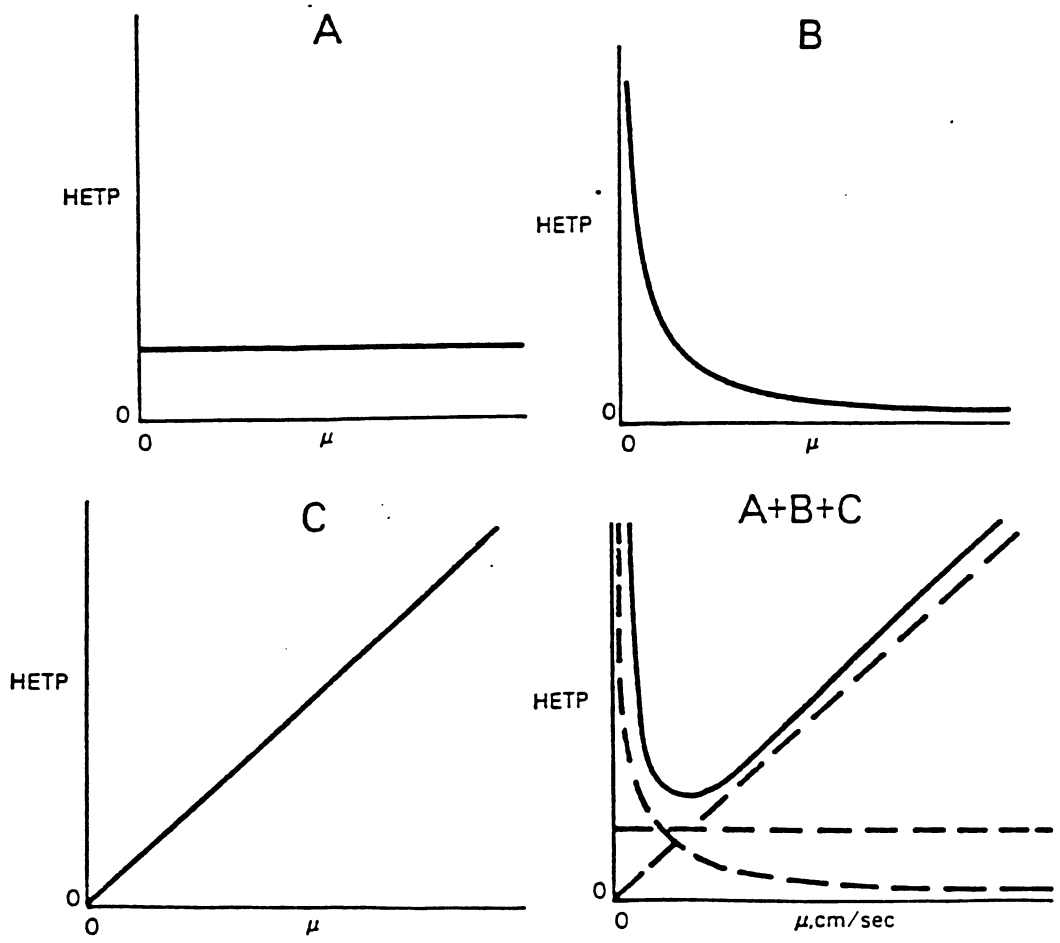


FIGURE 15

Even though there is a theoretical minimum in the van Deemter plot, it occurs at such a low flow rate for most LC columns that its use is impractical. Most flow rates used in liquid chromatography are set without reference to the van Deemter minimum. It turns out that a more important factor is the particle size. The smaller the particle, the flatter the slope of the van Deemter plot, because of improved mass transfer.

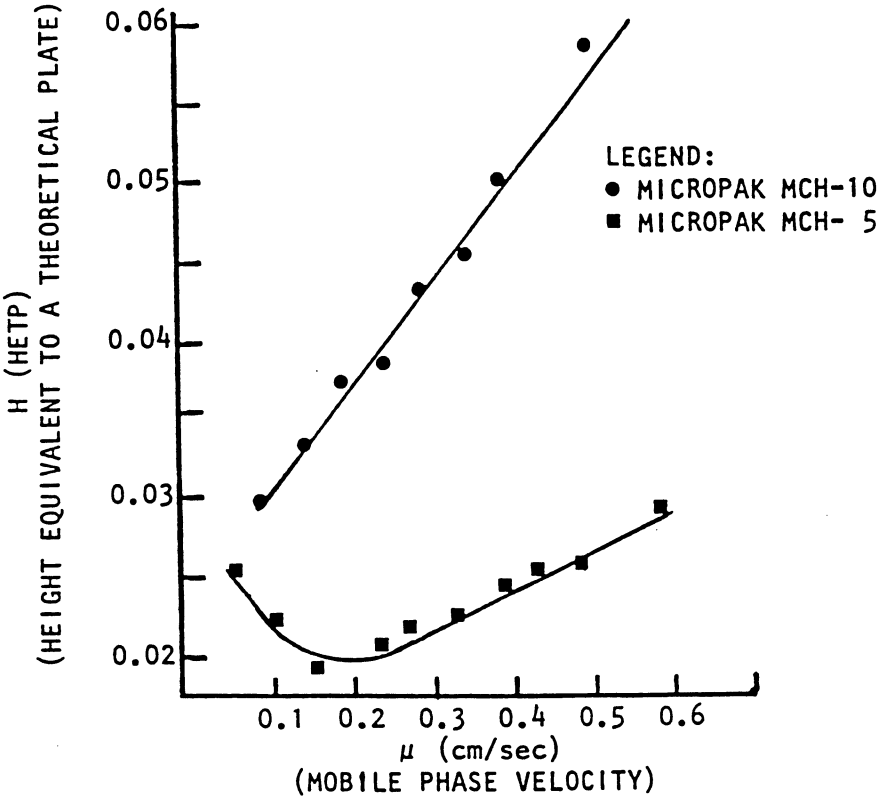


FIGURE 16

The Resolution Equation

The resolution between two components in a separation can be expressed as a function of retention, column selectivity, and column efficiency:

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

COLUMN EFFICIENCY — SELECTIVITY — RETENTION

The resulting value of R is exactly the same as in the earlier resolution equation. For example, two peaks at k' = 5 where $\alpha = 1.1$ and N = 5000 will be separated to baseline, since R = 1.47 (check it yourself). If the separation is not adequate, R (the resolution) must be increased by increasing either retention (k'), selectivity (α), or column efficiency (N).

Optimizing an LC Separation

The first step in optimizing an LC separation is to obtain adequate retention. Examination of the resolution equation above shows that resolution increases negligibly at k' values greater than 10. In other words, increasing retention from k' = 1 to k' - 10 will greatly improve an isocratic separation, but little improvement is seen for greater retentions.

Retention (and thus, k') is increased on any particular column by decreasing the solvent strength. For a given column, a strong solvent is one that moves a component through the column rapidly, and a weak solvent is one that moves a component through the column slowly. Mixtures of strong and weak solvents are used to adjust solvent strength, and thus, retention. If retention is very low or very high on a column, regardless of the solvent strength used, then a different column must be used.

To summarize:

To optimize the capacity factor, k':

> Adjust solvent strength

> Change the stationary phases

Because the mobile phase plays such a large part in liquid chromatography, selectivity can be altered in many ways other than changing the column, as is done in GC. For example, using different strong and weak solvents will often affect selectivity. Acetonitrile and water are often used in "reverse phase" separations. However, some mixtures and compounds require the use of methanol in addition to or in place of the acetonitrile to achieve the proper selectivity.

Additions of small amounts of solvent modifiers can also change selectivities. For example, addition of small amounts of tetrahydrofuran (THF) to reverse phase systems affects the nature of the stationary phase, and thus, the selectivity. If ionic or ionizable compounds are being separated, then modifiers that affect pH will change selectivity. Temperature affects chemical equilibria, and can also be used to change selectivity.

To summarize:

To optimize selectivity:

> Change mobile phase composition

> Use mobile phase additivies

> Change pH

> Change the stationary phase

> Change temperature

Retention and selectivity are relatively easily adjusted. Increasing column efficiency requires a change of hardware, in most cases. Column efficiency can be increased by using less viscous solvents and raising the column temperature to improve mass transfer, but these effects are relatively small. Similarly, the solvent flow rate can be decreased, but this increases the separation time with little improvement, especially with smaller particle sizes (5 μm and below).

The most effective way to change efficiency is to use a higher efficiency column, particularly if the present column is old and is not as efficient as when it was new. High efficiency columns are generally packed with small particles which are very uniformly sized.

Longer columns may also be used to improve column efficiency. However, it is better to couple two or more short columns in series than to use one long column of equivalent length. Long columns cannot be packed as well as short columns, and will never give the total efficiency of the combination of shorter columns.

To summarize:

To optimize column efficiency, N:

> Reduce flow rate

> Use 2 or more columns in series

> Use less viscous solvents

> Increase column temperature

> Decrease particle size

Appendix: Measure t_o for k' Calculations

The unretained peak time, t_o, is the time required for a completely unretained peak to pass through the LC column. Thus, it is **not** a retention time. A peak eluting at t_o is also referred to as eluting at the **void volume**, because t_o multiplied by the solvent flow rate equals the void volume of the column. The void volume consists of the volume between and within the particles. These two volumes are called **interstitial** and **pore** volumes, respectively.

Measuring t_o can be difficult. Many people use the solvent upset observed when injecting a sample in a small amount of solvent that differs in composition from the eluent. However, many modern UV detectors have minimized the effect of refractive index changes such that the upset has disappeared. The best way to measure t_o is with the use of a known unretained component.

Unretained components should be small, UV active molecules that can be predicted to show no retention on the column being used, especially if very strong solvents are used as eluents. For example, an ionic compound would be used with a reverse phase column. The unretained peak time and the void volume can be calculated, assuming that the size of the pores is larger than the test molecule and the flow velocity is low enough to allow permeation into the pores.

PUMPING SYSTEM REQUIREMENTS

Chemically inert

Develop 6000 psi

Pulseless

Flow rate reproducibility<1%

Small volume for rapid change of solvent composition

MATERIALS USED IN CONSTRUCTION OF LC SYSTEMS

Stainless steel 316 series

Teflon

Kel-F

Kalrez

Quartz detector windows

Sapphire and ruby

The list of materials above represents commonly employed materials for liquid chromatographs. Stainless steel exhibits high tensile stength required for high pressure applications (>1000 psi) and good corrosion resistance. (Halide ions, particularly in an acidic solvent, should be avoided). Teflon is often used to make seals between components and is often used for solvent or waste lines under low pressure (< 500 psi). KelF has a much higher mechanical strength with respect to teflon and is often employed in place of teflon. Kalrez is a chemically resistant fluoroelastomer. Quartz is used as windows for optical detectors. Pistons are made of sapphire and check balls are made of ruby to reduce wear.

Pumps

The purpose of the pumping system is to provide an accurately metered mixture of solvents to the column head in a well-regulated pulseless flow. The basic components of the hydraulics system of an HPLC are shown in Figure 1. The components include a pump, a proportioning device (gradient maker), solvent reservoirs and an injector. These devices are described in detail in this section for a number of pump types.

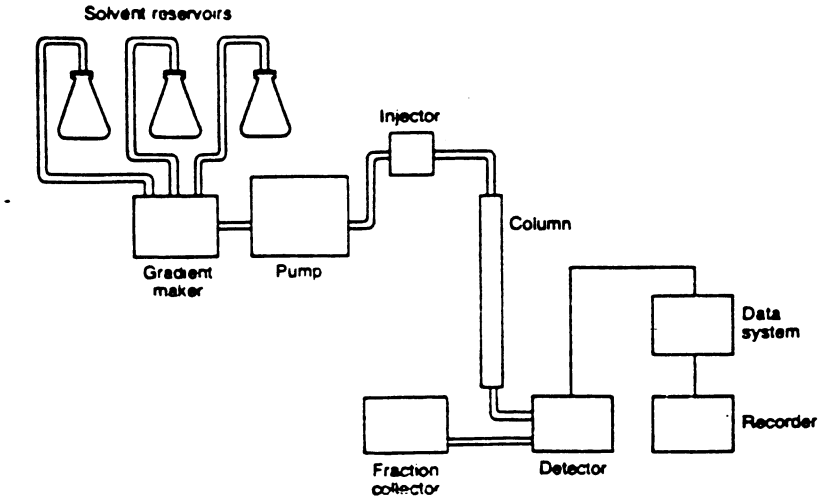


FIGURE 1

Every pumping system starts at the solvent reservoir. Solvent selection is a function of column selection and is covered in the column section. Solvents should be free of particulate and filtered if particulate matter is present, (for example: undissolved salts in buffers). The solvent line is fitted with a particulate filter (usually 2-10μ) in order to prevent material from entering the pump and abrading seals. This filter should be replaced or cleaned when priming becomes difficult. A simple test for plugged filters is to change the filters and observe whether or not priming is easier.

There are several types of pumps available, which include:

a) The holding coil type which consists of a large volume coiled tube containing a few hundred milliliters of solvent. The coil has a valve at each end to control the solvent during pressurization and solvent filling. The solvent is forced onto the head of the column using compressed gas. A variation of this uses a cylinder rather than a coiled tube. This is only discussed for historical significance because variations in gas pressure cause variations in solvent delivery rate.

b) Pneumatic amplifier type. This is a pneumatic amplifier consisting of a pump driven by compressed gas (usually less than 200 psi) contacting a large surface-area piston which contacts a smaller surface of solvent.

c) Diaphragm type. This is similar in operation to a piston driven pump, where the volume of a chamber increases and decreases, driving the fluid by the action of two check valves. However, the chamber is sealed, and the volume changes occur as a diaphragm on one side of the chamber is pushed in and pulled out. Two diaphragm pumps are required to form gradients.

d) Syringe pump types. These pumps operate using a screw gear that displaces a plunger through the solvent reservoir. These were used earlier, but they were very expensive. They may gain popularity again for microbore liquid chromatography because they are totally pulseless and can accurately delivery microliter quantities reproducibly.

e) Reciprocating types. This is the most common type of HPLC pumping system. A reciprocating pump consists of a moving piston which has direct contact with the solvents and is controlled by some sort of motor drive. Simple reciprocating pumps have constant piston speeds; thus, the time required for a fill stroke equals the time used for a pump stroke. Such pumps require extensive pulse damping. Because of this, a single reciprocating pump is often designed with two pistons, each working in opposition to the other.

Most reciprocating pumps have two check valves per piston. The solvent is drawn in through an inlet check valve into the piston chamber and then pushed out through an outlet check valve.

Two pumping systems (containing one or two pistons each) are required to form a gradient for many instruments. The Varian 5000 series pumps, however, require only a single piston for isocratic or gradient work.

There are a number of gradient pump designs. We will discuss the two major types, though there are many other types which are beyond the scope of this discussion. These types are high pressure mixing with a two pump delivery and low pressure mixing with single pump delivery.

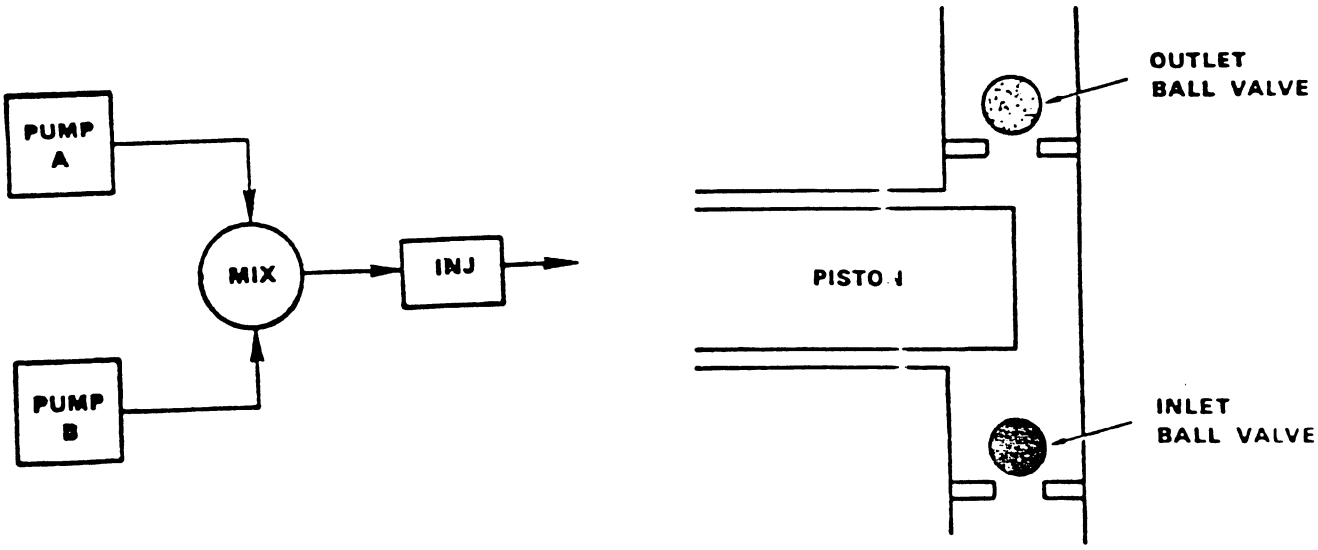


FIGURE 2

The first pumping design consists of a two pump high pressure mixing system. This pump design requires two separate pumps and a controller to do gradient elution. Each of the two pumps delivers separate solvents such as acetonitrile for one and water for the other, as in reverse phase chromatography. The controller's function is to control delivery speed of the separate solvents to form the gradient's composition, and to assure proper mixing with a dynamic mixer.

This type of system has four check valves on each pump, requiring eight check valves to do a binary gradient. The inlet check valves are susceptible to dirt and gases in L.C. solvents, which can lead to loss of pump prime or back flow into the solvent reservoir. Pumps using inlet ball valves require filtering and degassing of solvents to minimize such problems. In all pump designs, filtering is recommended whenever particulates are suspected.

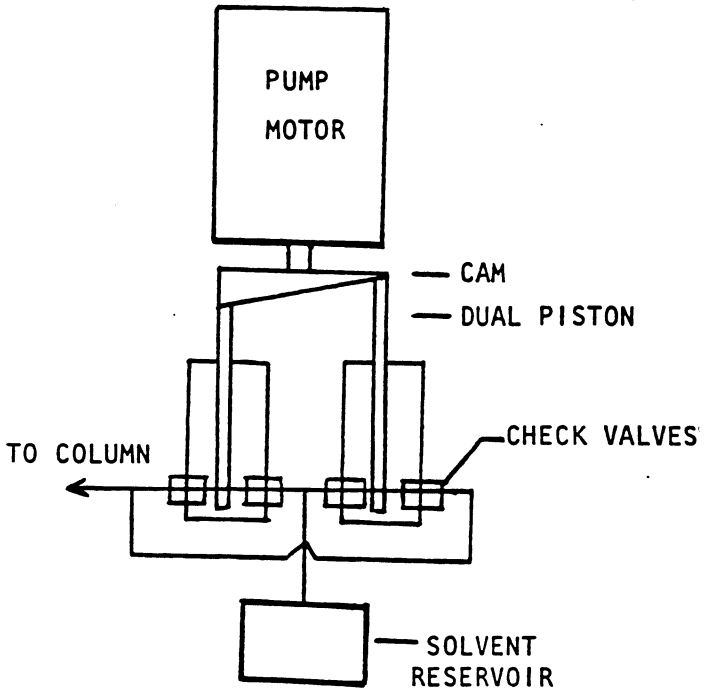


FIGURE 3

The figure above shows a schematic of the Varian Model 2000 pump. This pump has two pistons in each pump (two pumps are required for gradients). In the two piston design, we have a rotating cam where one piston is filling while the second is delivering solvent. The idea is that pulsations from each piston will cancel out by pumping in opposite directions. (one filling and one pumping.)

The second type of pump is the single piston low pressure proportioning type pump. An example of this type of pump is the Varian 5500 series pump. In order to do gradient elution, only one pump is needed.

This single pump has proportioning valves and no separate gradient controller. These proportioning valves allow the chromatograph to proportion from 1-100% of any of 3 solvents to form a ternary mixture, thereby eliminating the need for more than one pump to form the gradient. The proportioning valves are controlled by a microprocessor and are opened during the inlet cycle.

The inlet check valves, as used in the dual pump system, are replaced by a mechanically actuated inlet slider valve which eliminates the need for degassing or filtration except when particulates are suspected.

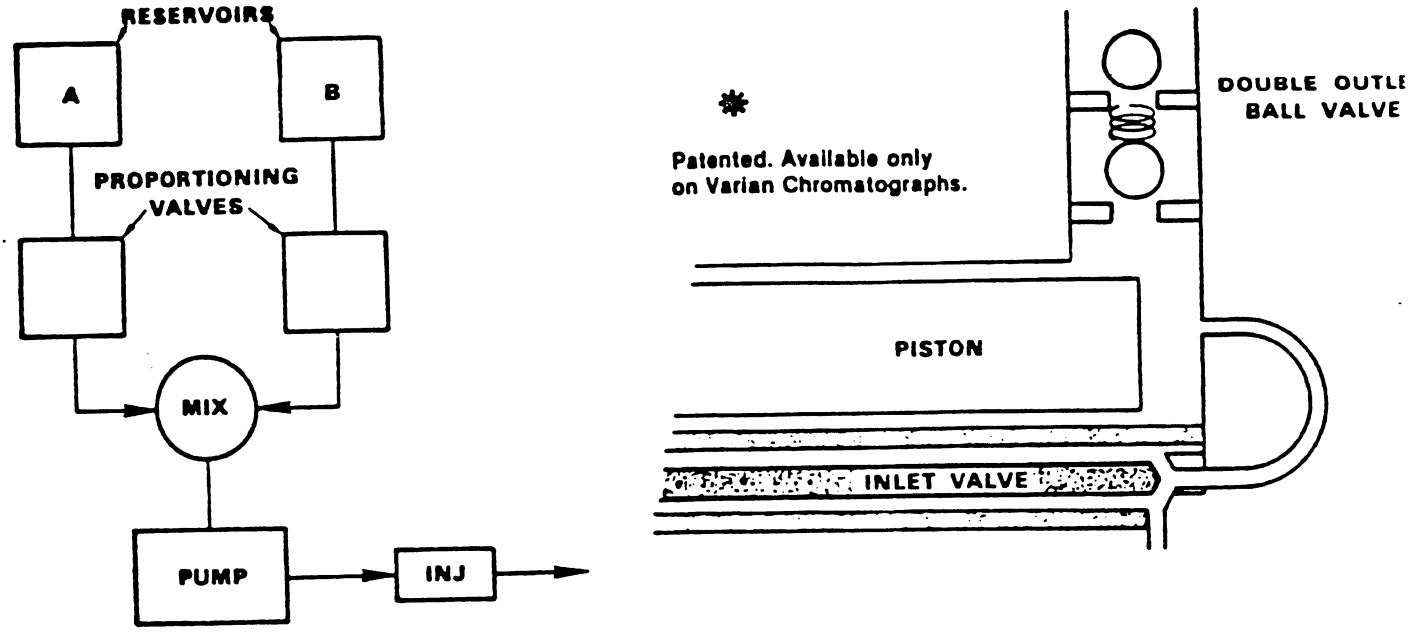


FIGURE 4

Figure 4 is a detailed schematic of the Varian Model 5000 pump. The proportioning valves admit solvent from the reservoirs to the pump. As many as three proportioning valves can be mounted in the inlet-valve head and any two (in binary systems) or all three (in ternary systems) can be opened to provide the solvent composition requested. During each fill stroke of the pump, one proportioning valve is always open. If more than one must be opened during a single stroke, they are opened sequentially. When the coil is not energized, a spring seals the orifice within the proportioning valve. Since the solvent is drawn from the reservoirs by the fill stroke of the pump, the solvent delivery system does not depend upon gravity or pressurized reservoirs.

The inlet-valve head, to which the proportioning valves are attached, contains the stainless-steel inlet-valve needle and the inlet-valve seat. During the fill stroke of the pump the valve is opened mechanically by means of a crankshaft, drawing solvent into the pump chamber. During the pump stroke the needle is seated by the pressure of spring washers to prevent back flow into the solvent reservoirs. The inlet-valve head is connected to the pump head by a 1/16" interconnect tube. The pump head contains the pump piston and the main piston chamber, and carries the check-valve assembly and a bleed valve used for priming the pump. The pump piston, which delivers 90 $\mu\ell$ of solvent during each stroke, is made of sapphire to reduce wear on the piston seals.

This design, which includes a variable-speed pump stroke and a rapid fill stroke, reduces the pulsations that would result if a constant angular velocity were translated directly into solvent flow.

The check-valve assembly, mounted on top of the pump head, closes the outlet line during the fill stroke of the pump, to prevent solvent back flow into the pump chamber. It includes a 2-μ filter, to remove particulate contaminates from the solvent, and two ruby-ball/sapphire-seat check valves. The first is spring loaded, the second actuated by solvent back-pressure.

Pumping Sequences

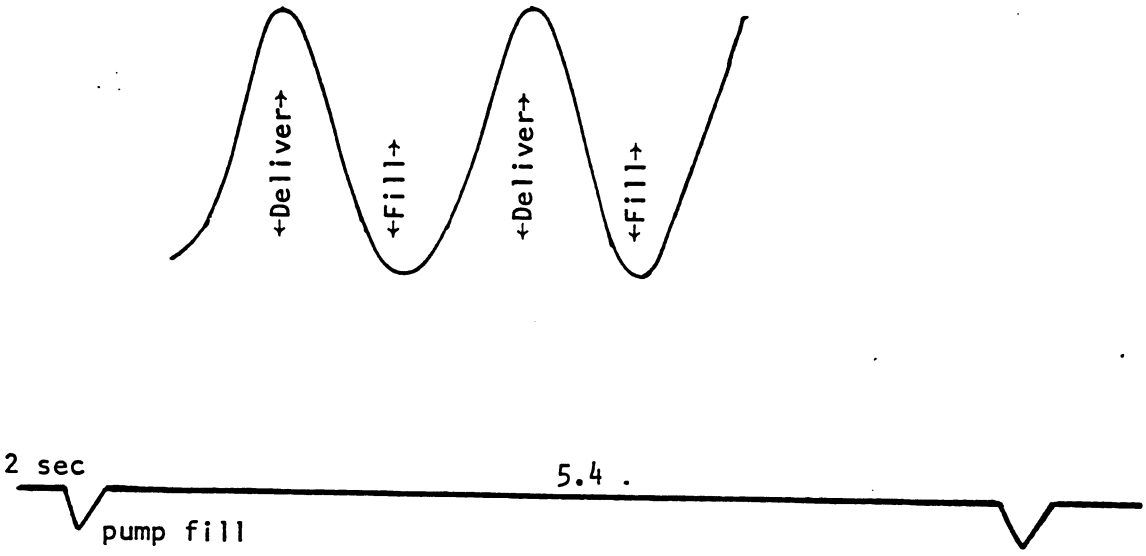


FIGURE 5

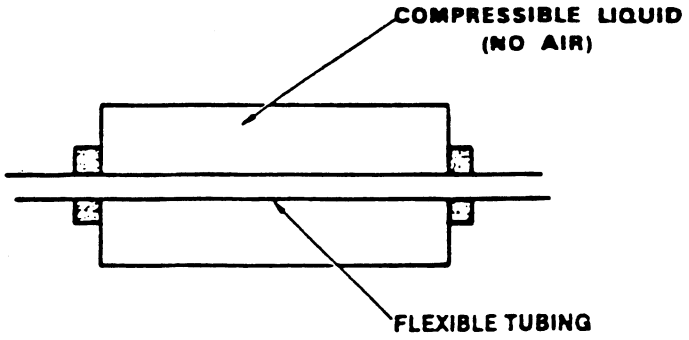
There are three different types of pumping sequences. A single piston, simple reciprocating pump has a sinusodial wave pumping pattern. Without any pulse damper we see an increase in pressure during delivery and decreased pressure during fill. This creates pulses, which was one of the problems with the first HPLC pumps. (see Figure 5)

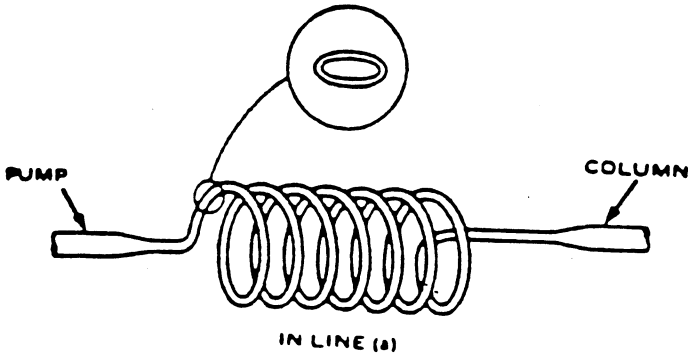
In the LC 5000 and 5500 series there is a built in pumping sequence controlled by a microprocessor. The pump has a very rapid fill stroke, filling the 90 l pump chamber in 200 milliseconds and then having a slow even delivery stroke. As the flow rate changes, the time of the delivery stroke changes (1 ml/min-5.4 seconds, 0.5 ml/min 10.8 seconds, 2 ml/min-2.7 seconds). The fill stroke is fixed at 200 milliseconds. This decreases the pulsations by having the pump spend a majority of its time delivering solvent and minimal amount of time in fill mode. (See Figure 5 B).

In the 2000 series, the design of the pump has a proportioning cam which minimizes the pulsations. The two piston pump allows one piston to be in a fill stroke on one side, and a delivery stroke on the other side, at the same time, which causes cancellation of the baseline pulsation. (See Figure 5 C)

Pulse Dampers

Compressed Liquid Type:





Coiled Tube Design

FIGURE 6

The pulse damper serves to remove any residual pump pulsations. There are a number of types. We will discuss two:

1) Compressed Liquid Type
2) Coiled Tube Design

The compressed liquid type consists of a flexible tube surrounded by a compressible liquid. During the pressurization portion of the pump cycle, the flexible tubing expands and energy is stored by compressing the liquid surrounding the tubing.

The second type is the coiled tube design. This consists of a large dead volume metal coil similar to a spring. The pulsing is absorbed by the resistance in the spring. This is the type of pulse damper which is found in the Model 2000. These coiled tube design dampers have to be installed in front of the solvent mixer for gradient elution.

Column By-Pass Valve

A small three way valve is installed between the pump and the column head. The function of this valve is to by-pass the column. When changing solvents, the pumping system should be flushed out with a miscible solvent. For example: If we were doing reverse phase, using acetonitrile and water, and we would like to change to a normal phase system, using hexane and methylene chloride, we would use an intermediate solvent, such as isopropanol, to wash the system out to waste, using the column by-pass valve.

Injector

SAMPLE INJECTION VALVE

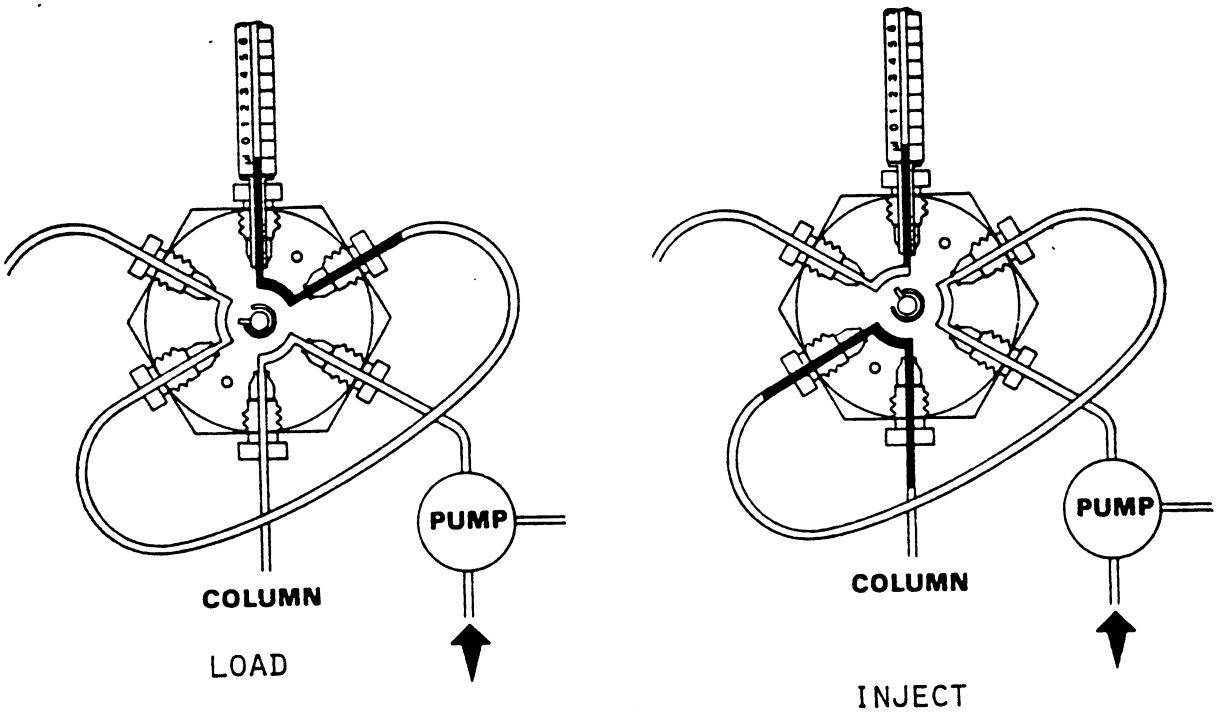


FIGURE 7

The above diagram shows one type of injector. Sample loops are most commonly 10-100 ul. Larger loops may contribute significantly to band broadening, but are used for semi-preparative chromatography or whenever efficiency is less important than loading capacity. If the loop is completely loaded with sample, precision can be 1.0% RSD. Partial loading of a loop results in poorer precision and is dependent on the operator's syringe technique. With care, one can achieve precision on the order of 1-2% RSD for partially loaded loops. Note that this assumes good control of flow rate and mobile phase composition and temperature. The true loop volume may vary as much as $\pm$ 10% of the stated volume and should be checked gravimetrically if loop sizes are changed during a series of sample runs. Most chromatographers prefer to load the sample loop partially or dilute the sample rather than change loops. Cross-contamination between sample injection can be significant (.5-10%) but is easily avoided by flushing between samples. One should check cross-contamination under the particular conditions being used. One

should avoid removing the syringe before the sample is injected since sample may drain out of the loop into the waste container. The length of the sample syringe needle must be long enough to seat near the valve core without damaging the surface. If the needle is too short, cross-contamination between samples or leakage around the needle can yield poor precision. If the syringe needle is too long, it will damage the valve core. Follow manufacturers' guidelines whenever selecting sample syringes. The injector handle should be turned quickly since flow to the column is momentarily blocked between the load and inject positions. Slow activation can cause a pressure surge in the system and an artifact on the chromatogram. In the worst case the pump may stop if the upper pressure limit is exceeded. All the sample valves contain a seal made of a polymer that must be replaced after extended use. A spare valve or seal replacement should be kept on hand to expedite repair.

Heaters

Reasons for Temperature Control in LC	Considerations Before Using Elevated Temperatures
• Maintain stable column temp.	• Column stability
• Improve efficiency selectivity	• Sample stability
• Shorten analysis time	• Reduced selectivity
• Dissolve sample	

Control of column temperature in LC is especially important if ambient temperature varies widely, e.g. 10°C in an 8-hour day. For example, experimental studies have shown that anthracene and naphthalene retention times vary about 1% if column temperature varies 1.5°C. Selectivity changes with temperature, but it is difficult to predict how such changes will affect resolution. Heating lowers the viscosity of LC solvents which results in higher efficiency. One should weigh such an increase against shortened column lifetime.

There are two types of heaters; contact heaters and convention ovens. The contact heaters consist of a block of aluminum containing heater coils and a temperature probe which is sealed inside the block for safety. This type is found in the LC 5000 and LC 5500 series. They will hold two columns each and have a temperature range from ambient to 150°C (a second block can be added if necessary when doing GPC with 4 columns.)

The second is the convection oven type. They will hold a large number of columns and have heating coils inside but are considerably more expensive than the contact heater design. This type of heater is found on the Model 2000 system and has a temperature range from ambient to 99°C. They require a nitrogen purge to eliminate any solvent vapor and a leak sensor for added safety.

Connectors and Fittings

ZERO DEAD VOLUME UNION

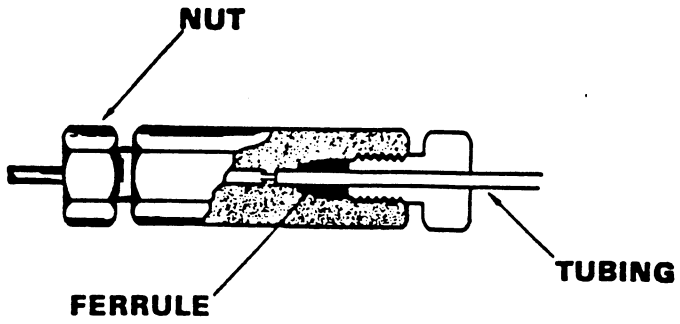


FIGURE 8

There are, unfortunately, a wide variety of nuts, ferrules, and connectors available. Very often nuts and ferrules are **not** interchangeable between manufacturers and care must be taken to use the proper fittings. For example, LC injectors generally require a long threaded nut and special ferrules because of their basic design. Long nuts can be substituted for short nuts, but not vice-versa. The shape of ferrules varies among manufacturers. Some require a back ferrule to seat. When making a fitting, make sure the ends of the tubing to be connected are clean and flat. A whet stone or fine metal file used carefully can be helpful. Be sure not to block the hole. The nut and ferrule are then slipped over the end of the tube. The smaller end of the tapered ferrule should be furthest away from the nut. The tubing, nut and ferrule are connected to the fitting to be made. The nut is tightened only after the tubing is pushed into the bottom of the fitting. A properly made connection will have about 2-3 mm of tubing extending beyond the ferrules. This length varies somewhat depending upon the manufacturing source.

LIQUID CHROMATOGRAPHY DETECTORS

There are many types of detectors available. The three most commonly employed are:

1. UV-Vis absorption
2. Refractive index (RI)
3. Fluorescence

Several other special purpose detectors are less commonly employed for specific types of analysis and will be discussed in special detector methodology.

A detector has two major functions:

1. To produce an electrical signal proportional to sample concentration or flux.
2. To generate little or no signal for eluent components and unwanted sample components.

It is extremely important to determine detector performance because it greatly affects the quantitation of components of interest. Detector performance is affected by:

1. Short term noise - should be measured with flowing solvent and influenced by detector time constant.
2. Long term noise and drift - this may be due to the detector warming up, column contamination, leaks, etc.

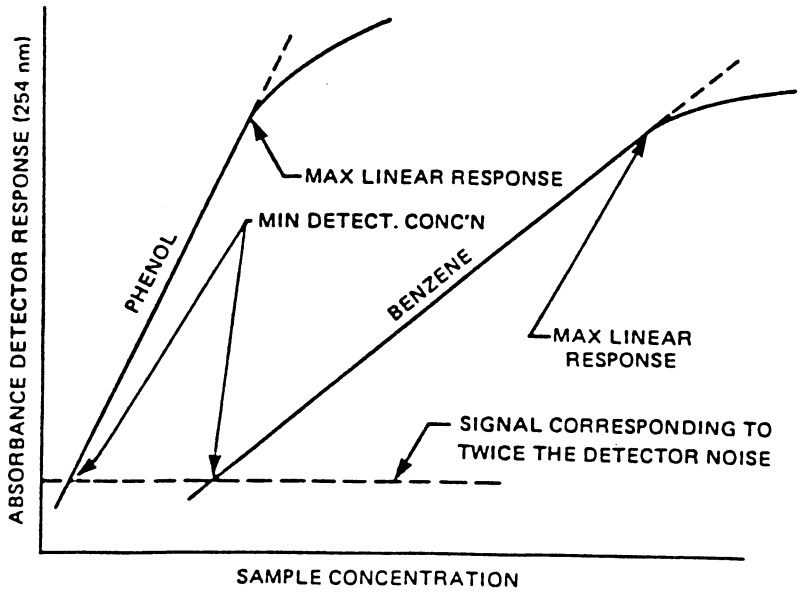
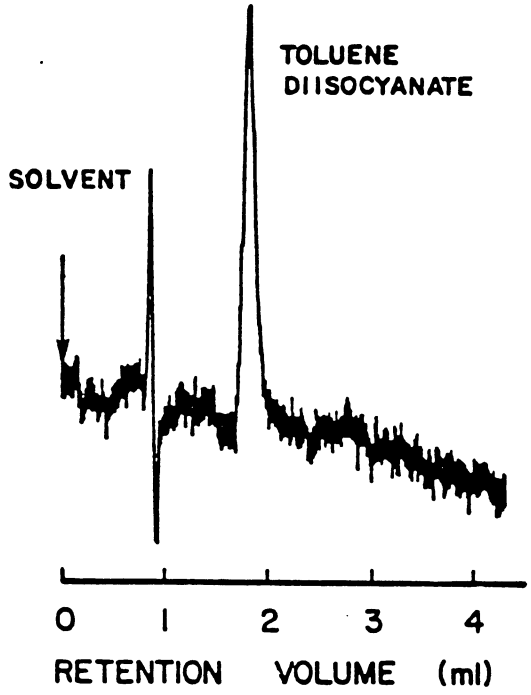


FIGURE 1

Cycling may occur due to temperature change from heating/air conditioning, drafts, etc.

The sensitivity of a component in a detector must be determined before quantitation.

A sensitivity plot is shown in Figure 1 for two compounds, phenol and benzene. The sensitivity is defined as the slope of the line passing through the experimental points from detector responses plotted vs. sample concentration. It is important to define the lower limits of the detector. The lower limits of the detector are affected by the signal/noise ratio. The minimal detectable quantity (MDQ) is defined as two times the noise level. Therefore the S/N ratio reflects the precision of quantitation S/N-2-5 is suitable for qualitative analysis and S/N = 10 for quantitation. In Figure 2 we show the determination of MDQ for Toluene Diisocyanate.



COLUMN: Silica Gel

SOLVENT: 5% CH_2Cl_2 in Hexane

FLOW RATE: 2 ml/min

DETECTION: 254 nm, 0.002 AUFS

MDQ = 2 X NOISE

$$S/N = \frac{SIGNAL}{NOISE}$$

S/N = 2 - 5 Suitable for qualitative analysis

S/N 10 for quantitive analysis

FIGURE 2

Determination of MDQ for Toluene Diisocyanate

There are a number of important detector considerations:

1. Extra column band-broadening effects which are affected by flow cell size, transfer tubing size and length and the misuse of zero dead volume fittings. We have discussed this in the hardware section of this course.

2. Time constant setting on the LC detector which controls the electronic filtering used to reduce noise levels. The Time Constant affects the noise level. As the time constant value increases, the filtering increases and the noise level decreases causing a decrease in sensitivity.

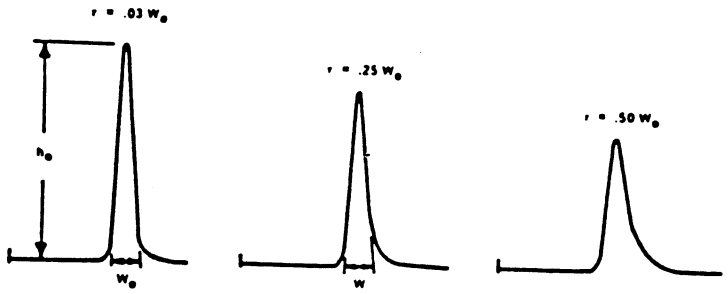


FIGURE 3A

Output Peaks of a UV Absorption Detector with Different Amounts of Filtering in the Output

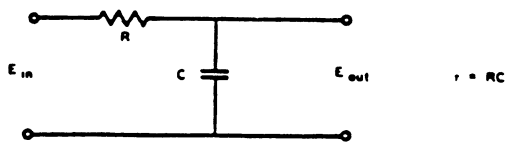


FIGURE 3B

Filter Circuit in Output of UV Detector

Figure 3A shows that if the time constant has too high a value peak distortion can occur. Therefore, it is critical to use a time constant of less than 1/3 the peak at half height. Recently, with very efficient columns, it has been necessary to use a value of .05 seconds or lower.

There are two main types of detectors: selective and universal. A universal detector is one which sees everything other than the solvent. A selective detector is a detector which can see a component over the response of solvent and the matrix. Increased absorption at a specific wavelength is due to chemical structure and in some cases a chemical reaction (e.g., post column reactor systems).

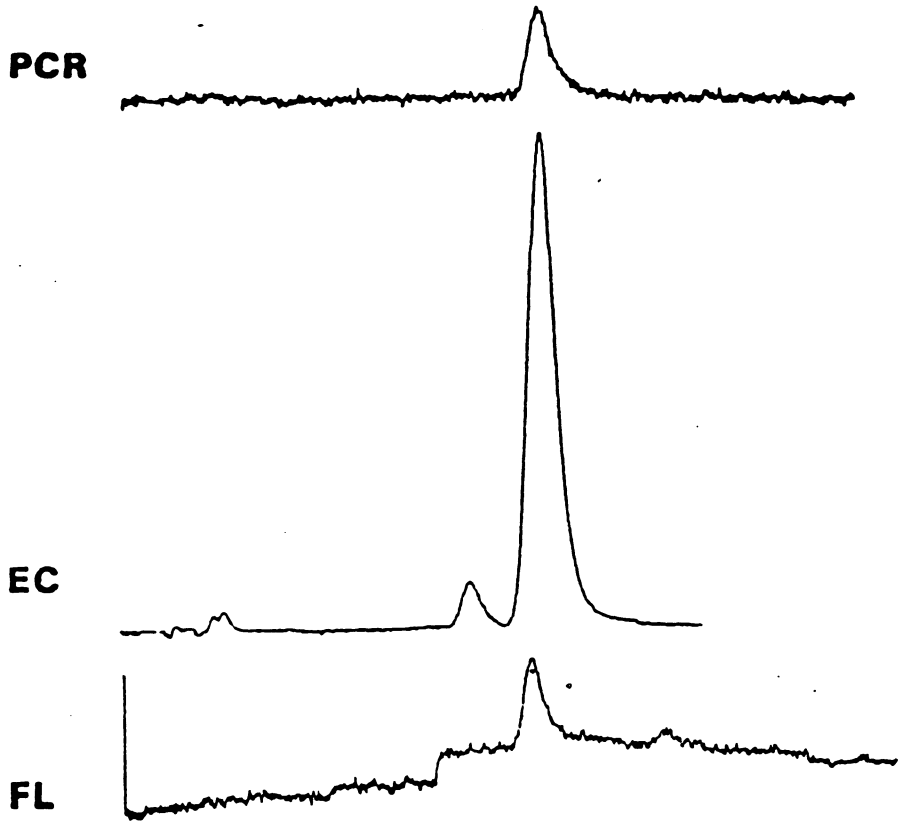


FIGURE 4

Examples of Peak and Baseline for VMA Standards in Low Normal Urine Range
(10.4 ng/injection or 10 µl injection of 1.04 mg/L std)

When choosing a liquid chromatographic detector, it is important to consider detector sensitivity. Detector selectivity is defined as the ability of a detector to see a component over the eluent and the background matrix. Figure 4 shows the response of VMA by three detection systems: UV, fluorescence and electrochemical. Note the baseline noise and peak size for the different detection systems with the EC having the highest response and the UV having no response. Figure 5 shows the VMA in urine. Note the matrix effect and the fact the only detection system which is selective over the matrix is the post column reactor (discussed in detail in the specific detectors section). Whenever you develop a method it is critical to check the detector selectivity of the component of interest over the matrix the sample is found in. The moral is always inject an unknown sample before completely optimizing method.

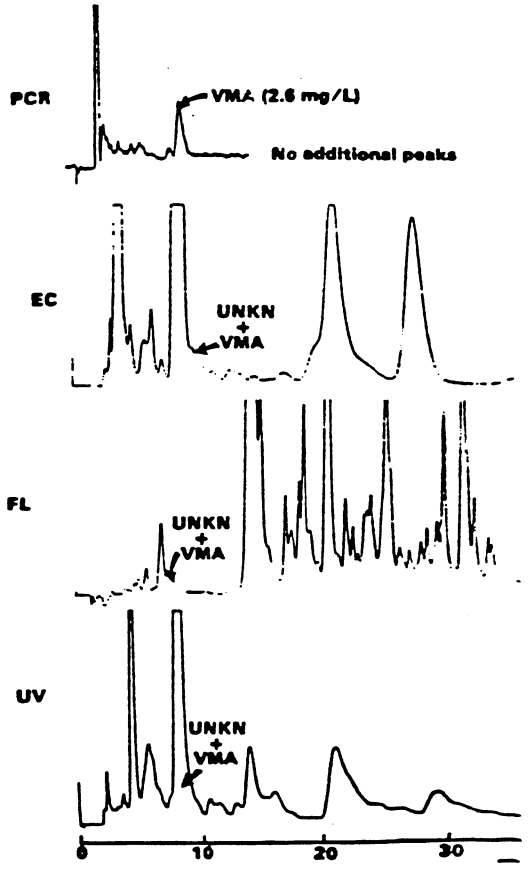


FIGURE 5

VMA in Urine Sample showing Detector Selectivity of
Post Column Reactor System

The most widely used detector in HPLC is the UV absorbance detector. UV detectors have the following advantages:

- High sensitivity for some compounds
- Good selectivity (variable)
- Easy to operate
- Nondestructive
- Gradient elution possible
- Nearly universal at low wavelengths (200 nm)

The principal of UV detectors is based on Beer's law. In Beer's law the log of the intensity of light through a reference path is ratioed to the intensity of light passing through the sample cell which is equal to the absorbance.

BEER'S LAW

$$\text{LOG}\left(\frac{\text{I REFERENCE}}{\text{I SAMPLE}}\right) = \text{EBC} = \text{ABSORBANCE (A)}$$

The absorbance is also equal to EBC where E is the molar absorptivity (molar extinction coefficient), B is the path length in cm, and C is the sample concentration. Since B is fixed in most LC detectors, only the type of compound and sample concentration affect the absorbance value. Molar extinction coefficents are a function of wavelength and molecular structure, and vary widely. This fact explains the sensitivity and selectivity that characterize UV absorbance measurements.

There are three major types of UV detectors:

a. Fixed wavelength - this type of detector has a single wavelength such as 254 nm using a mercury lamp. It has the advantages of being inexpensive, sensitive with low noise (less light scatter), and is widely cited in the literature. It is widely used in QC applications and routine methods but has the disadvantage of not being the optimum wavelength for many compounds and is generally limited in linearity.

b. Multiple wavelength detectors - these detectors are of intermediate cost using filters of various key wavelengths, some down to 200 nm when using a deuterium lamp. There are also multiple wavelength types which have multi-lamps that work down to 214 nm. They are somewhat inconvenient to change wavelengths.

c. Variable wavelength detectors. These detectors can have a two lamp system: a deuterium (UV) and tungsten (Visible), and have a monochromator allowing wavelength change. Recently they have been designed to use only the deuterium lamp covering the wavelength range from 190-600 nm. They are the most versatile detectors allowing easily selectable wavelengths to maximize selectivity and response for each component in the mixture. Some variable wavelength detectors have automated wavelength selection and auto zero capabilities which can be useful in methods development to increase selectivity and response. Some variable wavelength detectors employ diode arrays rather than a single phototube to give continuous readout of absorbance at a number of wavelengths. Their disadvantage is that they are more expensive, particularly when automated.

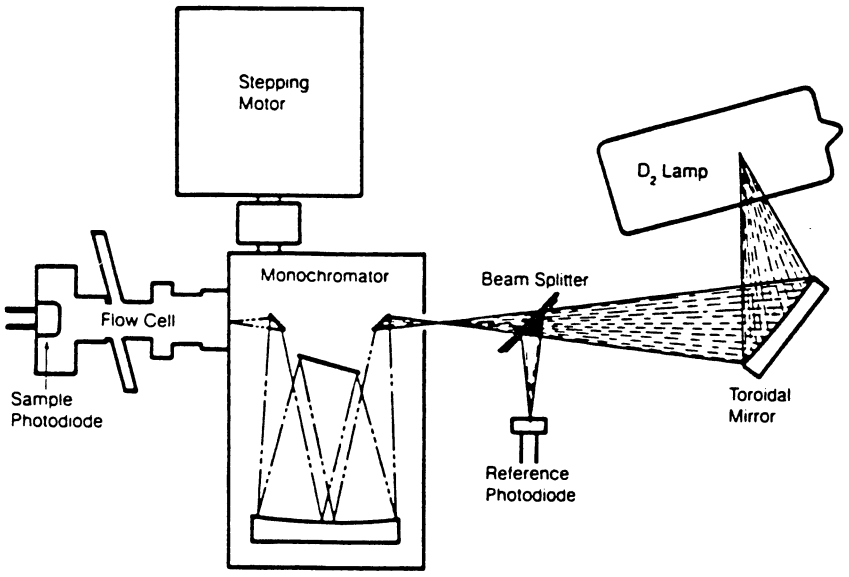


FIGURE 6

Figure 6 shows an example of a variable wavelength detector. The light from the deuterium lamp is directed by mirrors into a beam splitter with one beam going to the reference photo diode and the second into a monochromator. Light strikes the grating and is dispersed into its respective spectrum. The light is then directed out the exit slit and passed through the flow cell and into the sample photodiode.

The variable wavelength detector can go down to the far UV allowing the detection of quantities of low absorbing compounds such as olefins, phospholipds and carbohydrates.

Figure 7 shows the separation of olefins and demonstrates the increase in absorbance of approximately ten times at 192 nm over 200 nm.

LC OF MONO-OLEFINS

Conditions:
Column: MicroPak-CH
Mobile Phase: Acetonitrile
Flow Rate: 1 ml/min
Detector: Varichrom
Sample: 10 μg of each mono-olefin

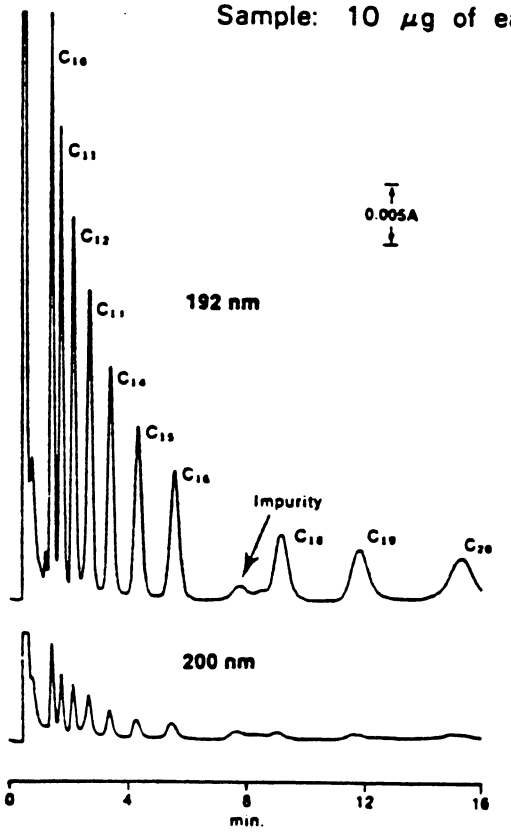


FIGURE 7

Background absorbance effects of the mobile phase affects the response of an individual compound. Each solvent used in HPLC has a UV cut-off which is defined at the wavelength at which the solvent absorbance reaches 1.0 AUF. Figure 8 shows the UV cut-off for common solvents used in HPLC. Since LC UV detectors become non-linear at this value, this represents the minimum practical wavelength at which one can work. Note that pure water is transparent throughout the UV, but the addition of other solutes e.g., buffer, ion pairing reagents, increases the cut-off wavelength. The moral of the story is to use the highest grade of salts or ion pairing reagents added to any mobile phase.

UV WAVELENGTH AT WHICH SOLVENT ABSORBTION EQUALS 1 AU

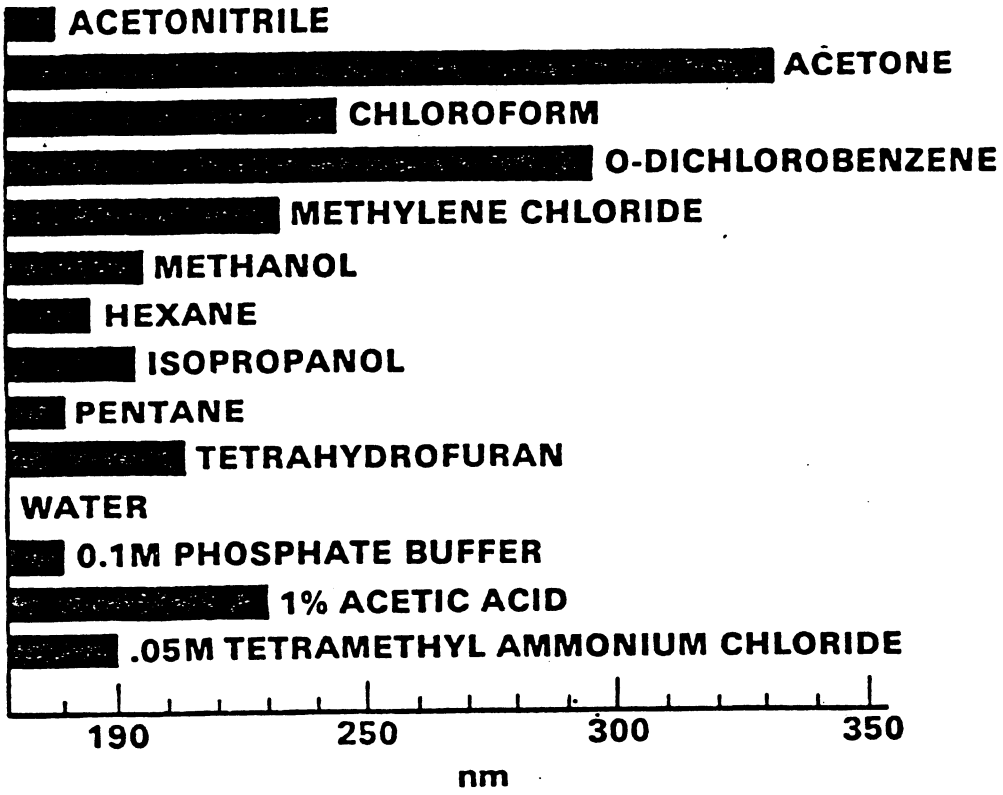


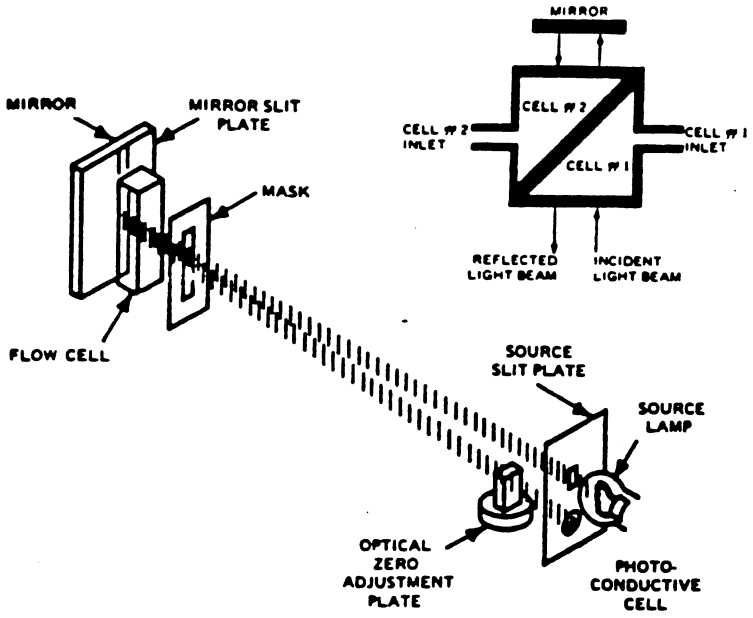
FIGURE 8

Refractive Index Monitor
The refractive index detector is a universal detector, which means it responds to everything, including the solvent. The basic principal of the RI is change in the refractive index in the presence of the sample.

There are two basic types of RI detectors:

1) Deflection type RI
2) Fresnal reflection type

The deflection type is shown in Figure 9. The cell is arranged so that equal change in the refractive index of the sample and reference cell causes a cancellation of deflection. When a sample passes through the cell the deflection occurring in each of the two light beams are additive. This type of detector is less sensitive than UV detectors.



Deflection Type RI Detector Optical Schematic.

FIGURE 9

The Fresnel Reflection type RI detector employs two beams of light from a tungsten lamp, passing through a glass prism. This light is reflected from a mirrored surface, hitting a dual photo cell. Two cells are found between the prism and the mirrored surface, one for reference and one for the sample. The detector has the capacity to measure any difference in the refraction of light between the sample cell and the reference cell. The Fresnel RI detector also has a number of prisms for different refractive index ranges.

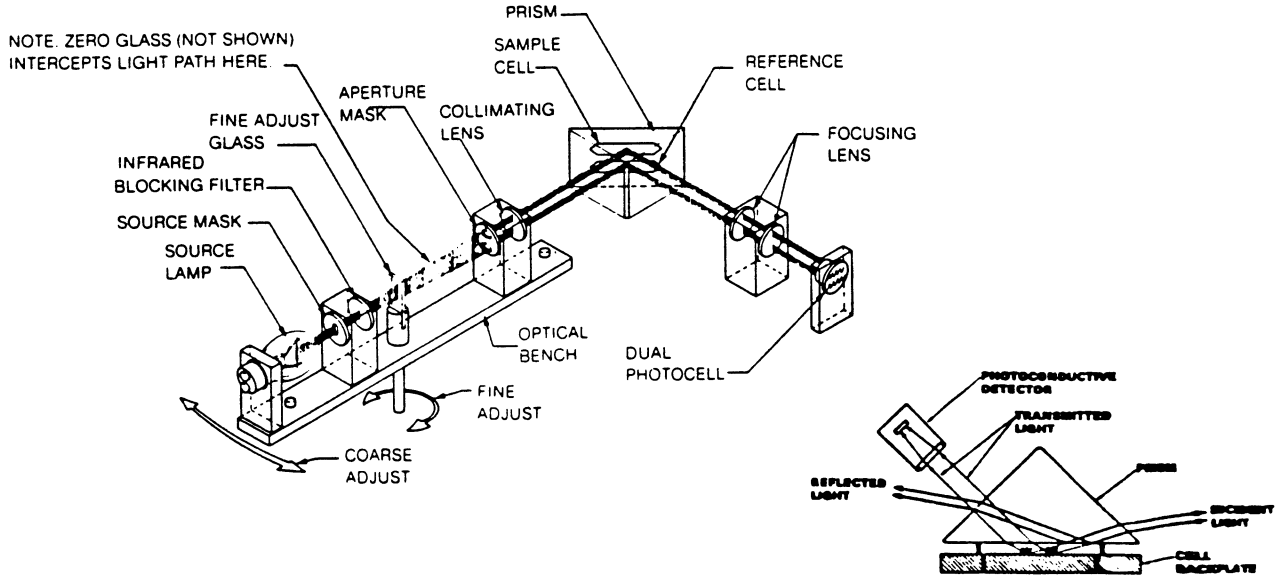


FIGURE 10

The refractive index detector is a non-destructive detector of moderate sensitivity ($\sim 10^{-6}$g minimum) and is commonly used for preparative work. The detector is incompatible with gradients because of the change in the refractive index of solvent during the gradient.

The refractive index detector is the most common detector used in size exclusion chromatography. Figure 11 shows the size exclusion separation of a complex hot melt adhesive HM-1. In the figure, the refractive index detector responds in both the positive and negative direction compared to the baseline, going positive when the solute has a higher RI than the solvent and negative when it has a lower RI than the solvent.

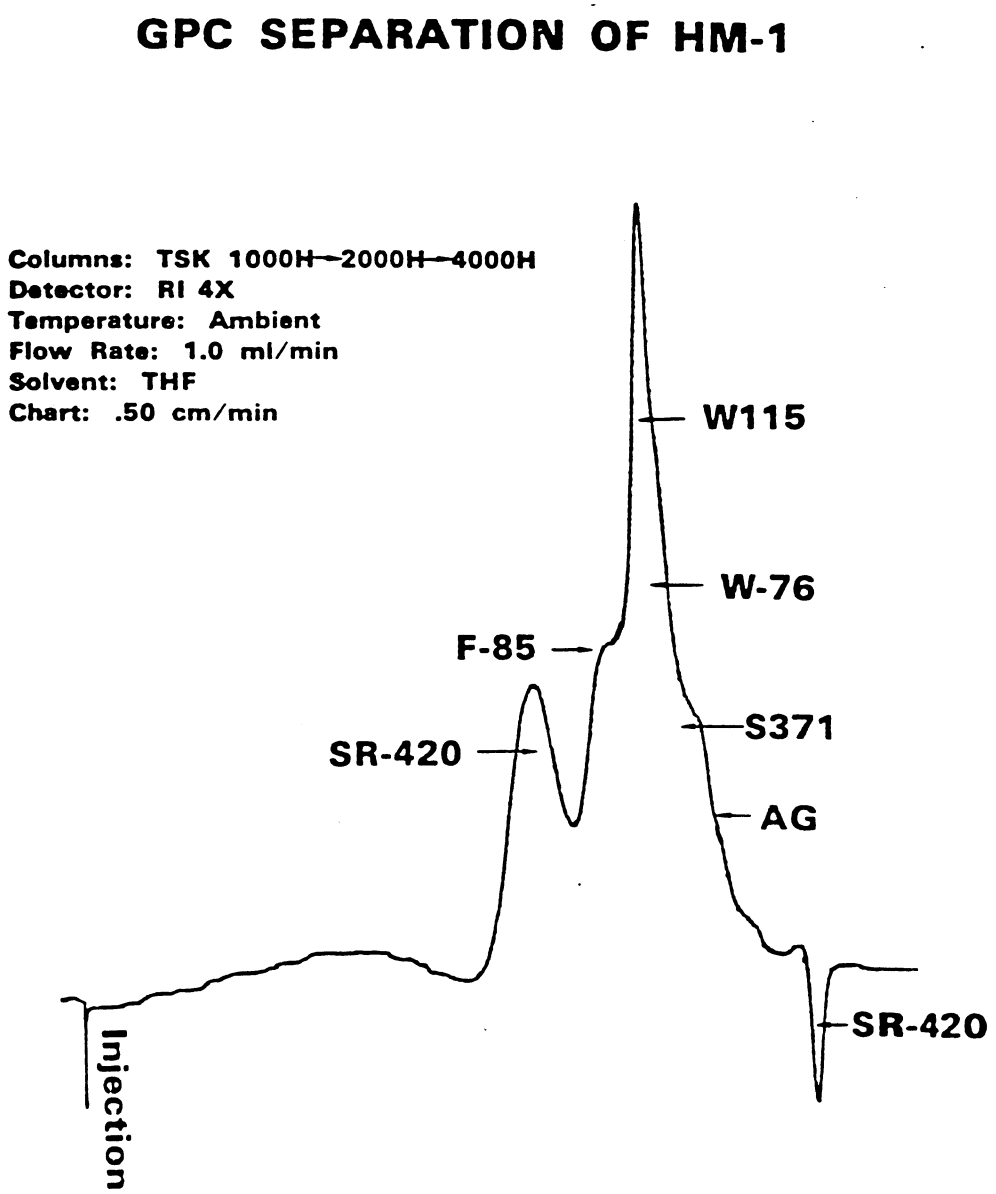


FIGURE 11

Carbohydrates have very low absorption in the ultraviolet range and are commonly detected using an RI monitor. Shown in Figure 12 is the separation of sugars found in the pentane extract of a hot dog.

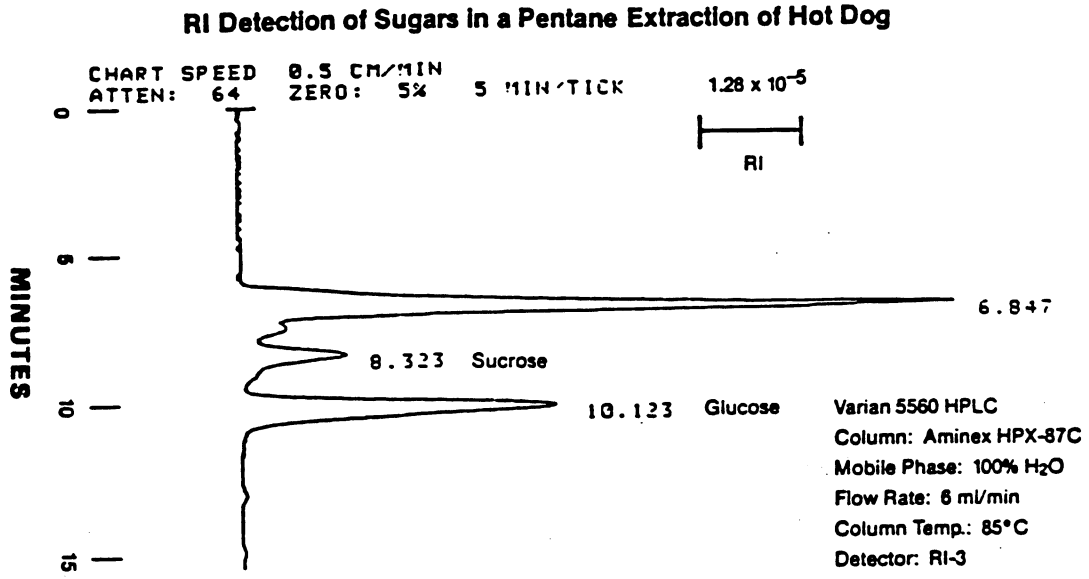


FIGURE 12

DO'S AND DONT'S OF RI DETECTORS

1. **NEVER** place a back pressure regulator on an RI detector.

2. **ALWAYS** pre-mix and **then** degas solvents.

3. After turning on your RI detector, let it warm up **AT LEAST** 8 hours before doing any chromatography.

4. Use a large ID small piece of Teflon tubing as a waste line.

5. When placing two detectors in series, **ALWAYS** place the RI detector last.

6. Keep your RI under the temperature-controlled conditions:

   A. Under conditions of constant temperature, the RI can be run dry or without circulating water bath.

   B. If not, use a water bath that keeps the temperature $\pm$ .5°C.

   C. For a **very** constant water bath, use tap water that comes from pipes that run **underground**. Hook up some tubing and turn on the water so a slow flow of water is passing around the cells.

7. Keep the RI cells clean - every manual will list a method of cleaning the cells.

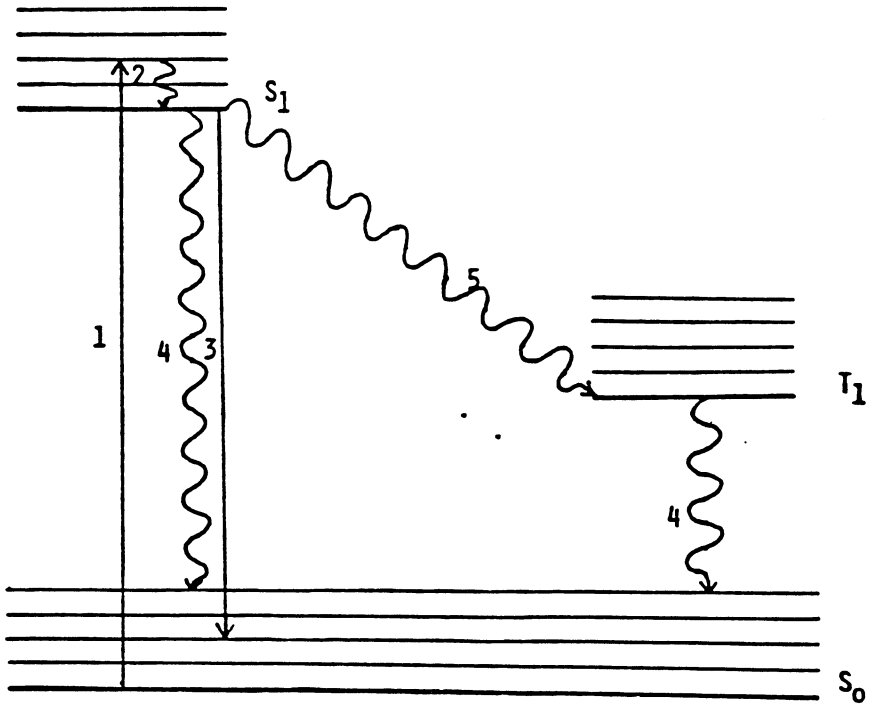
8. When filling the reference cell, **ALWAYS** use solvent that has eluted from the column. Allow solvent to flow through the column for 10 minutes, then fill the reference cell. **DO NOT** fill reference cells with solvent that **has not** passed through the column.

9. Make sure the cells appear to be the same color. If not, repeat the above procedure 2-3 times. If cells still are not the same color, clean the cells.

The Fluorescence Detector

The Fluorescence detector is based on the physical principle that some molecules have the capability of absorbing energy at a shorter wavelength to form an excited state and then drop back down to the ground level (essentially instantaneously) with the emission of that energy at a longer wavelength.

FLUORESCENCE PROCESSES



S_0 ground singlet state
S_1 first excited singlet state
T_1 triplet state
1 absorption
2 vibrational relaxation (non-radiative)
3 fluorescence
4 quenching
5 intersystem crossing

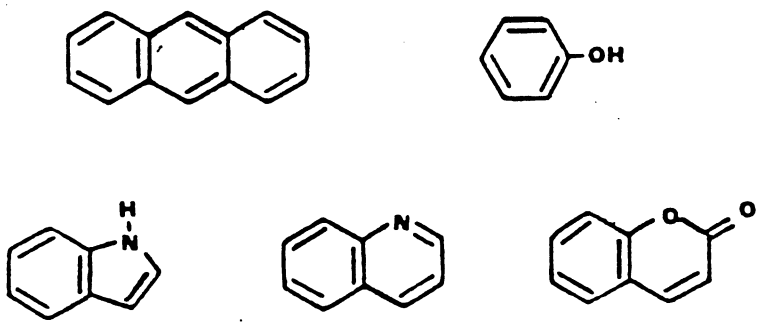
FIGURE 13

The major advantages of fluorescence detectors are their selectivity and sensitivity. The sensitivity of fluorescence detectors arises from the fact that the fluorescence produces a change in detected light level near zero background light. The selectivity is due to the selection of two wavelengths to perform fluorescence. The exciting wavelength is where the solute absorbs light and the emitting wavelength is the wavelength of the fluorescence radiation. Although many compounds may absorb energy at a given exciting wavelength, only a select few will emit radiation at the selected emission wavelength and, thus, interfering peaks will not be observed. The fluorescence detector does not respond to refractive index, can be used with strongly absorbing solvents, and is compatible with gradient elution.

The types of compounds which fluoresce include:

1. Native fluorescence compounds: These mostly occur if a compound possesses a conjugated cyclic structure such as a benzene ring or multiple rings, a good example being polynuclear aromatic hydrocarbons. The prediction of fluorescence can be difficult since it depends upon pH, solvent and substitution, etc. Electron donating functional groups such as NH2, NHR, and -OH on the aromatic ring tend to increase fluorescence. Shown below are the types of compounds which are fluorescent.

NATIVE FLUORESCENCE



EFFECTS OF SUBSTITUENTS ON FLUORESCENCE OF AROMATICS

Increase Intensity: -R, -OH, $-OCH_3$, $-OC_2H_5$, -CN, $-NH_2$, -NHR, $-NR_2$

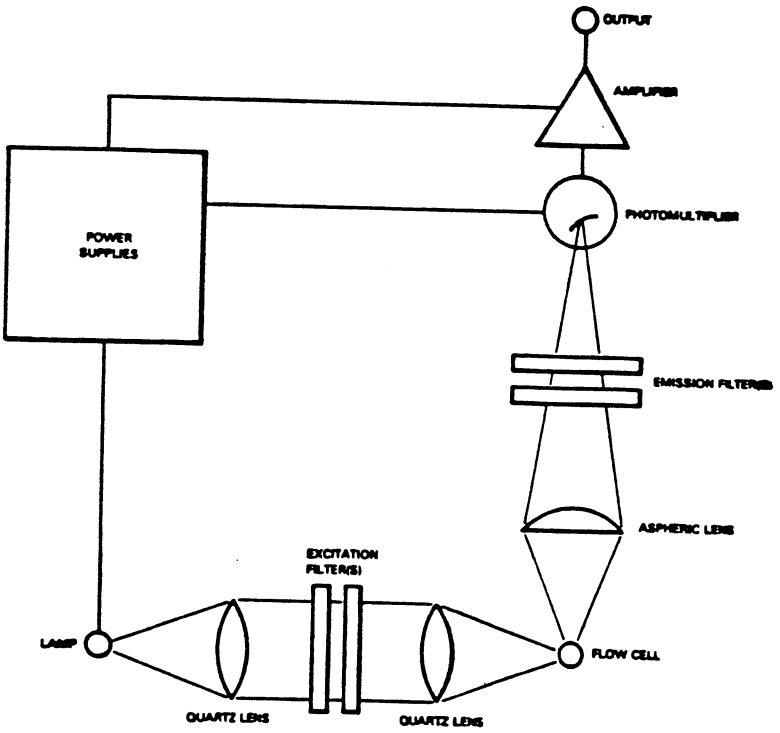
Decrease Intensity: $-CO_2H$, $-NO_2$, -NO, -SH, -F, -Cl, -Br, -I

FIGURE 14

2. Derivatized Fluorescence compounds: Compounds which do not have any native fluorescence can be derivatized, especially amine functionalities. The most popular fluorescence reagents are dansyl chloride, fluorescamine and o-phthalaldehyde.

There are two types of fluorescence detectors, the filter types and the monochromator type or spectrofluorimeter. The spectrofluorimeter will be more selective since the excitation and/or emission wavelength may be selected with narrow bandwidths. But there is a loss in sensitivity due to the monochromator and filter system and, in some cases, lower detection limits.

The filter units provide good wavelength selection, but in a somewhat less convenient manner. However, the use of filters in both excitation and emission offer sensitivity advantages, since filters generally transmit more light. Selectivity is reduced because of the wider bandwidths of typical filters. A filter fluorimeter costs half the price of monochrometer units. They are excellent for known fluorescence conditions and applications.



FLUORICHROM OPTICAL SCHEMATIC

FIGURE 15

Filters Used In Filter Fluorimeters

There are three types:

1) Glass Band Filters -
   Glass and filters have a broad transmission band. Band filters are commonly used for far UV excitation.

2) Glass Cut-Off Filters -
   These filters cut off all radiation below a given wavelength and pass up to 85% of the radiation above that wavelength (see Figure 16). They are used to collect fluoresence emission.

3) Interference Filters -
   Interference filters have relatively narrow transmission bands. These filters have relatively little leakage outside the bandpass, especially in the red. They are thus excellent for use as excitation and emission filters. See Figure 16.

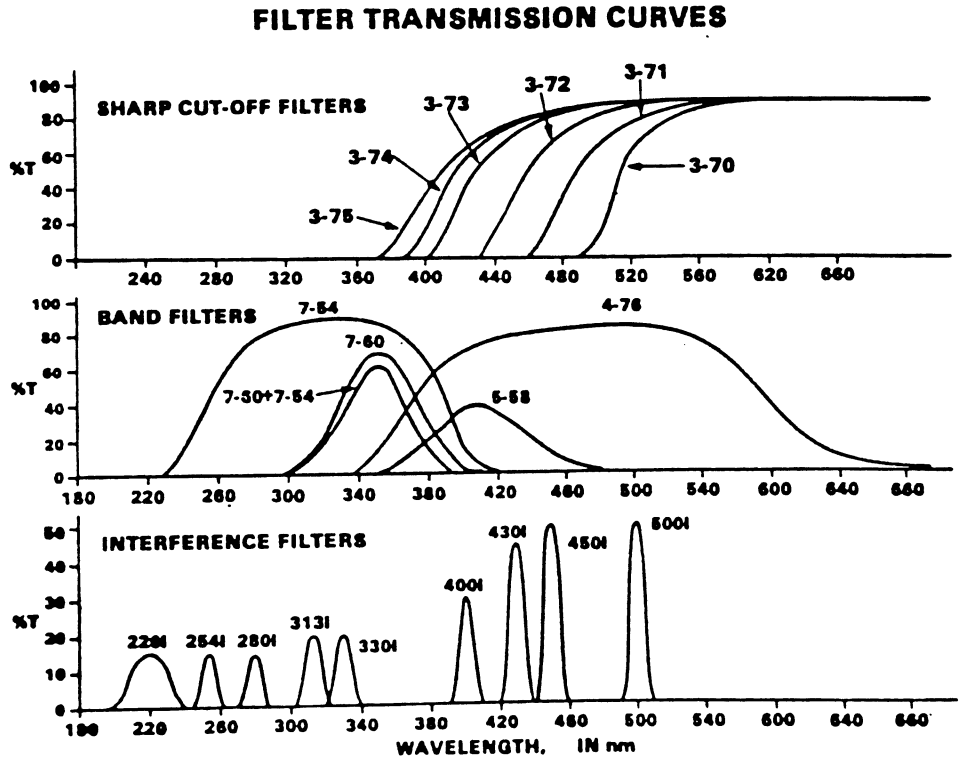


FIGURE 16

Choosing Fluorescent Experimental Conditions

To choose the excitation wavelength, you first have to choose a light source. There are three major types of excitation light sources, the Tungsten, Deuterium and Xenon. Shown below is the source intensity in the ultraviolet and visible range. Tungsten is good for excitation above 350 nm. Xenon is good for excitation above 300 nm. The second consideration is that compounds have more than one band of excitation. Shown in Figure 18A is the excitation spectrum of tryptophan. It is easy to see that the molar excitation is higher in the 225 nm range so a deuterium lamp source may be chosen, but one has to also consider the source intensity, because the Xenon light source may be better even though it is at a longer wavelength and has a lower molar extinction co-efficient, but a higher lamp intensity. Based on the excitation spectrum we could then choose an appropriate lamp and filter or excitation monochromator setting.

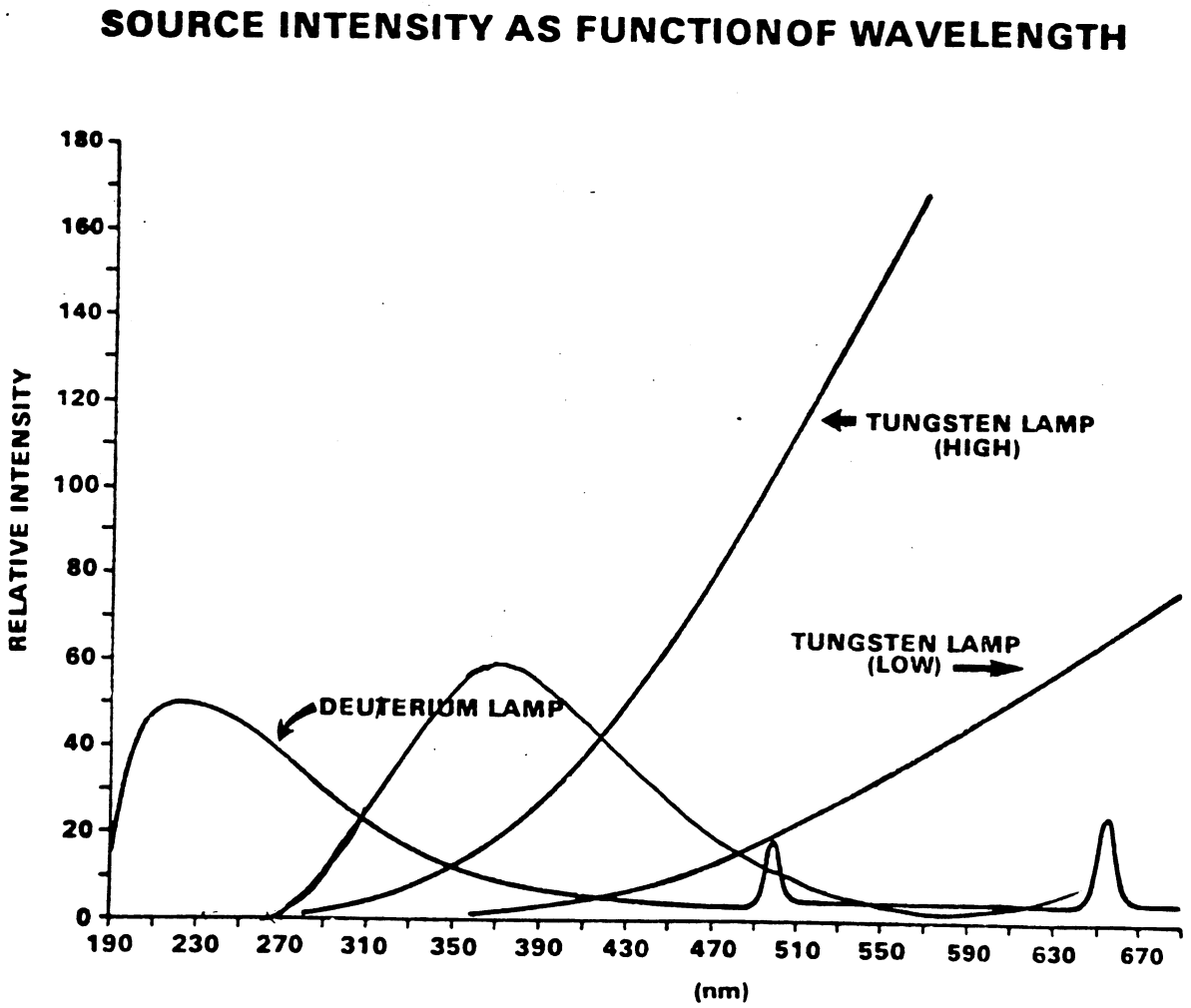
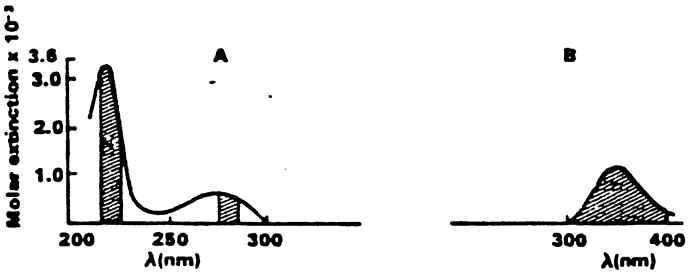


FIGURE 17



Fluorescent Excitation spectrum (A) and Emission Spectrum (B) of Tryptophan in aqueous solution. Shaded areas in Excitation spectrum show possible filter choices 220I or 280I. The entire emission spectrum (B) can be observed using a 7-60 filter. Note that a 330I could have been used as an emission filter but with diminished collection of the broad emission band.

FIGURE 18

We choose an emission filter or monochromator setting that collects fluorescence in the region relatively free of scattering from the emission spectrum. For example, we would choose a filter or monochromator setting of 350 nm, the maximum emission of tryptophan as seen in Figure 18B. We would have to use a trial and error method if we were using a filter fluorometer and did not have a fluorescence spectrum. For example, beginning at 50-100 nm (toward longer wavelengths) from the tail of the absorption curve various filter combinations should be tried. One should always check the background fluorescence and then the fluorescence of solute and solvent together to find the emission conditions.

COLUMN SELECTION

The selection of the separation mode and the correct column packing is the most critical step in the development of an analytical method. The selection of the mode and separation conditions can be carried out using some basic guidelines.

GUIDE TO LC MODE AND COLUMN SELECTION

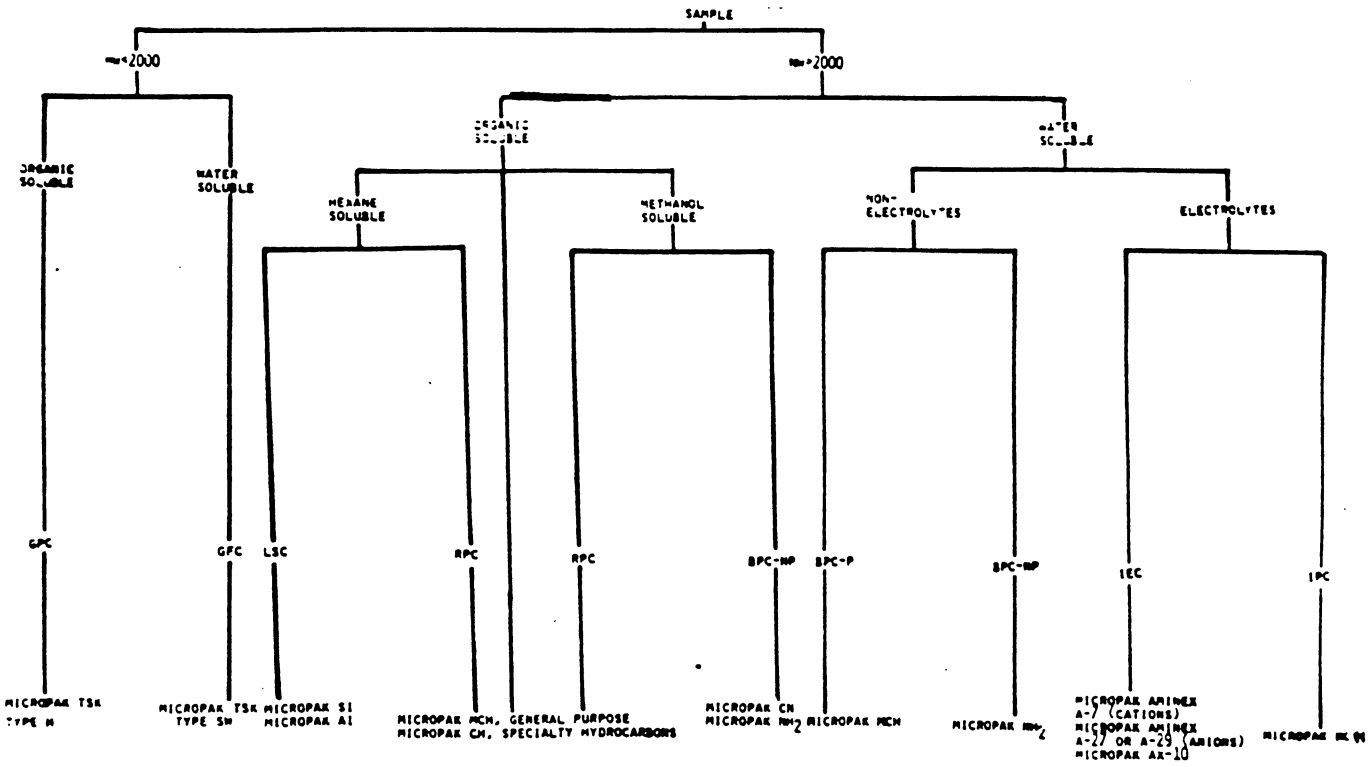


FIGURE 1

Selection of the LC Mode

1. Classify the sample according to physical properties which may include, (follow Figure 1 in the column selection guide).

   a. **Molecular Weight:**
   If the sample is greater than 2000 M.W. then you would go to a size exclusion technique. If it is less you would go to a partition technique (see Figure 1).

   b. **Solubility:**
   If the sample is greater than 2000 M.W. and if it is soluble in organic solvent then you would do gel permeation chromatography (GPC) but if it is water soluble use gel filtration chromatography (GFC). If the sample is less than 2000 M.W. then you will do partition chromatography and the type will be governed by the solubility. If the sample is organic soluble and also water soluble then go to Reverse Phase (RP) mode. If it is hexane soluble then you will have to do adsorption chromatography on silica gel. If the sample is insoluble in hexane and water but soluble in methanol then you can do normal phase chromatography (NP).

c. **Ionic Character**
If the sample is less than 2000 M.W. and water soluble the sample is considered an electrolyte and will have to be separated by ion exchange chromatography (IEC) or ion pairing chromatography.

We will discuss each one of these modes in detail in this section. In order that you do not re-invent the wheel, a method can often be found in a chromatography journal (list of chromatography journals and indexes found in Appendix 1). Molecular weight, solubility and structure can be found in reference manuals such as the Merck Index.

Selection of the Type of Column Packing:
You have to choose a column according to the resolution, efficiency, and capacity required for a specific type sample. For example, for a simple quality control sample containing two components you would need a much lower efficiency than a complex sample found in trace quantities containing 20 or 30 very similar components. There are a number of different types of packing materials used in liquid chromatography (see Figure 2).

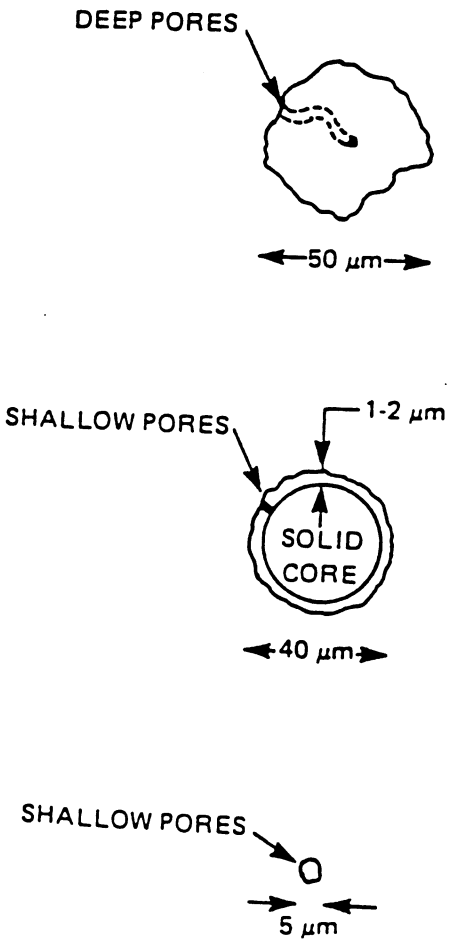


FIGURE 2

1. **Macro Porous:**
Usually on the order of 60 μm in diameter. Used mainly in preparative chromatography because they have high capacity, are inexpensive and easy to pack but have very low efficiency.

2. **Pellicular Packings:**
These consist of a glass bead coated with a thin layer of stationary phase. They are very efficient and easily packed (these are used in the universal guard columns) but have low sample capacity and are very expensive.

3. **Microparticle Packings:**
The most common types of packing used in modern chromatography come in 3, 5, or 10μ particle sizes. The smaller the diameter the higher the efficiency. The 10 μ has adequate resolution for most analytical separations while the 5μ is recommended for more demanding separations. The 5 μ has 2-3 times the number of theoretical plates compared to 10 μ but the back pressure rises about 2 to 3 times. Recently 3 μ columns have been introduced in short lengths for very fast high resolution separations. In choosing a particle size one should consider how clean his sample is because as the particle size decreases the capacity of a column for contamination and plugging increases. Also, because of the high pressure from the smaller particles, the flow rate must often be lower. Figure 3 shows a comparison of different particle size and its effect on efficiency.

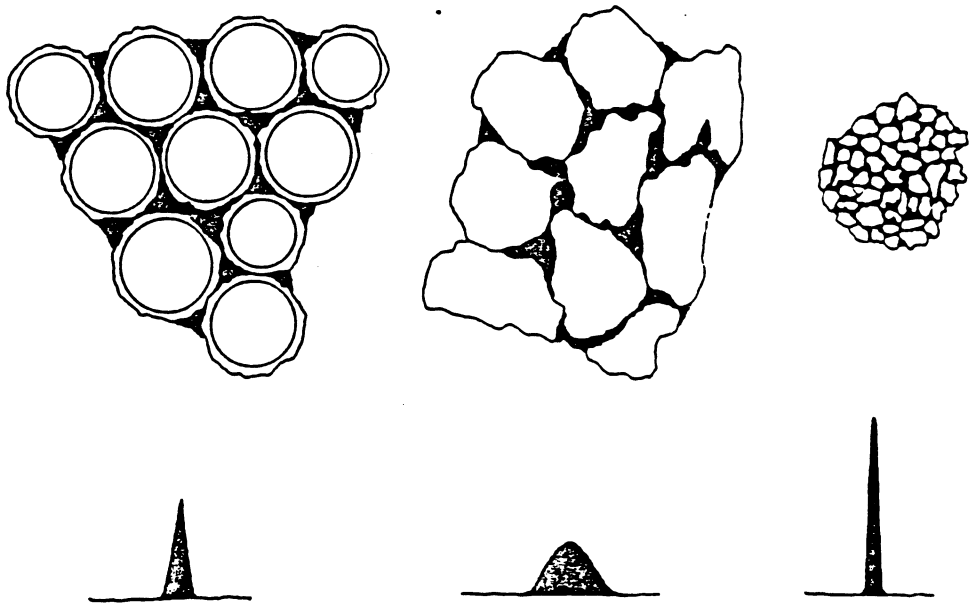
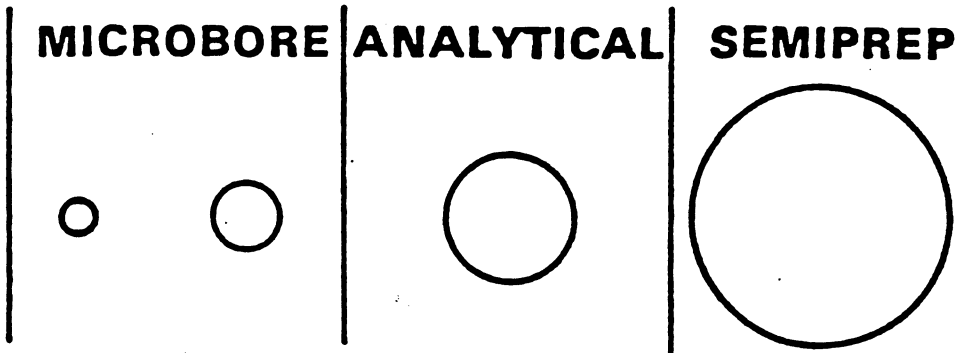


FIGURE 3

The Effect of Particle Size on Column Efficiency:
As the particle size decreases, the efficiency increases allowing more components to be separated per unit time.



COL. DIAM	1	2	4	8 mm
FLOW RATE	50	250	1,000	4,000 µl/min
SOLVENT USE/DAY	31	125	500	2,000 ml

FIGURE 4

Types of Chromatography

Selection of Column Dimensions:
Column diameters of prepacked columns range from 1 mm (special instrument requirements are necessary) to 8 mm i.d. The 4 mm is a general purpose diameter useful in general analytical and small scale preparative work (few mg). The capacity of the column increases as the diameter increases because the amount of stationary phase increases. As the column diameter decreases, capacity decreases, but smaller amounts of solvents can be used. As pointed out in the theory lecture, as the column length increases, the resolution increases. Usually, the separation is carried out on a shorter column; if a longer one is needed, a second one is put in series. The figure shows the different column types. The 1 and 2 mm are considered microbore columns for high resolution, low loading separations. They use much less solvent but require more clean up and special instrumental design to overcome extra-column bandbroadening effects but give the highest efficiencies. The 4 mm is a standard column but has been produced in short length and with small particles for a technique called fast LC. A standard column is between 15 and 30 cm. The 15 cm length is recommended for simpler separations requiring only a few thousand theoretical plates. It uses less solvent and allows for faster separations, but if more theoretical plates are required a 30 cm length could be used.

The 8 mm column is called a semi-preparative column and is used to separate milligram quantities of sample. As the diameter of the column decreases the amount of component per volume of solvent increases dramatically. The big advantage of going to smaller diameter columns is the about -20 time increase in detector response with 1 mm vs. 4.6 mm I.D. of column and, of course, less solvent used. Figure 5 shows the separation of polynuclear aromatics on 1 mm, 2mm and 4.6 mm columns showing the increase in response.

Once the column has been chosen we have to choose a mobile phase, mobile phase composition, flow rate, sample concentration and volume, etc. The next sections will pertain to making the best selection. It should be noted that some samples can be done by more than one mode of chromatography.

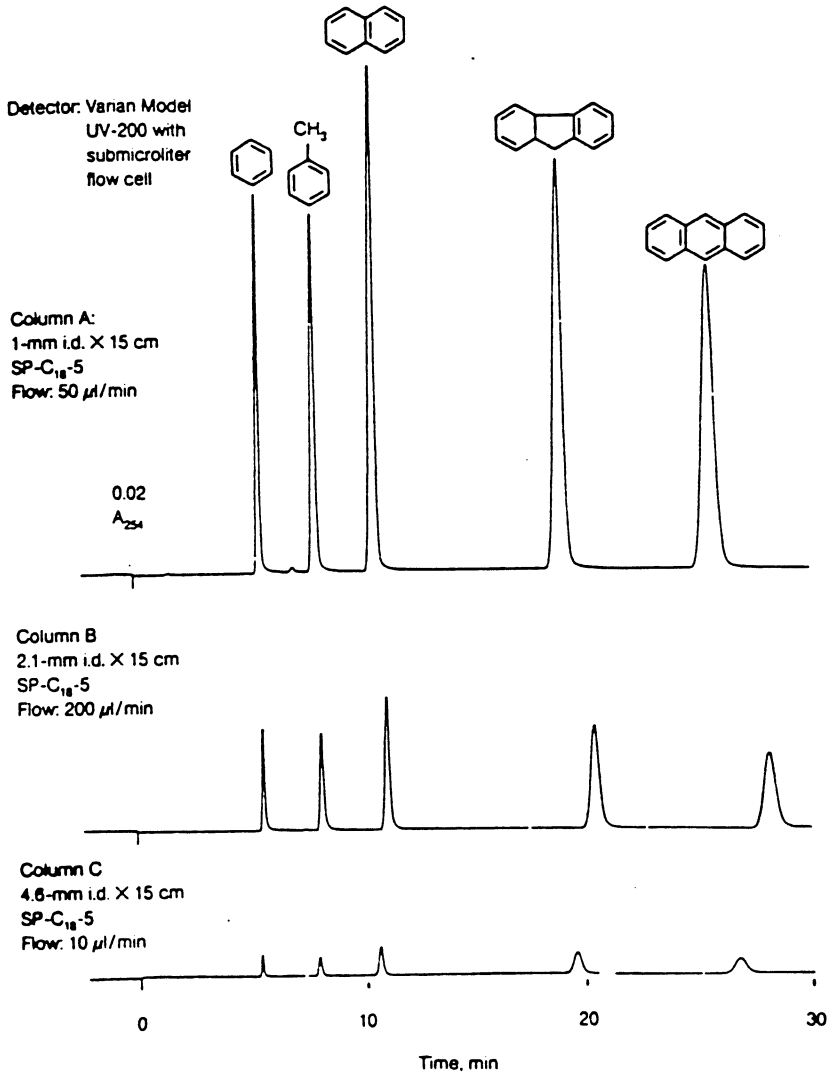


FIGURE 5

Figure 5. Detectability differences of Microbore and conventional LC columns with a UV detector. Mobile phase: 55% acetonitrile in water. Injection of 1-µl containing 4 µg benzene, 4 µg toluene, 0.5 µg naphthalene, 0.2 µg fluorene, 0.05 µg anthracene.

ADSORPTION CHROMATOGRAPHY

Liquid-solid chromatography, or absorption chromatography, was the first mode of HPLC to develop. It represented a transfer of technology from thin layer chromatography (with some modification in mobile phases) to packed columns and HPLC pumping systems. Today separations in this mode are primarily done on silica rather than alumina columns. LSC has been applied to numerous class separations of non-ionic organic compounds over the years. Many separations have been achieved on silica which are not possible on other modes.

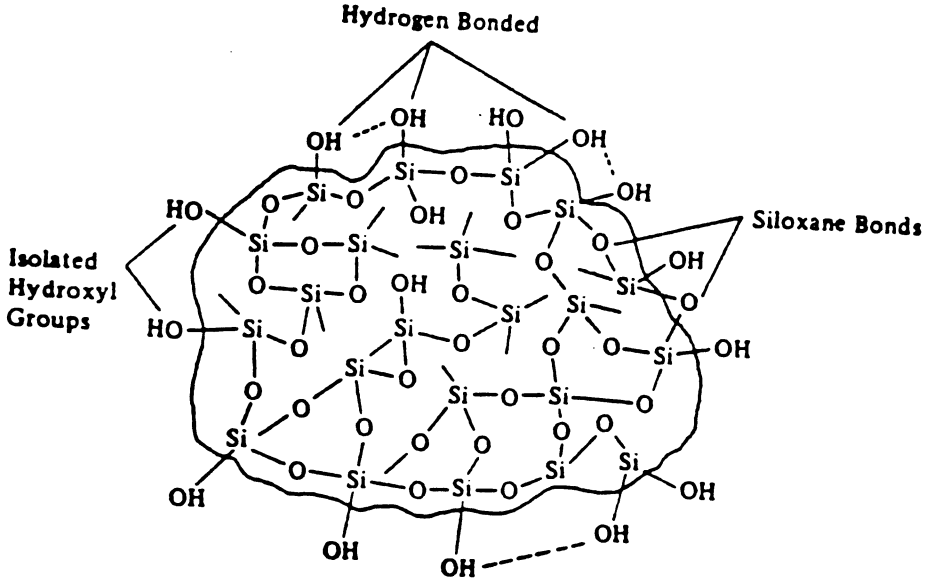


FIGURE 1

The structure of silica gel showing surface silanol groups.

The adsorption mechanism of silica gel involves polar compounds hydrogen bonding to active silanol groups on the surface of the silica. This polar-polar interaction is controlled using non-polar solvents such as hexane or heptane (weak solvents) and moderately strong solvents such as methylene chloride and THF. Strong modifiers such as small amounts of IPA, methanol and acetic acid are added to change column selectivity. The sample interaction is controlled by sample polarity and stereochemistry of the compound. The stronger the polarity of the compound the stronger the solvent needed to elute it.

A polarity scale has been set up for solvents called the "Eluotropic Series", which is a measure of relative polarity between the mobile phase and the sample relative to the retention of alumina (Figure 2). The more polar solvents such as alcohols have the highest value and non-polar solvents have the lowest value. Therefore, to elute a very polar compound would take a very polar solvent.

Solvent	ϵ°
n-Pentane	0.00
Isooctane	0.01
Cyclohexane	0.04
Carbon Tetrachloride	0.18
Xylene	0.26
Toluene	0.29
Benzene	0.32
Ethyl Ether	0.38
Chloroform	0.40
Methylene Chrloride	0.42
Tetra Hydrofuran	0.45
Acetone	0.56
Ethyl Acetate	0.58
Aniline	0.62
Acetonitrile	0.65
i-Propanol	0.82
Ethanol	0.88
Methanol	0.95
Acetic Acid	large

FIGURE 2

Eluotropic Series for Alumina (Silica has a similar rank order)

An example of the effect of solvent strength is shown in the retention of toluene 2,4-diisocyanate on silica gel. In this case hexane is the weak solvent and methylene chloride is the strong solvent. In Figure 3 we see at 5% $CH_2C\ell_2$ in Hexane we have a retention time of 2 minutes. If we increase the solvent strength we decrease retention to 1 minute as seen in the second chromatogram, where we have 50% $CH_2C\ell_2$ in Hexane. We can therefore increase or decrease the retention time of Toluene diisocyanate by the percentage of $CH_2C\ell_2$ in the mobile phase.

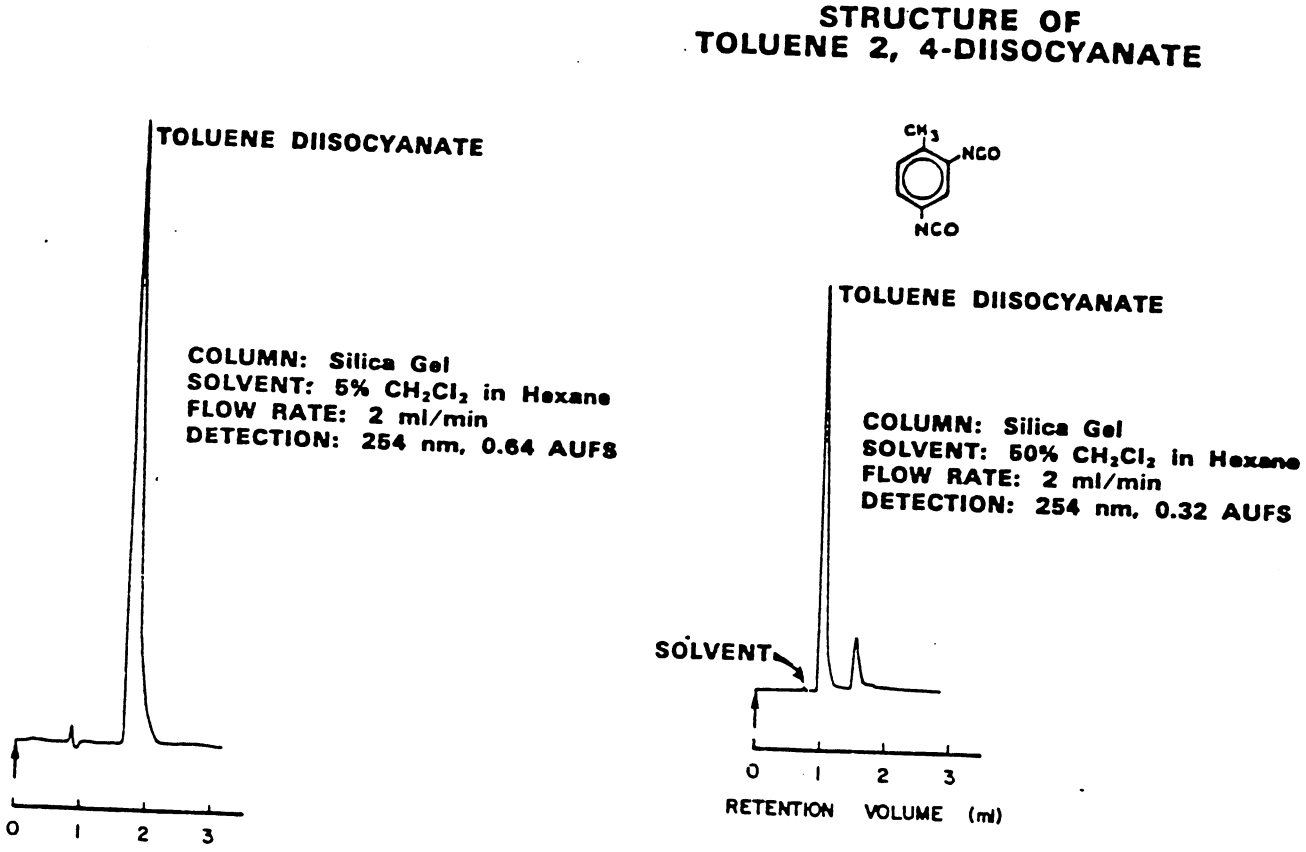
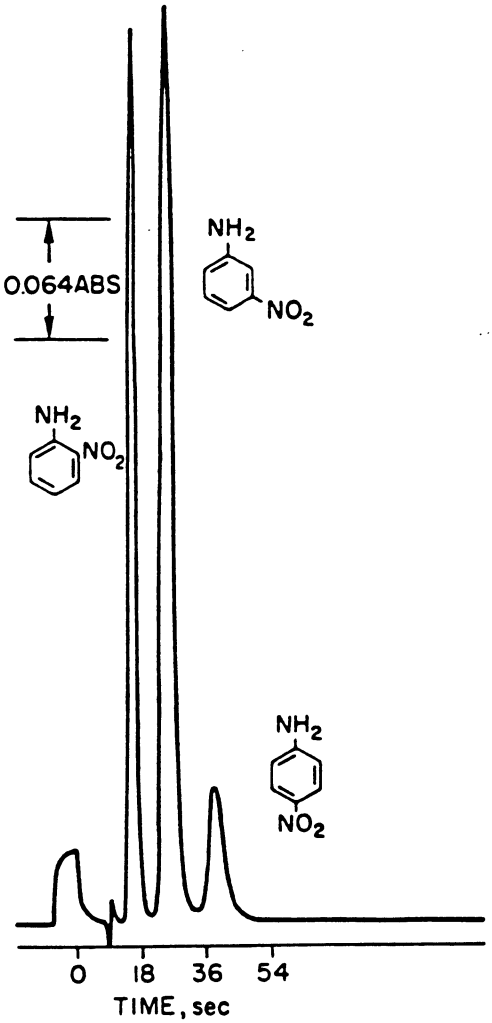


FIGURE 3

The effect of increasing solvent strength on the retention of toluene 2,4-Diisocyanate on Silica Gel

Stereochemistry is very important to the interaction of compounds with silica gel. Figure 3 shows how hydroquinone (para form) interacts at two sites and catechol (ortho form) interacts at one site onto silica packing material. Based on the number of sites of interaction we would expect the catechol to elute first followed by the hydroquinone, which has a higher affinity for the packing material.

An example of where stereochemistry plays a major role in the separation mechanism is in the separation of Nitroaniline Isomers on Alumina (no longer used). The elution orders the ortho, meta and then para isomers due to sites of interaction.

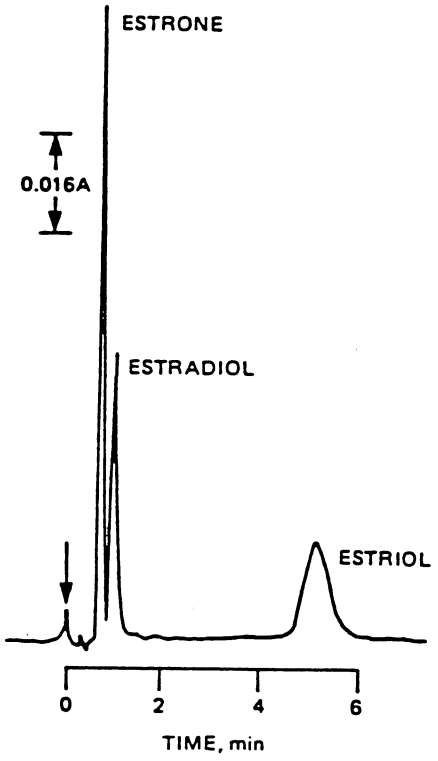


LSC Separation of Nitroaniline Isomers on 10 μm alumina. Column: 10 μm LiChrosorb Alox T. Dimensions: 15 cm x 2.4 mm. Mobile phase: 40% CH_2Cl_2 in hexane. Flow rate: 100 ml h^{-1}. Sample size: 1 μl. Sample concentration: 1 mg ml^{-1} in methylene chloride. Detector: UV. (Reprinted with permission from R. E. Majors, Anal. Chem. **45**. *755 (1973). Copyright by the American Chemical Society.)*

FIGURE 4

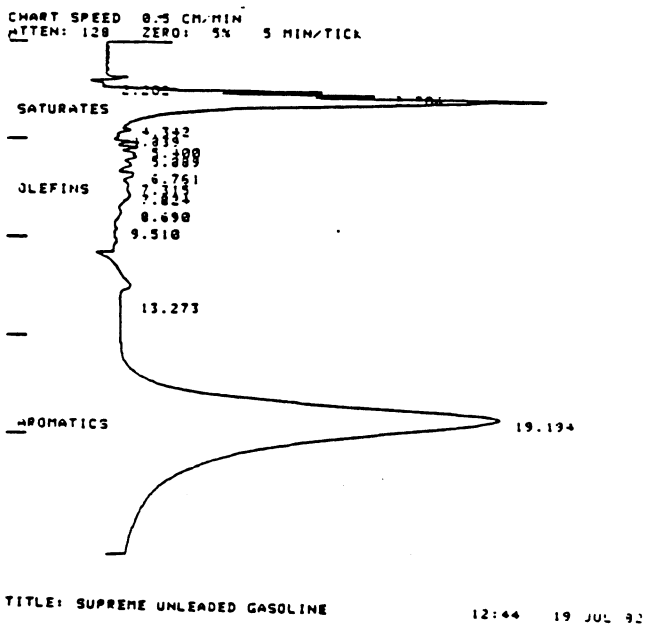
Adsorption is the preferred method for many water sensitive compounds, isomers (both geometrical and stereo), and multi-functional compounds with a similar carbon skeleton (i.e. aromatic isomers), and class separations (e.g. non-polars from polars).

Shown below (Figure 5) is the separation of estrogen steroids. The elution order is in order of polarity: estrone having one hydroxyl, estradiol having two hydroxyls, and estriol having three hydroxyls, and the longest retention time. It should also be noted that 5% isopropanol is added into the mobile phase as modifier to change selectivity.



Separation of Estrogen Steroids. Column: MicroPak® Si-10 (Silica). Dimensions: 25 cm x 2.2 mm. Mobile phase: 5% isopropanol, 5% methylene chloride, 90% hexane. Flow rate. 123 ml h^{-1}. Sample Size: 4 µg each. Detector: UV 254 nm.

FIGURE 5



Column:	MicroPak SI-5 4mm x 30 cm
Column Activation:	130°C for 30 minutes
Solvent:	FC-72
Flow Rate:	2ml/min
Sample:	Supreme unleaded gasoline
Detector:	RI-3, with ultra low index prism
Loading:	5 µl neat
Backflush Time:	10.5 minutes

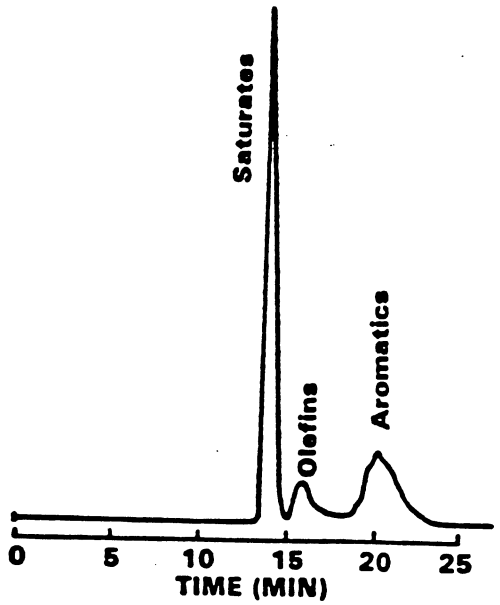
FIGURE 6

Figure 6 shows the separation of hydrocarbons in gasoline using a fluorocarbon mobile phase on activated silica. Activated silica is produced by removing water from the column by drying with dry nitrogen in a GC oven and using a dryer column in the LC to assure that the solvent is dry. Water in the mobile phase causes loss of retention. The amount of water can vary causing poor reproducibility.

Figure 7 shows the separation of a gasoline sample; note there is AgNO3 in the mobile phase. This modifier allows better class separation on silica. This type of chromatography is termed argentation chromatography and is also done in the reverse phase mode for the separation of Olefins.

HPLC ANALYSIS OF GASOLINE SAMPLES

SAMPLE: Gasoline
COLUMN: Silica Gel Column (7.5 mm x 50 cm) + Silica Gel Coated with $AgNO_3$ (7.5 mm x 4 cm)
MOBILE PHASE: CCl_4
DETECTOR: Infrared (6.9 μm — 1440 cm^{-1})

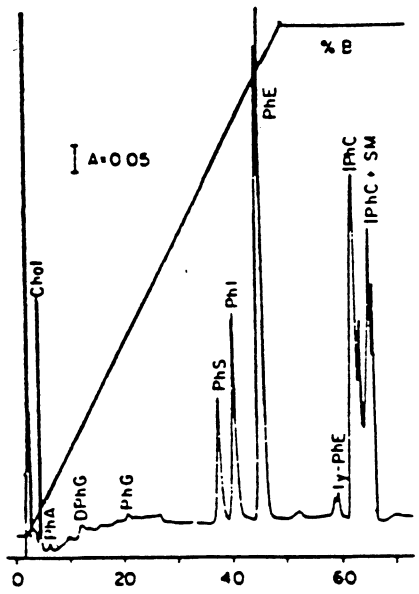


Reference: *J. Chrom.* 208 (1981) 429-432

FIGURE 7

In petrochemical separations, silica gel is the only packing giving enough class separation, as shown in the above examples.

Phospholipids, which are important membrane components, are separated very well on Silica gel using isopropanol, methanol and acetonitrile. This is an excellent example of a multi-functional component class separation. (See Figure 8, next page).



SEPARATION OF LIPID EXTRACT FROM RAT LIVER ON MICROPAK SI-5

Source: Yendrzejtz, J. R., Berry, G., and Segal, S., *J. Chromatogr.*, *225*, 319 (1981).

FIGURE 8

Today silica has found its place mainly in specialized class and isomer separations because it has the following deficiencies as compared to other packings:

1. If silanol activity is too strong, peak tailing and even irreversible adsorption is possible.

2. Irreversible adsorption can yield column deactivation or plugging.

3. Silica activity is usually controlled or modified with fixed water or alcohol content. However, the concentration may be carefully controlled using drying techniques and dryer columns. Deactivation will cause decreased retention times, therefore, it is important to dry sample prior to injection.

4. The slightly acidic functions on the silica may behave as catalytic sites and adversely affect the solute.

5. Gradient elution is difficult because silica gel responds slowly to some changes in mobile phase composition.

Silica gel has some unique selectivity for isomers and class separation but is not usually practical for most separations. Because of the above problems with adsorption chromatography, bonded phases are used for a majority of separations.

BONDED PHASE CHROMATOGRAPHY

Early in the development of HPLC, silica gel support materials were coated with organic liquids similar to GC liquid phases (e.g. Carbowaxes, DEGS, OV series) to change their partitioning properties. The sample partitioned between the two phases (immiscible solvents): the mobile phase and the coated stationary phase; very much like a solvent-solvent extraction. In practice the mobile phase was saturated with the stationary phase which made it impossible to do gradient elution and the temperature had to be controlled. This technique led to bonded phase packing materials.

These bonded phase packing material supports are prepared by the reaction of microparticulate silica gel, which possesses reactive silanol groups, with organochloro or organoalkoxy-silanes (Figure 1). The R group, which may be alkyl or aryl, with or without other functional groups such as -CN, will determine the final physical properties. Although the term BPC is applied to Normal bonded phases (primarily -NH2 or -CN) and reverse phases (C-18 packings), other packings such as silica based ion exchangers (e.g. AX-5, AX-300) and size exclusion packings (e.g. TSK SW series) are synthesized by a similar procedure. Silica gel as a base material has the following physical properties:

a. Small particles (3, 5, 10 μ)

b. High surface area

c. Controlled pore size (almost all supports are microporous silica)

d. Rigid with good mechanical strength for withstanding high pressures, which makes it excellent for bonded phase microparticle packing materials.

These bonded phase materials take advantage of silica's physical strengths while minimizing the disadvantages such as troublesome silanols. The silica based bonded phase columns are limited to the pH range of 2-7.5. Above pH 7.5 the silica dissolves and below pH 2 siloxane bond cleavage causes loss of bonded phase.

Typical Reactions for Making Bonded Phases

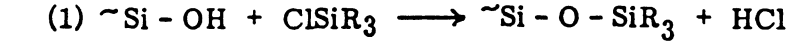


FIGURE 1

Functional groups that are bonded onto the silica include:

Reverse Phase:

$-C_{18}$, C_8, $-C_2$

Normal Phase:

$C_3H_6NH_2$,$-C_2H_4CN$

Ion Exchange:

$-SO_4^-$

$-NH_3+$

Gel Permeation:

- diols

Other types of bonded phases are those which are bonded onto polystyrene divinyl benzene. These types of phases are most common in size exclusion chromatography (e.g. TSK PW, H types) and ion exchange (e.g. MicroPak SAX-10). Recently columns have been developed for reverse phase chromatography which have polystyrene divinyl benzene backbones. This overcomes the pH limitations of RP silica base columns.

Normal phase chromatography is called "normal" because polar packings such as silica, were used exclusively until the invention of bonded non-polar packing (hydrophobic) or reverse phase. Normal bonded phases are polar groups such as cyano or amino and sometimes diol (see figure below).

NORMAL PHASE BP

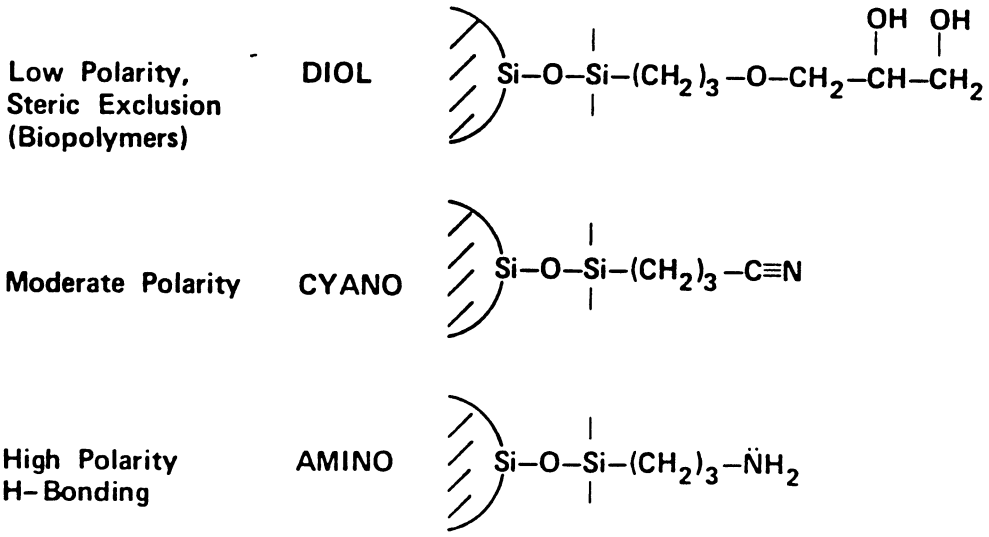
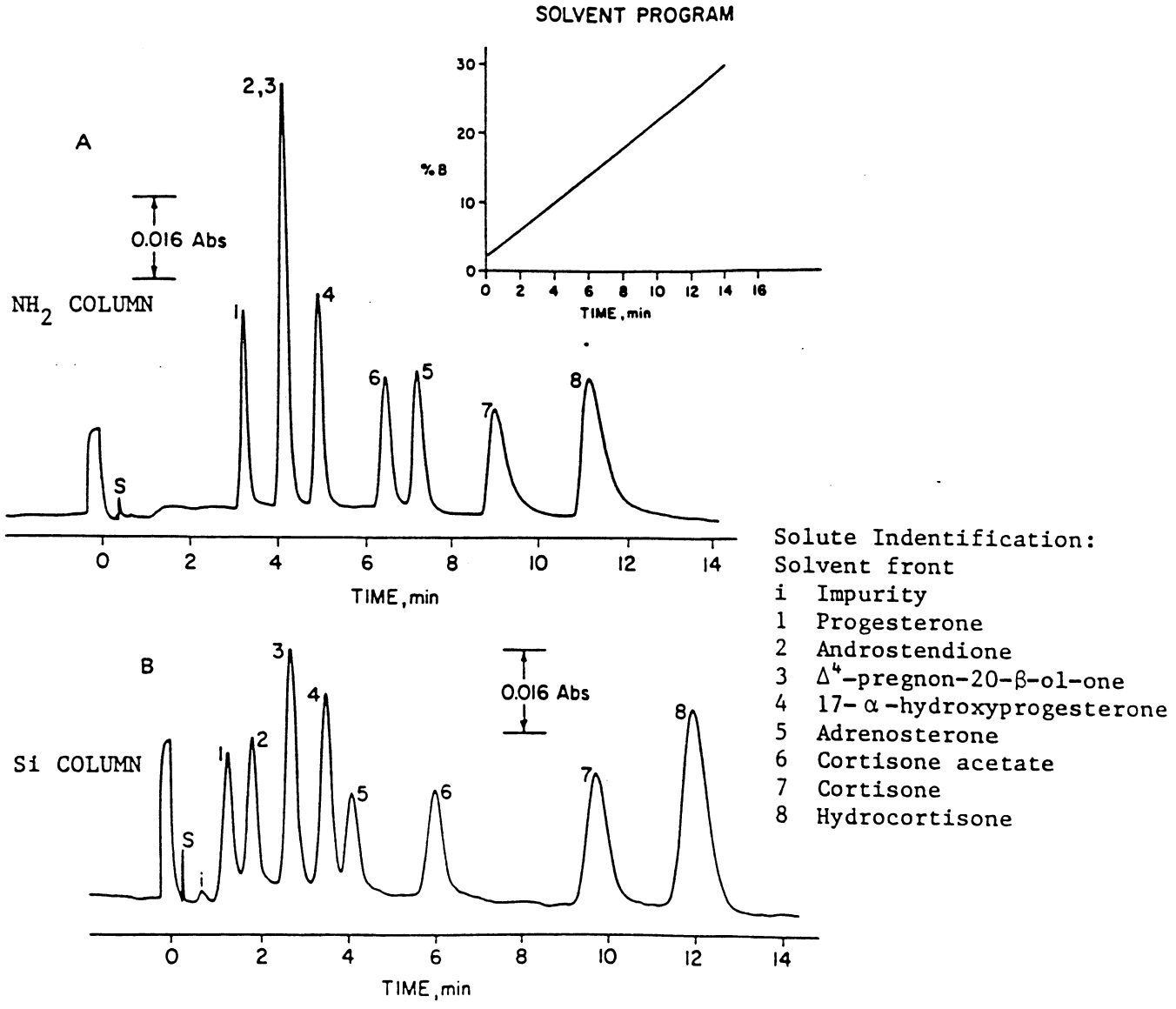


FIGURE 2

The basic mechanism of normal phase chromatography is the interaction of polar compounds with a polar packing material, controlled by an amount of non-polar and semi-polar solvents such as hexane and methylene chloride. Isopropanol and ether are used as modifiers in the mobile phase at concentrations of around 1-2%. The solute retention increases as its polarity increases and decreases as the polarity of the solvent increases. The mobile phase is usually less polar than the stationary phase.

The normal phase packing materials have similar selectivity to silica columns, as shown in Figure 3 for the separation of steroids on both the silica and amino propyl column. Elution order is not identical, but is similar with the silica having better selectivity in this case.

COMPARATIVE STEROID SEPARATION ON MICROPAK-NH_2 AND MICROPAK Si-10

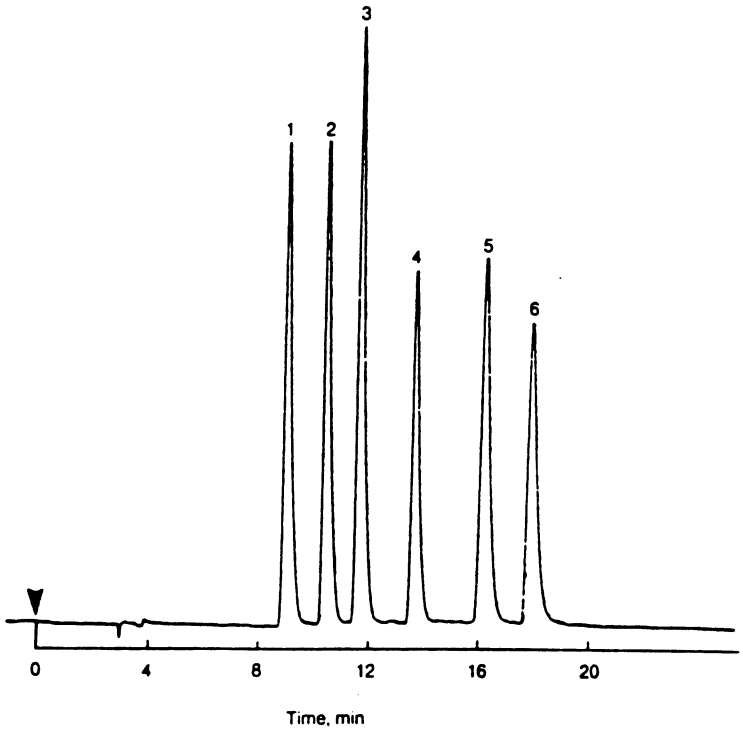


Solute Indentification:
Solvent front
i Impurity
1 Progesterone
2 Androstendione
3 Δ^4-pregnon-20-β-ol-one
4 17-α-hydroxyprogesterone
5 Adrenosterone
6 Cortisone acetate
7 Cortisone
8 Hydrocortisone

FIGURE 3

The weakly polar packings consist of diol, dimethylamino or nitro. The diol has shown promise for the separation of organic acids and certain polymeric oligomers but the biggest application is for aqueous size exclusion chromatography. The nitro functionality has shown selectivity for aromatics and the dimethylamino has been used as a weakly basic anion exchanger for the separation of weak acids and phenols. These packing materials are not very popular and are not as significant as the amino and cyano functionalities.

The cyanopropyl functionalities show moderate polarity and less retention when substituted for silica, but have similar selectivity. The advantage is more rapid regeneration following gradient elution or mobile phase composition changes. The cyano column shows fewer side reactions than the amino and less tailing with polar solutes due to elimination of acidic silanols during chemical bonding. The cyanopropyl has good selectivity for compounds having double bonds. Shown in Figure 4 is the separation of ketosteroids; an example of the separation of double bonded isomers on the cyanopropyl columns.

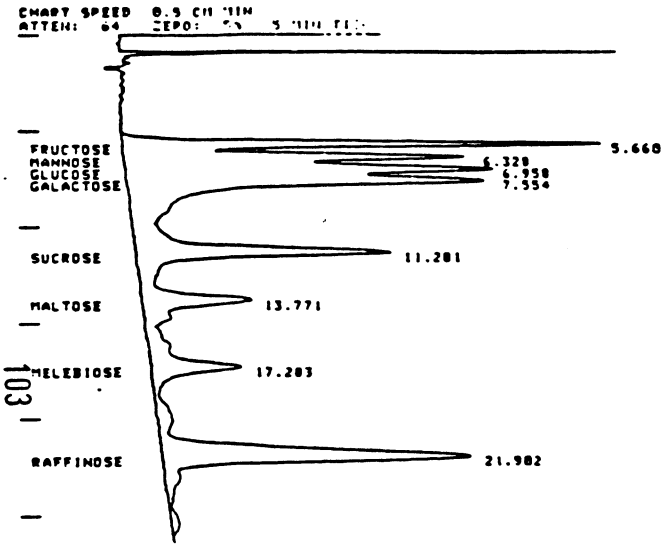


Separation of ketosteroids by normal bonded phase chromatography. Column: MicroPak CN-10 (Varian); mobile phase: A = hexane, B = 33% isopropanol in dichloromethane; gradient: 15-75%B in 20 min; flow rate: 1 mL/min; detector: = 254nm, 0.32 Aufs.

FIGURE 4

The aminopropyl is the highly polar bonded phase with stronger retention than silica. It is a unique column because it can be used as a basic normal phase packing or as a weak anion exchanger (example: Nucleotides). The amino column can be used for the separation of very polar compounds which are not retained on reverse phase packings. An example of carbohydrate separation is shown in Figure 5 using acetonitrile water mixtures. Although such solvents are used in RP the amino column is "normal phase" phenomenon because as the water increases the retention time decreases; this is opposite to the RP system.

SEPARATION OF SUGAR STANDARDS ON MICROPAK NH_2-10



RECALC
TITLE: 8 SUGAR STANDARDS 10:25 18 DEC 80

CHANNEL NO: 1 SAMPLE: 8 STANDARDS METHOD: SUGARS

PEAK NO	PEAK NAME	RESULT	TIME (MIN)	TIME OFFSET	AREA COUNTS	SEP CODE	WID (SEC)
1	FRUCTOSE	12.1506	5.660	-0.010	949922	BV	13.35
2	MANNOSE	10.9911	6.328	-0.012	859273	VV	24.20
3	GLUCOSE	13.5401	6.958	-0.012	1058560	VV	29.00
4	GALACTOSE	20.1981	7.554	0.004	1578450	VV	42.10
5	SUCROSE	14.3176	11.281	0.001	1119340	VV	32.30
6	MALTOSE	5.8888	13.771	0.001	454130	VV	43.25
7	MELEBIOSE	5.2628	17.293	0.003	411911	VV	51.35
8	RAFFINOSE	17.7329	21.982	0.002	1386350	VV	46.30
TOTALS:		100.0000		-0.023	7817940		

DETECTED PKS: 14 REJECTED PKS: 6

MULTIPLIER: 1.00000

NOISE: 20.6 OFFSET: 8647

FIGURE 5

The amino columns are very reactive and some caution should be maintained. The primary amino group can condense with reactive aldehyes and ketones forming Schiff bases. Compounds containing carbonyl groups, such as acetone or ketosteroids (Figure 6 shows this separation on CN-10), should be avoided. When using a derivative for primary amines the unreacted reagent should be removed before injection. Primary amines can undergo oxidation. Thus, degassing of mobile phase, especially if used at elevated temperatures, is recommended. Peroxides in the mobile phase in solvents like tetrahydrofuran and chloroform can be detrimental to aminopropyl columns.

Both cyano and amino columns have been coupled with silica columns for hydrocarbon group separation. Shown below is solvent refined coal where we are able to separate parafin, napthenes, mono-olefins, aromatics, PAH's and polars (requires a backflush off silica). This is an example of a normal class separation. The silica is required for increased selectivity for the separation of the napthenes and the mono-olefins.

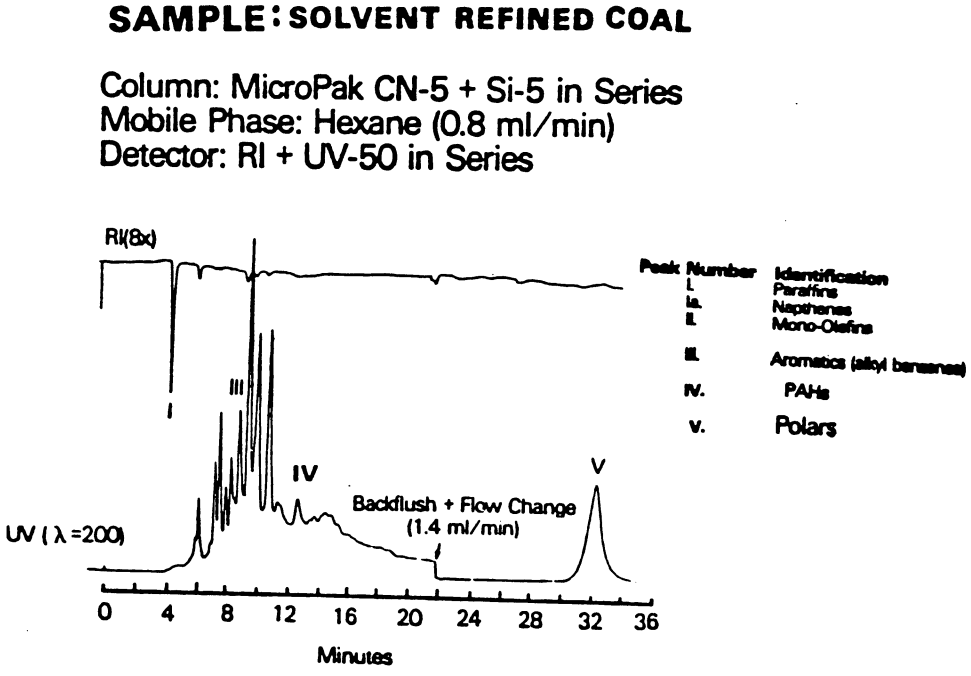


FIGURE 6

Normal phase chromatography has many advantages which are listed below:

1. Tailing and irreversible retentions are minimized.

2. It is unnecessary to control the level of water unless unreacted silanols remain.

3. Equilibrate rapidly so compatible with gradient analysis and quickly regenerated to initial conditions.

4. Compatible with large variety of solvents; both aqueous and organic.

5. Wide variety of polarity in surface functionalities.

6. You can obtain class separation which cannot be obtained with reverse phase.

7. Ability to separate isomers (great significance to the pharmaceutical chemistry).

8. Ability to separate highly hydrophilic species which cannot be retained on reverse phase columns (e.g. saccharides).

9. Use predominately organic solvent based mobile phase avoiding silica dissolution problems.

10. Mobile phase volatility allowing simpler, more efficient concentration after separation and fraction collection.

11. Ability to separate solutes that break down in aqueous solutions.

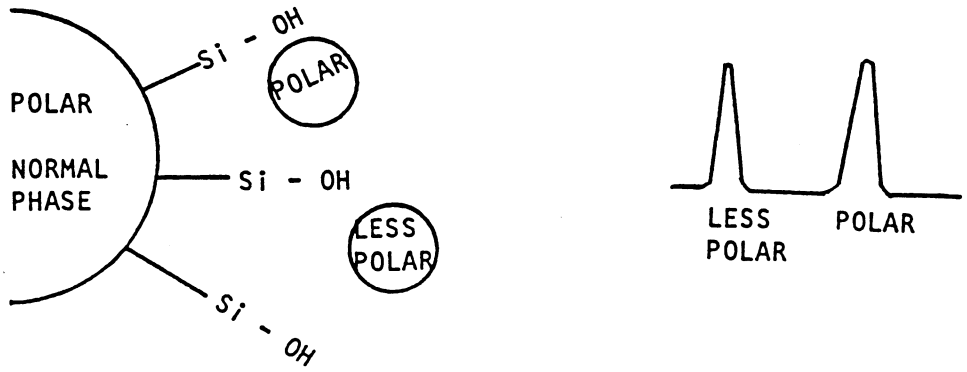
The greatest disadvantages with normal bonded phase chromatography are the high cost of organic solvents and instability of some normal phase packings.

REVERSE PHASE CHROMATOGRAPHY

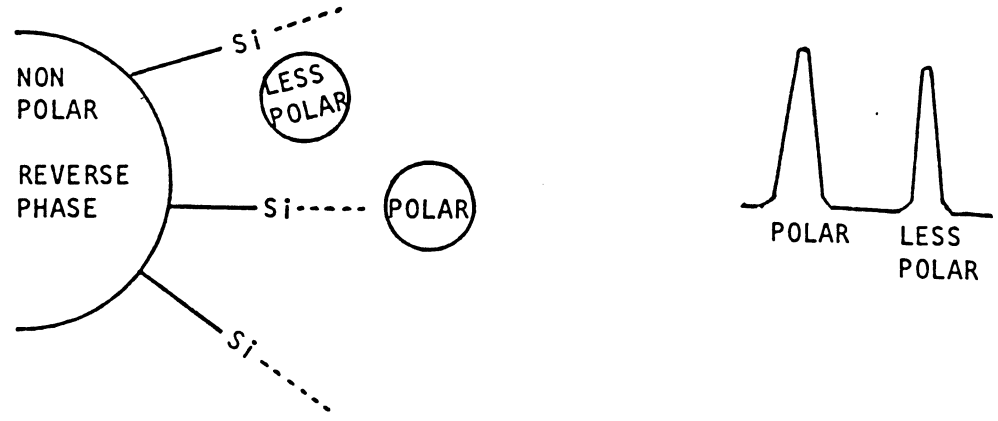
The term "Reverse Phase" chromatography originates from the fact that RP separations are based on the reverse of the normal phase interactions. Figure 1 shows a comparison of the mechanism of reverse and normal phase chromatography. Normal phase chromatography functions because of polar-polar interactions, while reverse phase depends on non-polar-non-polar interactions. In reverse phase chromatography we use more polar solvents to control the retention of the solute, like water, acetonitrile and methanol.

Water is more polar than acetonitrile or methanol. As the water concentration increases it forces the components onto the column; as the acetonitrile concentration increases it displaces the components from the packing because it is less polar than water

NORMAL VS REVERSE PHASE



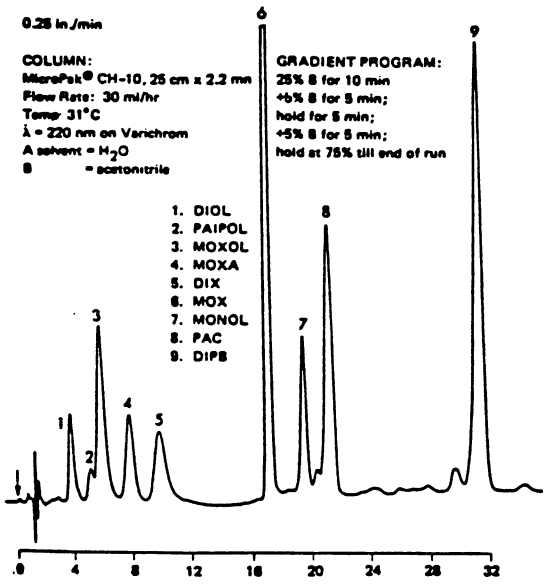
Weak Solvent - As % increases RT increases (less polar)
Strong Solvent - As % increases RT decreases (more polar)



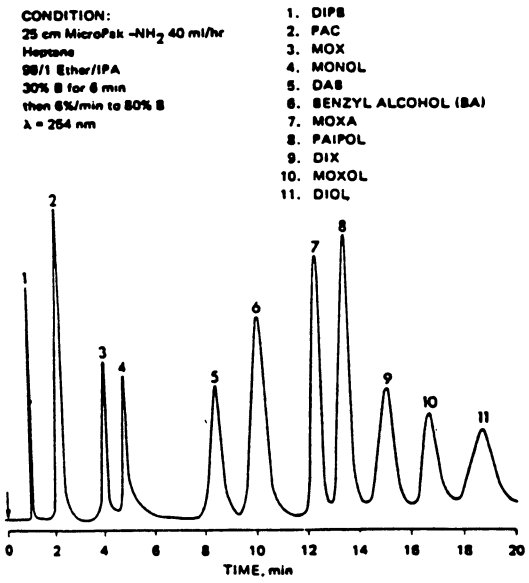
Weak Solvent - As % increases RT increases (more polar)
Strong Solvent - As % increases RT decreases (less polar)

FIGURE 1

Figure 2 shows a comparison of reverse phase to normal phase for the separations of hydroperoxides. On the normal phase column (NH_2 - column), the most polar components have the longest retention and less polar components have shorter retention. On the reverse phase column the opposite elution order occurs with the most polar eluting first and least polar last. Thus, non-polar components have higher affinity for non-polar packings like reverse phase and polar components have higher affinity for polar packings like those of normal phase columns.



Separation of Hydroperoxides of MicroPak-MCH Column.



Separation of Hydroperoxides of MicroPak-NH_2 Column.

FIGURE 2

Reverse phase packing materials are hydrophobic and contain carbon chains of varying lengths. Below is a table of typical packing types with octadecylsilane being the most common:

TYPICAL BONDED PHASES FOR RPC

Functionality	Estimate of usage	Type	General use
--Si-- $C_{18}H_{17}$	85%	Octadecylsilane (ODS)	Best general reverse phase, polymeric separations of very nonpolar molecules; monomeric; best for polar and semi-polar molecules, ion pair, ion suppression.
--Si-- C_8H_{17}	8%	Octylsilane	Lower retention than C_{18}+ good for moderately polar compounds, ion pair.
--Si--$(CN_3)_2$	4%	Dimethylsilane	Least retentive; very polar compounds, beware of bonded phase stability.
--Si--Ø	2%	Phenyl	Selectivity for aromatic compounds; useful for peptides.
--Si--C_3H_7CN	1%	Cyano	Used mostly in normal phase work. Has found some use in analysis of tricyclic antidepressants.

FIGURE 3

Reverse phase chromatography is the most popular HPLC technique for a number of reasons:

1. The columns are stable and rapidly come to equilibrium after changes in mobile phase composition.

2. Elution order is predictable; the more hydrophobic the solute the greater the retention.

3. Water is the primary solvent in most RP techniques.

4. Special selectivities can be obtained by addition of small amounts of modifiers.

5. RP chromatography has several forms: regular, ion suppression, controlled ionization, ion pair, and metal chelate addition.

The flexibility of reverse phase chromatography is probably the prime reason for its popularity, allowing use of one column for the separation of non-polar, polar, ionizable, and ionic solutes. Reverse phase mobile phases can be understood by understanding the physical properties of the molecule being separated. There are two major considerations to be taken into account:

1. Hydrophobicity
2. Ionic character

The primary separation mechanism in reverse phase is the hydrophobic interaction of the solute with a non-polar stationary phase. In some cases, especially with ionized species, we add mobile phase modifiers to make them neutral and allow hydrophobic interaction.

Since RPC is in such wide use an entire lexicon has developed with the technique. Table 4 lists these techniques and their typical mobile phases. We will discuss each of these techniques of reverse phase in this section.

LEXICON OF REVERSED-PHASE TECHNIQUES

Primary Name	Other Name	Typical Mobile Phase
Regular	"Normal"	A. Water + water miscible organic solvent, e.g., acetonitrile, methonal, dioxane
Ionization Control	Solvophobic Chromatography	B. As in A) + buffer
Ion Suppression		C. As in A) plus acid or base; For weak acids, as in A) plus acid (e.g., phosphoric, perchloric) For weak bases, as in A) plus base (e.g., carbonate, dilute NH_3)
Ion Pair		D. For cations, as in B) plus alkyl sulfonate or sulfate (e.g., C_7 sulfonate) For anions, as in B) plus tetraalkylammonium salt (e.g., tetrabutylammonium chloride)
Secondary Chemical Equilibria	e.g., Argentation Chromatography (Ag+)	E. As in B) plus metal chelates, chiral reagents, silver ion (for olefins), ligands
NARP	Non-Aqueous Reverse Phase	F. Acetonitrile or methanol + tetrahydrofuran or methylene chloride

TABLE 4

Regular Reverse Phase:
Regular reverse phase is the simplest of the RP techniques. It is based on the simple interaction of the solute with a hydrophobic stationary phase, using typical solvents like methanol-water or acetonitrile-water mixtures. Acetonitrile is favored for chromatographic efficiency, lower potential for outgassing when mixed with water, and lower UV cut-off (190 nm). Methanol, on the other hand, is cheaper, less toxic (still can cause blindness), and has a reasonably low cut-off (205 nm). Most chromatographers view methanol as a weaker (more polar or less non-polar) solvent than acetonitrile but often do not recognize their differences in selectivity.

Mixtures of water-MeOH and water-MeCN can be adjusted to approximately the same strength in terms of average retention times of solutes. Although the averages of the mixture may be similar, individual retention times (or elution order) may differ significantly by changing from water-MeOH to water-MeCN. Figure 5 shows a typical optimization experiment for a regular reverse phase separation of polynuclear aromatics.

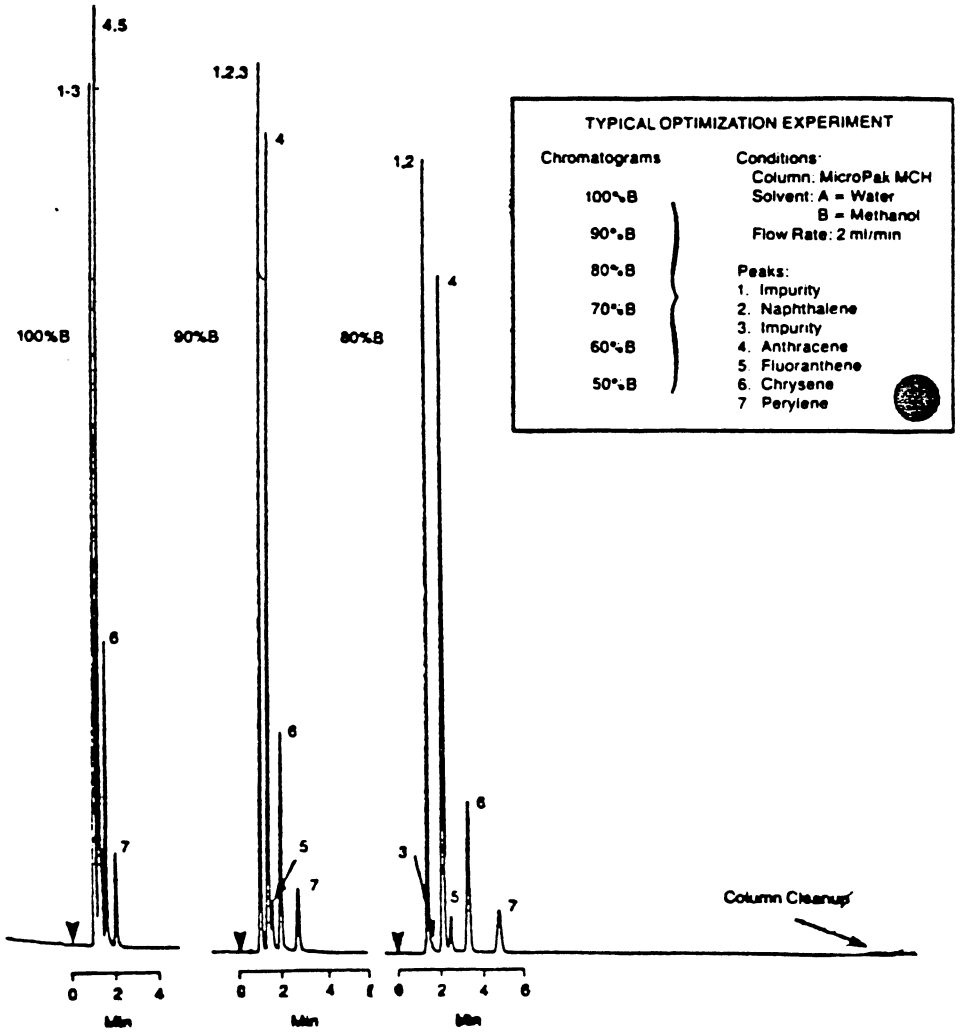
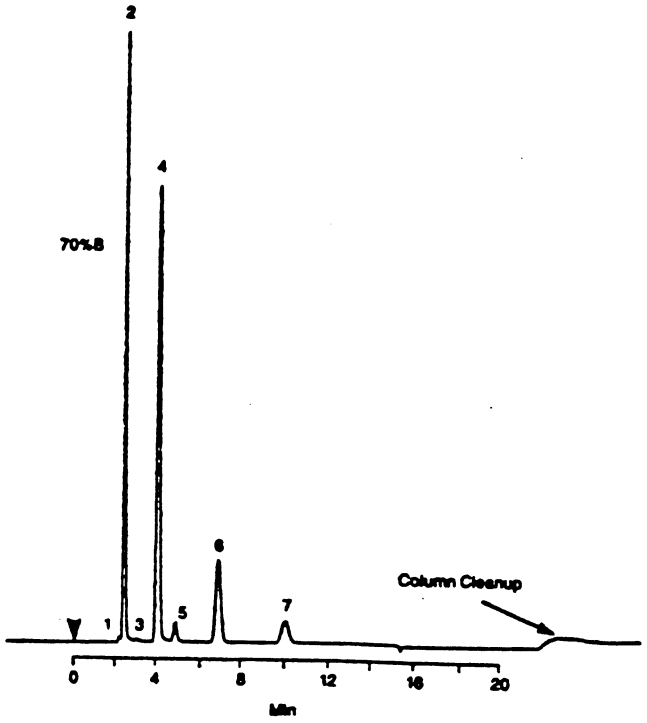
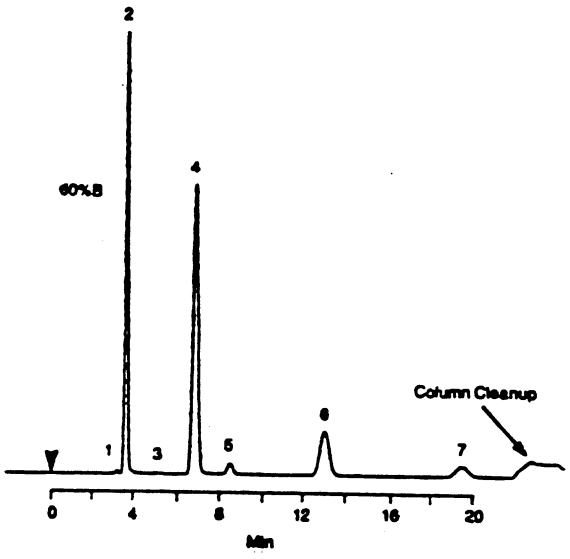
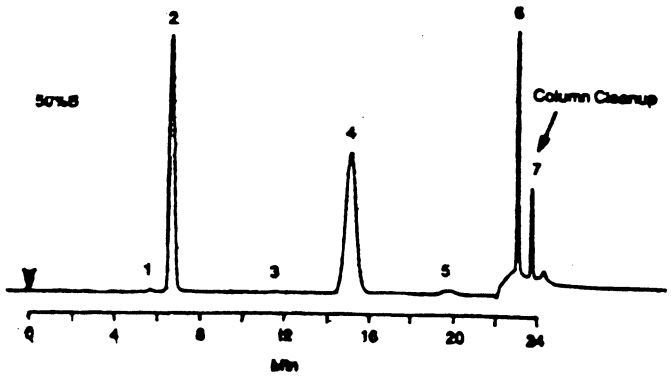


FIGURE 5


70%B
Column Cleanup
0
4
8
12
16
20
Min


60%B
Column Cleanup
0
4
8
12
16
20
Min


50%B
Column Cleanup
0
4
8
12
16
20
24
Min

To optimize a solvent system for regular RP we would start with 100% of a strong solvent such as methanol and decrease the methanol concentration in 10 or 20% increments until a k' between 1 and 10 for the components of interest is obtained. Selectivity should be at least 1.2 between peaks. In the mixture in Figure 5 we have seven components; we want good resolution of all components. At 100% methanol we have poor resolution of 1-3 and 4-5; only 6 and 7 are resolved. At 90% and 80% methanol we see the 4 and 5 beginning to resolve but 1 and 2 are eluting as a single-peak. At 60% we see baseline resolution of all components so it might be the solvent system of choice. Note we went to 100% methanol at the end of the run to clean up the column. At 50% methanol peaks 6 and 7 eluted during the clean-up step. Because of the long elution time with the 60% composition we may prefer to use a gradient program to do this separation. A possible program we could use could be 50% - 100% over 10 minutes, but it would be necessary to have an equilibration step in the program to come back to original conditions. An error that novices make is not to re-equilibrate the column to initial conditions, which leads to non-reproducible results.

Secondary Solvent Effects in Reverse Phase (Modifiers):

Organic Modifiers Used in RPC*

Solvent	
Ethylene glycol	
Methanol	Strength
DMSO	Increases
Ethanol	
Acetonitrile	
DMF	
Dioxane	
Isopropanol	
Tetrahydrofuran	

*Listed in order of increasing strength. Thus, at a fixed composition, say 50% organic modifier in water, a solute would elute earlier as the organic solvent was replaced by a solvent below it in the table.

FIGURE 7

In reverse phase chromatography we usually use a binary system of water-MeOH or water-MeCN, but recent literature shows an increased use of water-MeOH-MeCN mixtures to effect separations that were not possible with either binary mixture. Selectivities can also be modified by the addition of small amounts of very strong solvents such as isopropanol, tetrahydrofuran (THF), or methylene chloride (dichloromethane). Figure 7 lists organic modifiers used in reverse phase chromatography.

Shown in Figure 8 (next page) are the effects of THF in a methanol/water mixture for a phenol and benzyl alcohol separation. The addition of THF increases the solvent strength for the benzyl alcohol and decrease the solvent strength for the phenol. This addition is common to modify selectivity for complex mixtures.

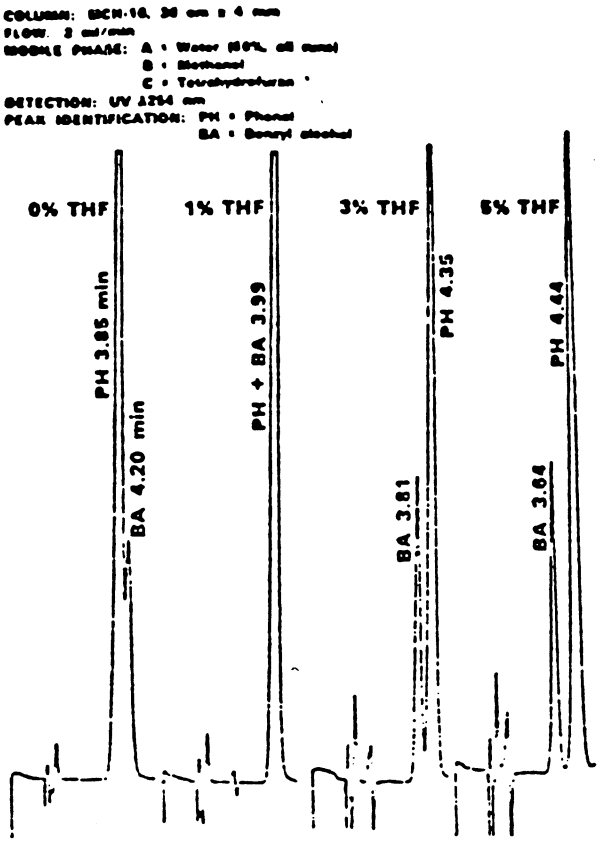


FIGURE 8
Secondary Solvent effects in a reversed-phase separation

COLUMN: MicroPak MCH-10, 4 mm x 30 cm
MOBILE PHASE: 1% Tetrahydrofuran
24% Acetonitrile
75% H_2O
FLOW: 2 ml/min
TEMPERATURE: 35°C
DETECTION: 200 nm, 0.1 AUFS

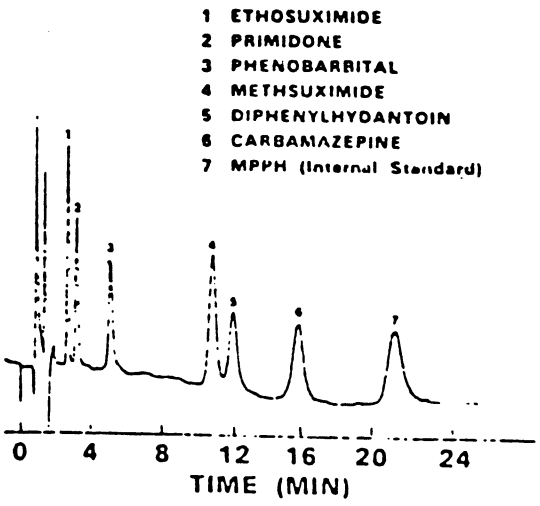
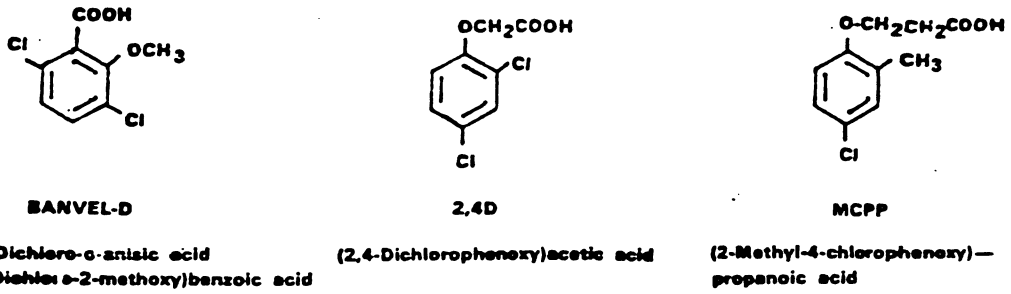


FIGURE 9

Separation of anti-convulsant drugs in a series using a ternary mobile phase

Figure 9 shows the effects of modifier on an anti-convulsant separation. Without the addition of a small amount of THF this separation would be difficult. Addition of a third solvent is called the secondary solvent effect and is best exploited with polar compounds having groups such as ^{-}OH, NO_2 or ^{-}CN. Numerous other mobile phase modifiers can be used as long as they do not create unusually high back-pressures (high viscosity) and do not interfere with detection (UV absorbing impurities or unacceptable UV cut-off).

Ion Suppression in Reverse Phase Chromatography:
If a compound is highly polar, ionizable, or ionic, its retention on a non-polar RP column will be very poor with regular mobile phases. The mobile phase can be modified in a variety of ways to achieve acceptable retention of these compounds. Shown in Figure 10 are three chlorinated pesticides each having a $^{-}$COOH moiety which needs to be protonated in order for these compounds to be separated on a reverse phase column. A small amount of Acetic acid or phosphoric acid is added to the mobile phase in order to suppress ionization and increase retention or improve peak shape. This protonation is termed ion suppression.



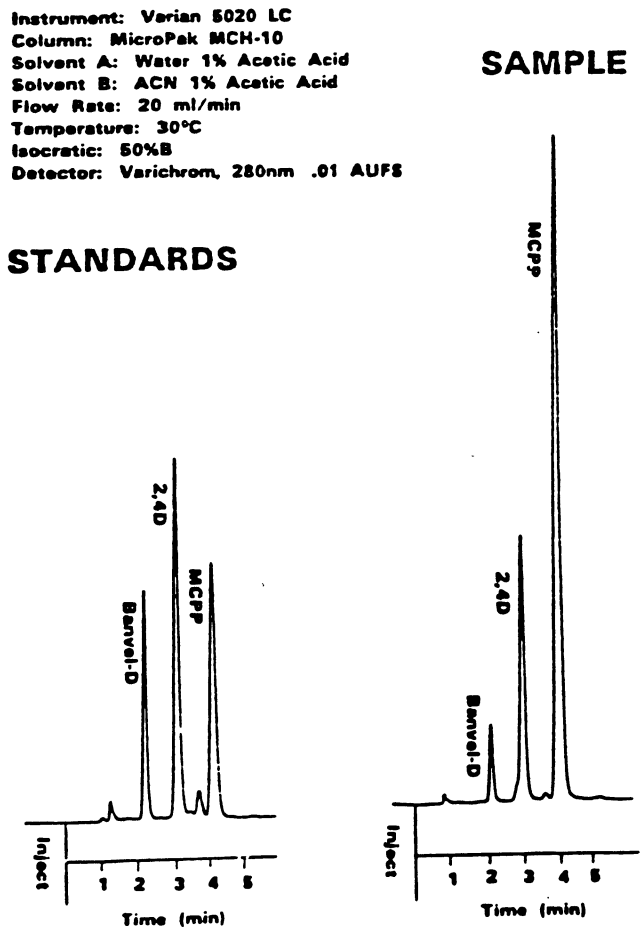


FIGURE 10

In order to control protonation a buffer system can be used. Buffer systems for use in HPLC have the following requirements:

1. Be of high purity as not to interfere with UV absorption.
2. Be highly soluble in the mobile phase. CAUTION MUST BE EXERCISED TO AVOID PRECIPITATION.
3. Have good buffering capacity at the pH required for protonation of the solute.
4. Be UV compatible with analysis system.
5. Be easily removable from the mobile phase if doing preparative LC. (e.g., $(NH_4)\ CO_3$)
6. Be cautious of buffers which attack stainless steel, e.g. LiC1, or the packing (as when the pH is too high as occurs with TMA sometimes).
7. Examples of buffers used in reverse phase include formic acid, acetic acid, phosphoric acid, succinic acid and ammonium acetate.

A second example of where Ion suppression is used in the separation of phenols is shown in Figure 11.

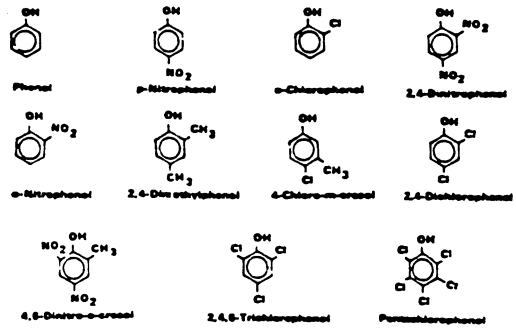


FIGURE 11
Reverse Phase Separation of Phenols

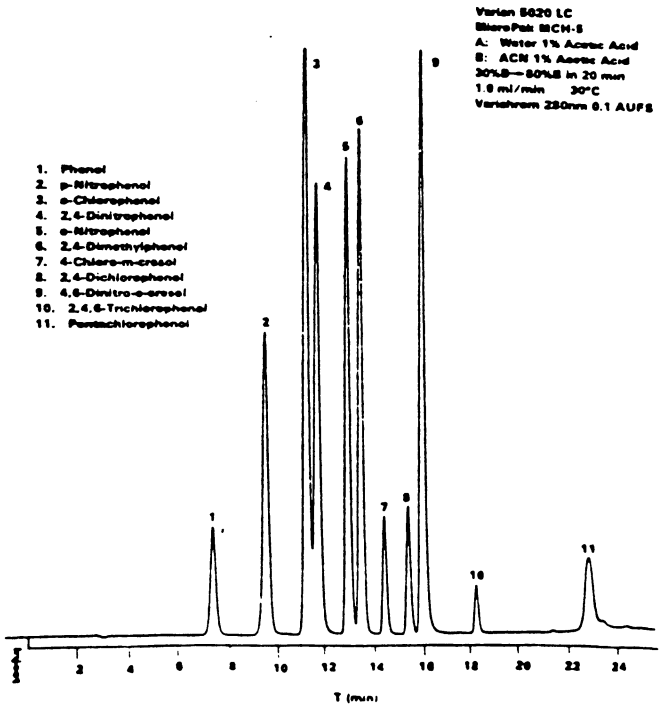


Figure 12 shows the effect of pH on the degree of ionization for 8-chlorotheophylline. The separation is of theobromine and caffeine in cocoa with 8-chlorotheophylline as internal standard. The acetonitrile is held constant for optimum separation of theobromine and caffeine. The 8-chlorotheophylline retention is greatly affected by pH. As the pH decreases, the degree of ionization decreases allowing more retention.

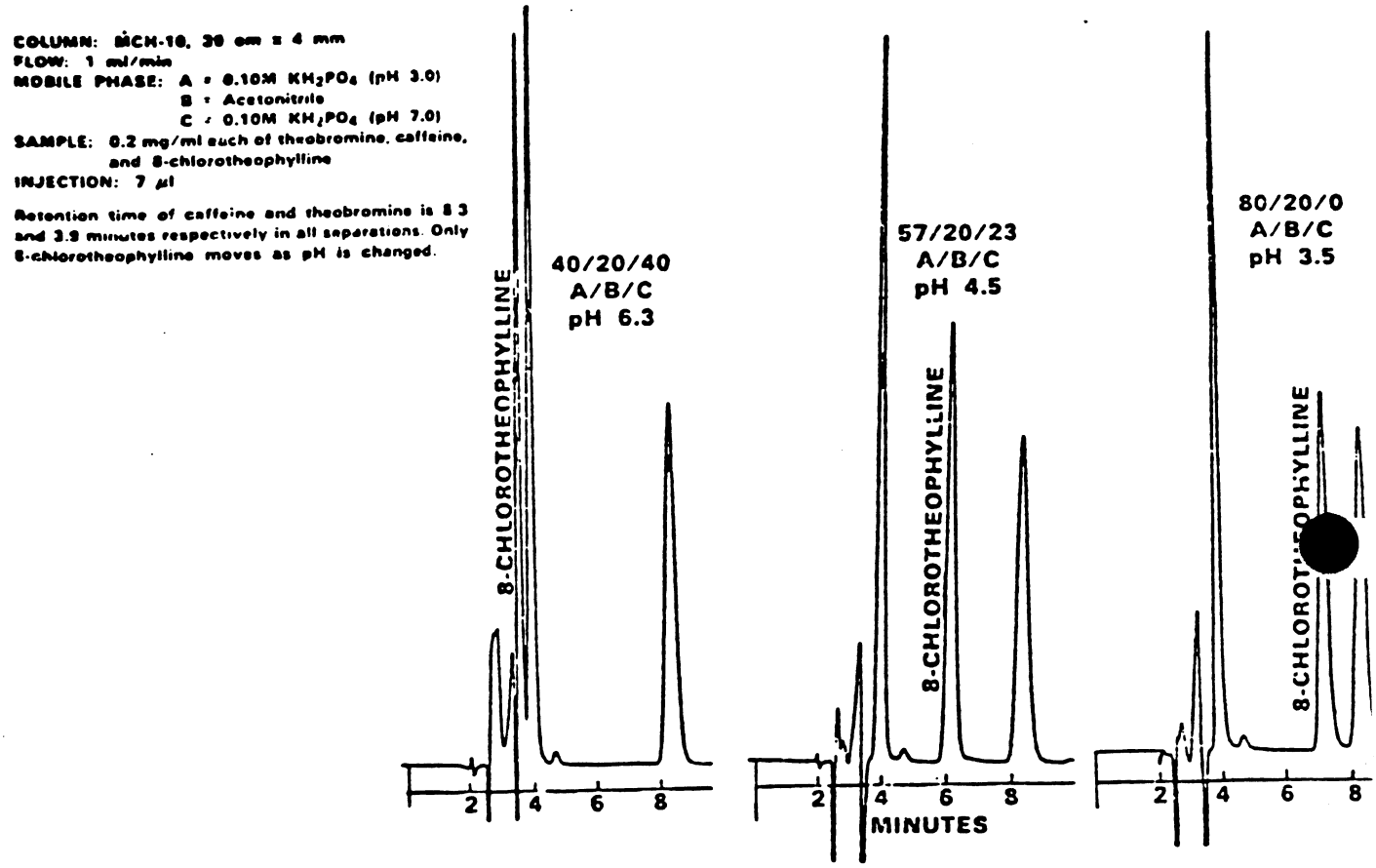


FIGURE 12
Method development with a ternary solvent system

Ion Pairing in Reverse Phase Chromatography:
Ion pairing reagents can influence the retention of certain ionizable or ionic solutes on RP columns (RP-IPC). Ion pairing effectively eliminates the charge on the solute molecules while adding alkyl (non-polar) character to increase retention.

Ion pairing reagents are listed in the table on the following page.

Cationic	Main Applications
Quaternary amines, e.g., tetramethyl, tetrabutyl, palmityl trimethyl ammonium ion	For strong or weak acids Sulfonated dyes, carboxylic acids
Tertiary amines, e.g. trioctylamine	Sulfonates

Anionic	Main Applications
Alkyl and aryl sulfonates, e.g., methane, butane, pentane hexane, Benzalkonium salts, catechol- heptane, or octane sulfonate, amines	For strong and weak bases
Camphorsulfonic acid	
Perchloric acid	Forms very strong ion pairs with a wide range of basic solutes
Alkyl sulfates	Similar to sulfonic acids, yields different selectivities
Lauryl sulfates	

There are a number of proposed models of ion pairing. We will discuss two of these. The first model, called the ion pair model, postulates that solute molecule forms an ion pair with the counterion (IP reagent) in the mobile phase. This uncharged ion pair then partitions into the lipophilic ("fat loving") stationary phase.

Anionic Ion Pairing:

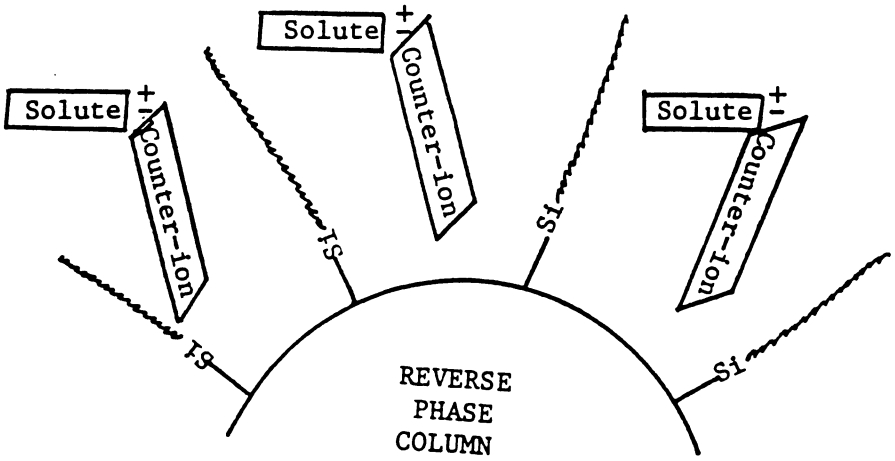
$SOLUTE^+$	+	$COUNTERION^-$	→	SOLUTE COUNTERION
POSITIVE CHARGE		NEGATIVE CHARGE		NEUTRAL ION PAIR

Cationic Ion Pairing:

$SOLUTE^-$	+	$COUNTERION^+$	→	SOLUTE COUNTERION
NEGATIVE CHARGE		POSITIVE CHARGE		NEUTRAL ION PAIR

The second model, called the ion-exchange model, postulates that the counterion partitions into the stationary phase with its ionic group oriented at the surface. The column then behaves like an ion exchange column.

Ion Exchange Model:



Factors controlling retention in RP-IPC include:

1. **Type of Counterion:**
   The better the ability to ion pair, the longer the retention.

2. **Size of Counterion:**
   The longer the chain length (more hydrophobic), the greater the retention, e.g., with heptane sulfonic acid versus lauryl sulfate, the mobile phase with lauryl sulfate will give longer retention.

3. **Concentration of Counterion:**
   Usually between 0.005-0.01M. Increasing the concentration increases retention, up to a limit, because it affects the equilbrum allowing full ion pair information. However, too high a concentration will cause decreased retention.

4. **pH:**
   Retention increases as pH is adjusted to maximize the concentration of the ionic form of the solute. For benzoic acid (pka 5; weak acid) we would want to adjust the pH to 6 or 7 to keep the acid dissociated to allow ion pairing. For TMA we would want a low pH to maximize ionization.

5. **Type of Concentration of Organic Modifier:**
   Retention decreases with increasing lipophilic nature and increasing concentration, (same as regular RP).

Practical considerations in doing ion pairing chromatography:

1. **Purity of Ion Pairing Reagents:**
It is best to purchase chromatography grade ion pairing reagents. It may be necessary to clean up the reagent prior to use by running through an old reverse phase column in a concentrated form and then dilute for use. (.1 to .5 M at 1-2 ml/min on RPC, and then dilute with distilled water). The addition of an ion pairing reagent may increase the UV cut-off, so the purer the better.

2. **Solubility of the Ion Pair Regeant:**
Precipitation can occur if the ion pairing reagent is insoluble in the organic modifier. The type of concentration is critical to preventing precipitation and plugging of your HPLC system. It is wise to check the solubility of the proposed counterion in the least polar chromatographic solvent. Methanol is widely used in ion pairing chromatography because of good solubility of the reagents. Quaternary amines used as counterions are readily soluble in methanol but are very soluble in acetonitrile, as well.

   When doing gradient elution chromatography, check the solubility of the reagents in the maximum organic concentration required prior to running the system. It is common to premix the solvent as not to exceed this concentration even at 100% programmed value, (e.g. 50% acetonitrile is maximum concentration without precipitation for a particular reagent. Therefore, we would premix the B solvent (50% water, 50% acetonitrile) as to not exceed that value even at 100% B solvent composition.

3. **Column Allocation in RP-IPC:**
Ion pairing reagents can permanently alter the column and change its selectivities, so it is recommended that columns be allocated for a particular ion pairing reagent. At all times pH restrictions should be maintained for silica gel columns (pH 2-7.5)

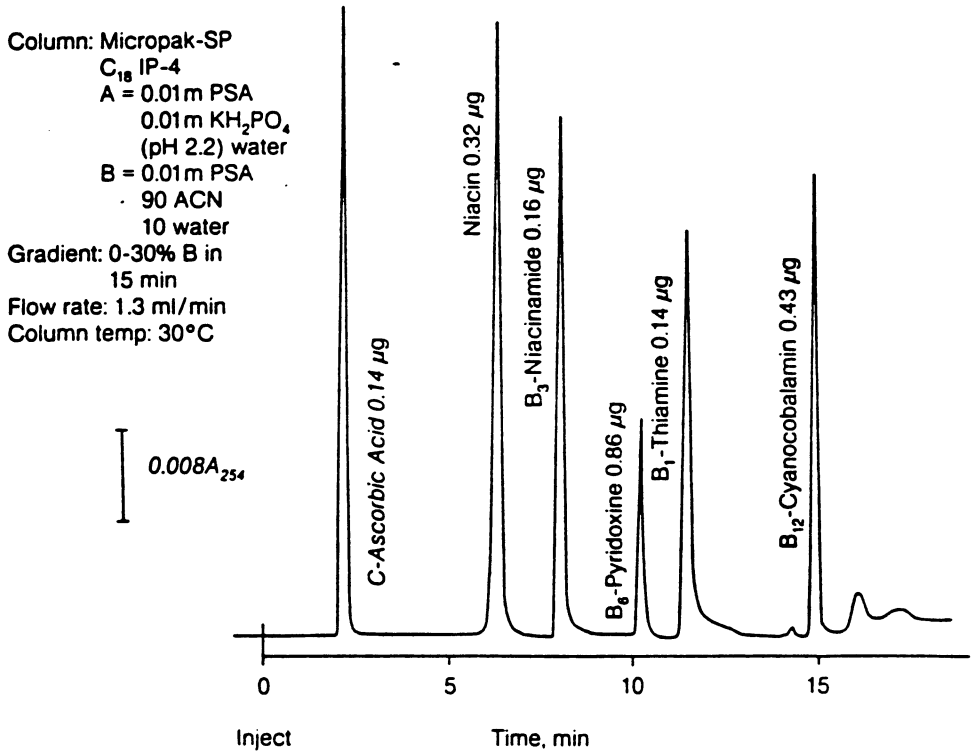


FIGURE 13

Figure 13 shows the separation of water soluble vitamins using ion pairing. A gradient is used to increase solvent strength and reduce retention times but the strong solvent was premixed in the composition 90 parts methanol: 10 parts water, containing 0.01M pentanesulfonic acid and 0.1 citric acid to prevent precipitation of both the buffer and the ion pairing agent. This is an example of the use of an anionic ion pairing agent.

Shown in Figure 14A (next page) is the separation of dyes using TMA. Note that the TMA is dissolved in methanol. If we increase the carbon chain length on the ion pairing reagent we will increase the hydrophobicity of the pair as shown in Figure 14B. This is the same separation using TBA rather than TMA and there is a substantial increase in retention time and different selectivity. No separation occurs for yellow #5 and red #2. In Figure 14C we have the same conditions, but have increased the chain length of the ion pairing reagent to five carbons. Resolution has been completely lost. It is recommended to purchase a set of both cationic and anionic ion pairing reagents in order to get the best selectivity for the shortest analysis.

SEPARATION OF DYES USING ION PAIRING CHROMATOGRAPHY

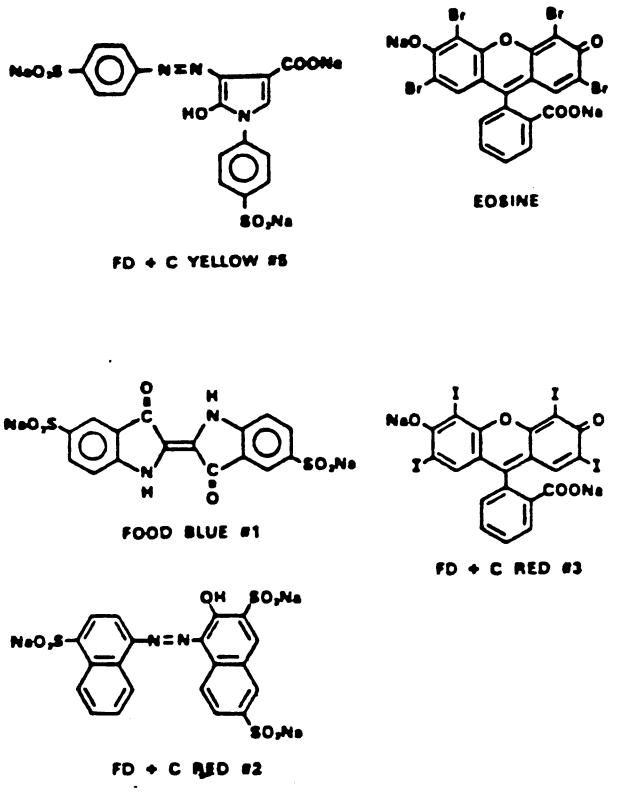


FIGURE 14: Structures of Dyes

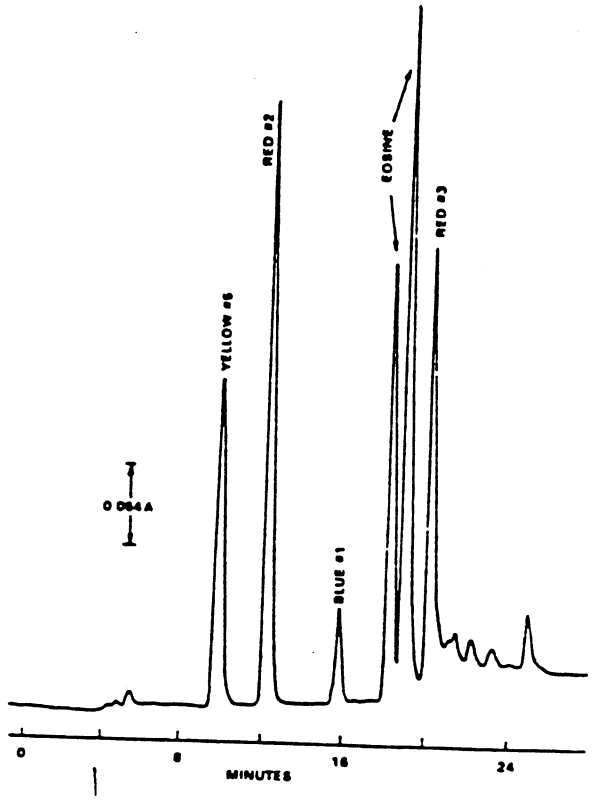


FIGURE 14A: RP-IPC separation of dyes using TMA; Column: 25 cm x 2.2 mm MicropPak -MCH. Solvents: A = 0.01M TMACl in MeOH; B = 0.01M TMACl in H_2O (pH 6.5 with 0.001M $NH_4H_2PO_4$). Gradient profile: 20% MeOH to 99% MeOH at + 3%/min. Flow: 0.5 ml/min. Detection at 254nm: 0.64 AUFS.

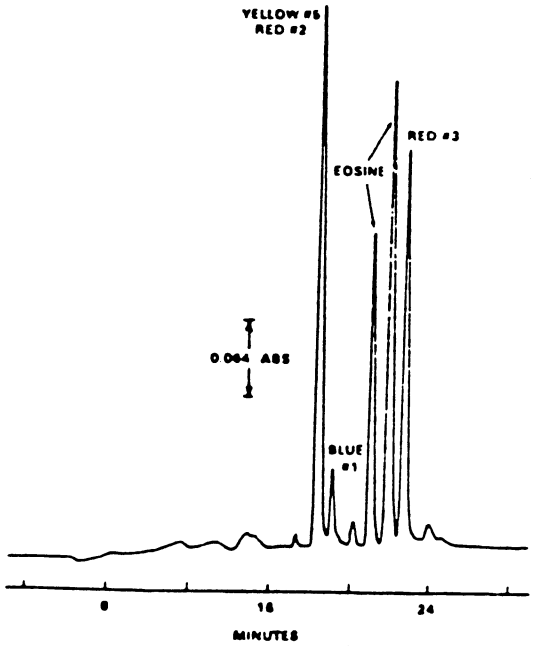


FIGURE 14B: RP-IPC separation of dyes using TBA; conditions same as Figure 7 except 0.01M TBA.

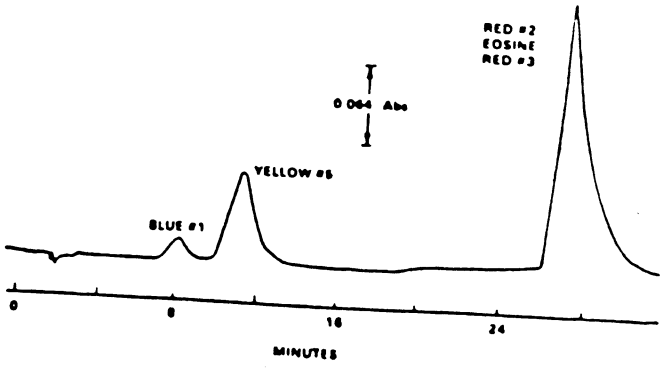
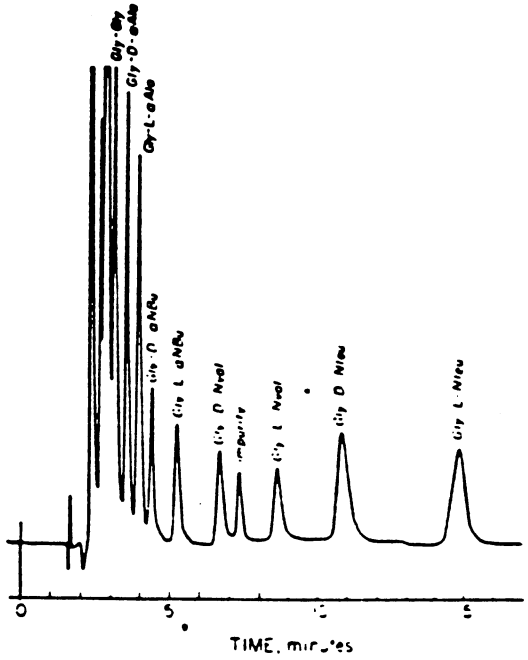


FIGURE 14C: RP-IPC separation of dyes using PMA; conditions same as Figure 7 except isocratic at 80% MeOH and 0.01M PMA.

Metal Chelates in Reverse Phase Chromatography:
The addition of metal chelates to mobile phases can lead to selectivities not found in conventional ion pairing. One application is the separation of optical isomers of dansyl amino acids. The chelating agent consists of a metal ion complexed with triamine chelates forming a complex cationic counterion which possesses hydrophobic properties (reference–W. Linder et al. J. Chromatog. 185:323–44 (1979).

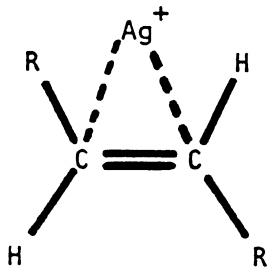


Separation of DNS-glycyl-D, L-amino acid dipeptides using metal chelate mobile phase additive. Conditions: 0.8 mM C_3-C_8-dien-Ni(II), 0.19M ammonium acetate, pH 9.0, acetonitrile-water (35:65 V/V), flow rate 1 ml/min, 30°C. Columns: 15 cm x 4.6 mm, 5 µm Hypersil bonded with n-octyl groups. Reproduced from reference 96 with permission.

FIGURE 15

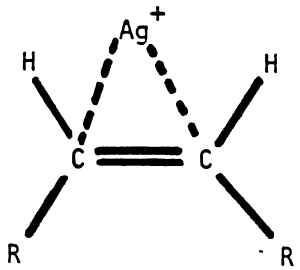
Argentation Chromatography on Reverse Phase:
Another form of chromatography where a metal is added is "argentation" chromatography. Argentation means adding $AgNO_3$ into the mobile phase. Silver has high affinity for double bonds and affects the ionic character of the compound, therefore decreasing the interaction with the stationary phase. The TRANS molecule is sterically hindered and therefore undergoes less change, which gives a long retention time. The CIS molecule has no steric hindrance and a higher charge, due to the silver ion. Therefore, the retention time is shorter.

ARGENTATION CHROMATOGRAPHY ON RP



TRANS

Steric Hindrance



CIS

No Steric Hindrance

Shown below is an olefin separation on a reverse phase column where $AgNO_3$ has been added into the mobile phase. The CIS compounds elute first (higher charge) and the TRANS compounds elute second (lower charge).

COLUMN: LICHROSORB RP8

SOLVENT: IPA/H_2O

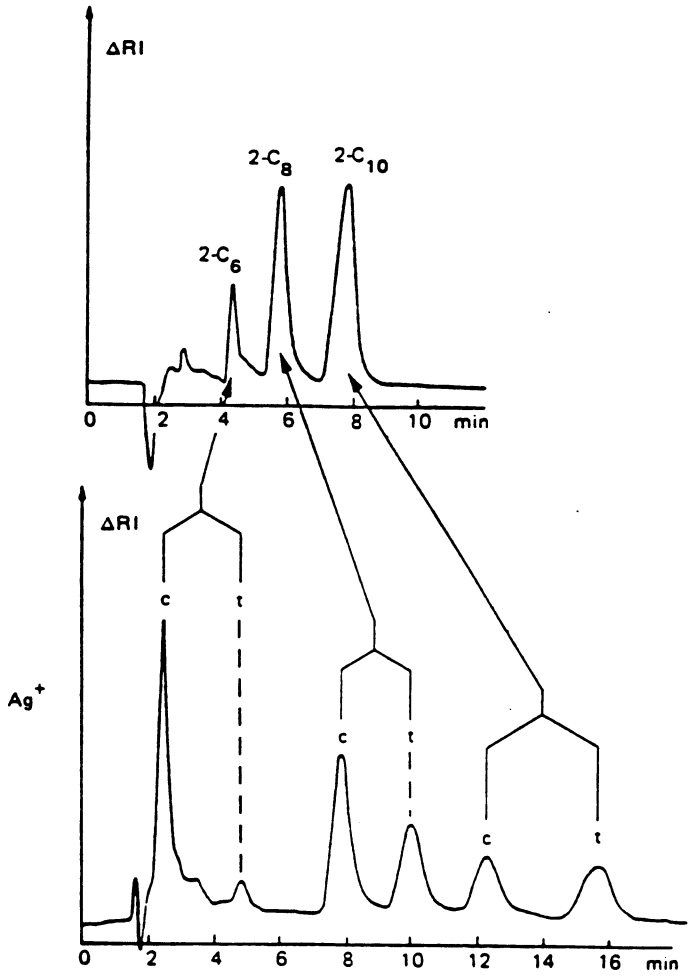
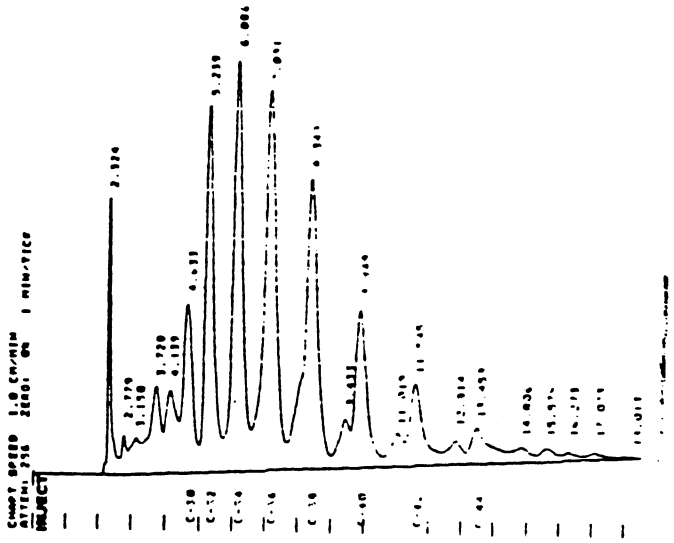


FIGURE 16

The Use of $AgNO_3$ Solution to Resolve CIS/TRANS Olefins
(Reprinted with permission from Schomberg and Zegarski, J. Chromatog. 114, 174 (1975). Copyright Elsevier Scientific Publishing Company

Non Aqueous Reverse Phase:
Some components are completely insoluble in water and only sparsely soluble in acetonitrile and methanol. In these cases one can do "non-aqueous reverse phase chromatography": where methanol and acetonitrile are weak solvents and tetrahydrofuran is the strong solvent. Shown below is a separation of triglycerides using IPA/acetonitrile/tetrahydrofuran mobile phase.



TITLE: REV PHASE TRIGL... MIXTURE

CHANNEL NO: 1 SAMPLE: COCONUT METHOD: TOM1

PEAK NO	PEAK NAME	RESULT	TIME (MIN)	APEAK COUNTS	SEP CODE
1		3.7632	2.324	856030	BV
2		4.0575	3.723	921758	VV
3		3.5895	4.133	815434	VV
4	C-30	6.6733	4.633	1517250	VV
5	C-32	12.6607	5.233	2876120	VV
6	C-34	16.6354	6.025	3824490	VV
7	C-36	19.8357	7.041	4506720	VV
8	C-38	16.9137	8.343	3842260	VV
9	C-40	7.6456	9.844	1736860	VV
10	C-42	4.9710	11.544	1129270	VV
11	C-44	3.0402	13.453	690640	VV
TOTALS		99.9934		22716800	

NOTES:
COLUMN: MICROPAK MCH-5
MOBILE PHASE: GRADIENT IPA ACN THF.
UV DET AT 220NM

HPLC analysis of coconut oil by NARP (35). Instrument: Varian Vista 54, column: MicroPak MCH-5; mobile phase: isopropyl-alcohol/acetonitrile/tetrahydrofuran gradient; detection: UV-50, λ = 220 nm, 0.5 AUFS; sample: trigyiceride components identified by carbon number at base of chromatogram.

FIGURE 17

ION EXCHANGE CHROMATOGRAPHY

Ion Exchange is one of the earliest modes of Chromatography. In commercial water softeners, ion exchange occurs between metal ions in water and hydrogen ions in the resin. Ion exchange chromatography is common in the life sciences because many compounds found in organisms are ionized or ionizable.

The basic principle of Ion Exchange is the exchange of solute ions with counter ions on the oppositely charged stationary phase. The stationary phase consists of a covalently bound moiety with a fixed ionic state which is in association with a counter ion of opposite charge. The mobile phase also contains counter ions. The basis of separation is the competition of the sample ions and the counter ions for the oppositely charged stationary phase.

If the stationary phase is positively charged, it is called an Anion exchanger; exchange occurs between a negatively charged counter ion and a negatively charged solute ion. If the stationary phase is negatively charged, it is called a Cation exchanger; exchange occurs between positively charged counter ions and positively charged solute ions. The basis of separation is the equilibrium of the ionized sample ion between the stationary phase and the mobile phase. This depends on the relative affinity of the counter ion. Figure 1 below outlines this mechanism.

ION EXCHANGE MECHANISM

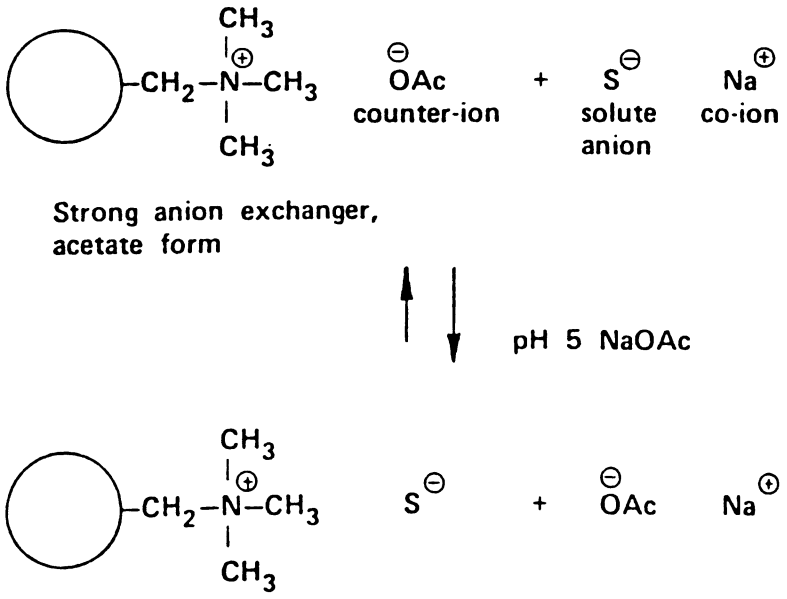


FIGURE 1

The parameters which affect the retention on an Ion Exchange column are:

1. **Nature of the Counter Ion:**
The selectivity of the column is dependent on the type of counter ion. Counter ions have a range of selectivities; for example, for a cation exchange resin the series from strongest to weakest is (K, NH4, Na, H, Li). For an anion exchanger it is as follows, from strongest to weakest: (citrate, SO4, PO4, CI, formate, acetate, OH). The relative affinity of solutes will depend upon the counter ion. The stronger the counter ion the less the retention.

2. **Concentration of the Counter Ion:**
The higher the concentration of counter ion the shorter the retention, since the counter ion can more effectively compete with solute for association with the stationary phase. It is common to run a gradient of counter ion concentration.

3. **Nature of the Sample Ion:**
To have maximum retention on an ion exchange column it is necessary to have the solute in its fully ionized form. In many cases, the degree of ionization is dependent on the pH. For example, organic acids have pK's of 2-5 for the carboxyl group. If protonated, the acid will not participate in ion exchange, but at high pH it will be negatively charged and will have affinity for a positively charged stationary phase. It is common to run pH gradients to separate mixtures in ion exchange.

4. **Non Ion Exchange Effects:**
It is possible to have hydrophobic interactions which contribute to separation. Many ion exchange separations involve mixed modes.

5. **Temperature:**
Elevated temperature is often used in ion exchange chromatography of small molecules to improve mass transfer kinetics and affect selectivity. An example of this is shown in Figure 2 for the separation of carbohydrates.

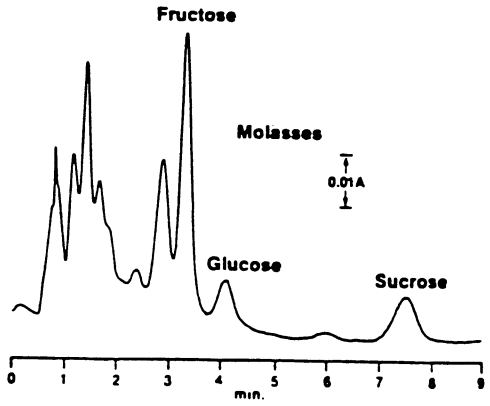


FIGURE 2

There are three types of packing materials in Ion Exchange: resin based, silica microparticles, and silica pellicular beads. All these packings have charged moeities attached to the ion exchange support.

The resin based ion exchanger is the oldest type, being first used in the separation of amino acids. The resin consists of cross-linked polystyrene/divinyl benzene with charged moeities such as quaternary ammonium groups for anion exchange resins and sulfonic acid groups for cation exchange resins. Figure 3 shows the structure of the resins. Resin based ion exchangers are usually strong ion exchangers. Resin based ion exchangers have the following disadvantages:

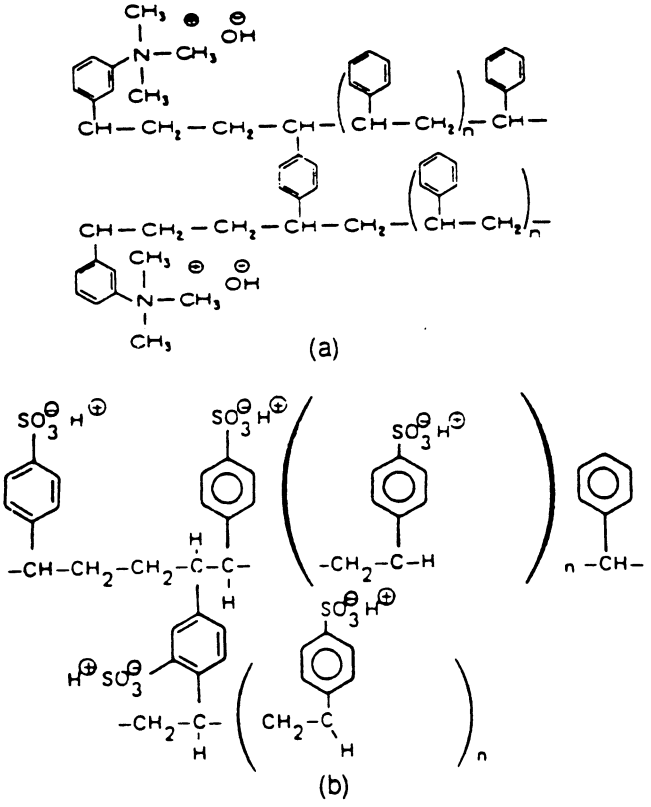
1. Low mechanical strength, which is governed by the degree of cross-linking of S/DVB. Compression of the packing material can be a problem at high pressures or high flow rates.

2. Resin can shrink and swell depending on the counter ion. In some cases it can be difficult to change counter ions. To change a counter ion requires repacking the column.

3. Resins cannot be operated with a wide range of modifiers.

4. There can be batch to batch variations.

The main advantage of the resin based ion exchangers is that they can be operated over a wide pH range, unlike silica based ion exchangers.



Ion exchange resin structures. (a) anion exchange; (b) cation exchange.

FIGURE 3

The pellicular based ion exchangers consist of a solid inert core (glass or silica) with a porous crust, which contains a resin polymer or silica on the surface. These types of ion exchangers are used mainly to dry pack guard columns for silica microparticle based ion exchangers. They have very high efficiencies but low capacities and poor mass transfer characteristics.

The silica based ion exchangers are the most widely used. They consist of 5 or 10 μ silica bonded to an organic chain carrying either a positive or negative charge very similar to other bonded phases. Being silica based they have the disadvantage of a pH limit of 2 to 8. These columns are slurry packed like other bonded phases. The columns have the following advantages:

1. The ion exchange group is bonded onto rigid silica; therefore, the columns have good mechanical stability.

2. It is possible to change the counter ion without shrinkage or swelling without unpacking the column.

3. You can use a wide range of organic modifiers, keeping in mind the solubility limits of the counter ion. (The columns can be stored in organic solvent after the buffer has been washed off).

4. These columns have a high capacity (it approaches that of the resins 2-10 millieq/gram of packing).

5. Good mass transfer.

6. There are a number of cation and anion bonded phases and even a number of particle and pore sizes. (See Figure 4 for structures).

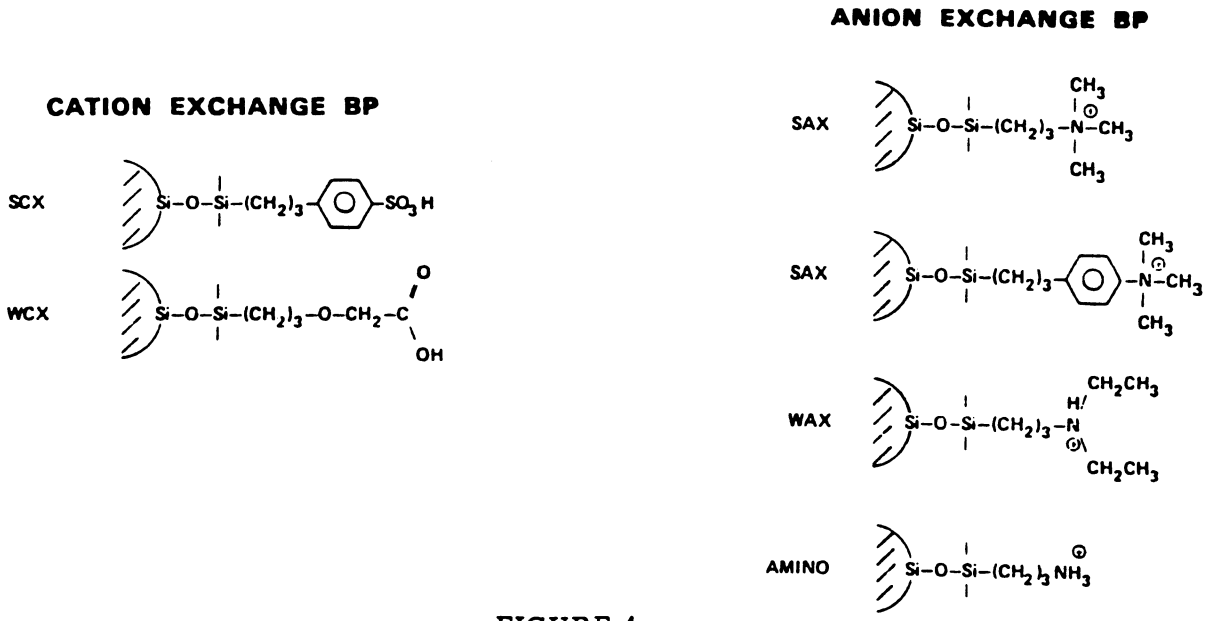


FIGURE 4
STRUCTURE OF ION EXCHANGE PACKINGS

SCX - Strong cation exchanger, WCX-weak cation exchanger, SAX-strong anion exchanger, WAX weak anion exchanger, amino moiety.

Applications:
Ion exchange has found widespread application in the routine analysis of nucleic acids. Figure 5 shows the separation of nucleotides, nucleosides and bases of a silica based weak anion exchanger. This column is compatible with mixed organic/aqueous mobile phases. The mono, di and tri phosphate nucleotides can be separated using gradient elution with phosphate buffers as the mobile phase. The retention time can be decreased by increasing the concentration of the strong buffer. The less polar nucleosides and bases can be separated using a mixed acetonitrile/buffer mobile phase. The acetonitrile decreases the hydrophobic interactions. Two sequential gradients are used: from acetonitrile/buffer to low molarity buffer to high molarity buffer. Flow programming was also used.

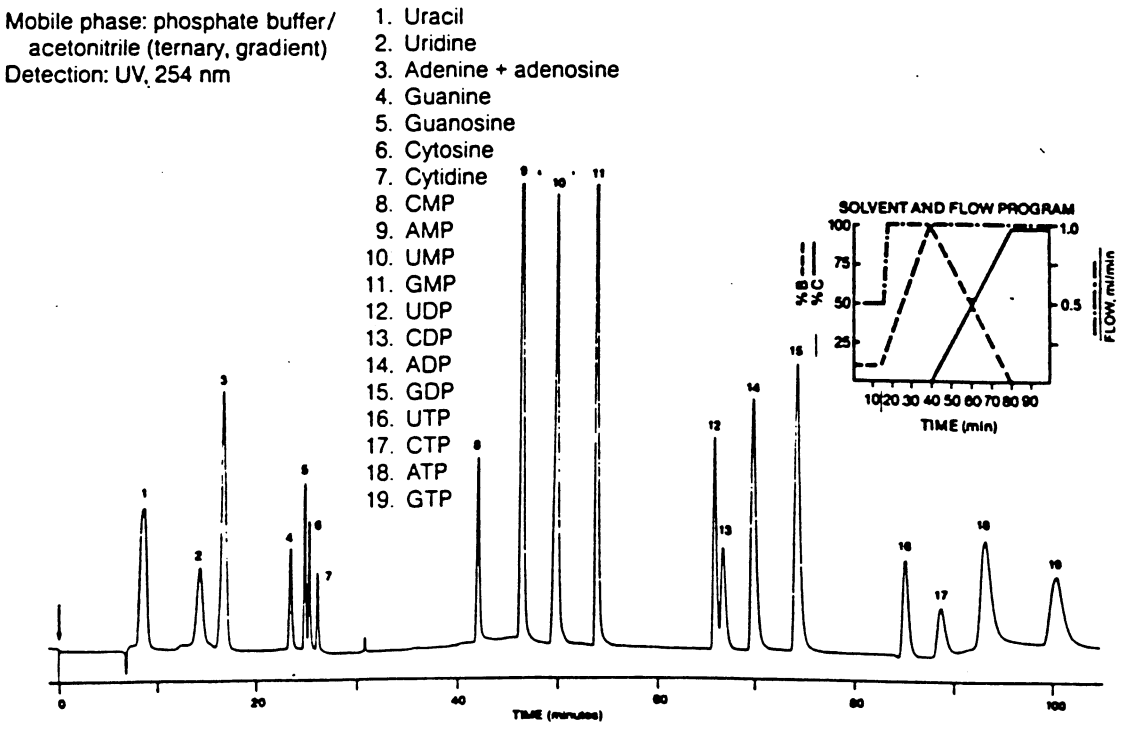


FIGURE 5

Anion exchange separation of nucleotides, nucleosides, and bases.

Tricarboxylic acids are metabolically important intermediates in the citric acid cycle. Mixtures of di and tricarboxylic acids can be easily resolved using an ionic strength gradient, as shown in Figure 6. As expected for an anion exchange separation, these compounds at pH 3.5 elute in order of increasing number of carboxyl groups (increasing charge). Note the citric, aconitic, and isocitric acids are not separated.

GRADIENT SEPARATION OF DI- AND TRICARBOXYLIC ACIDS

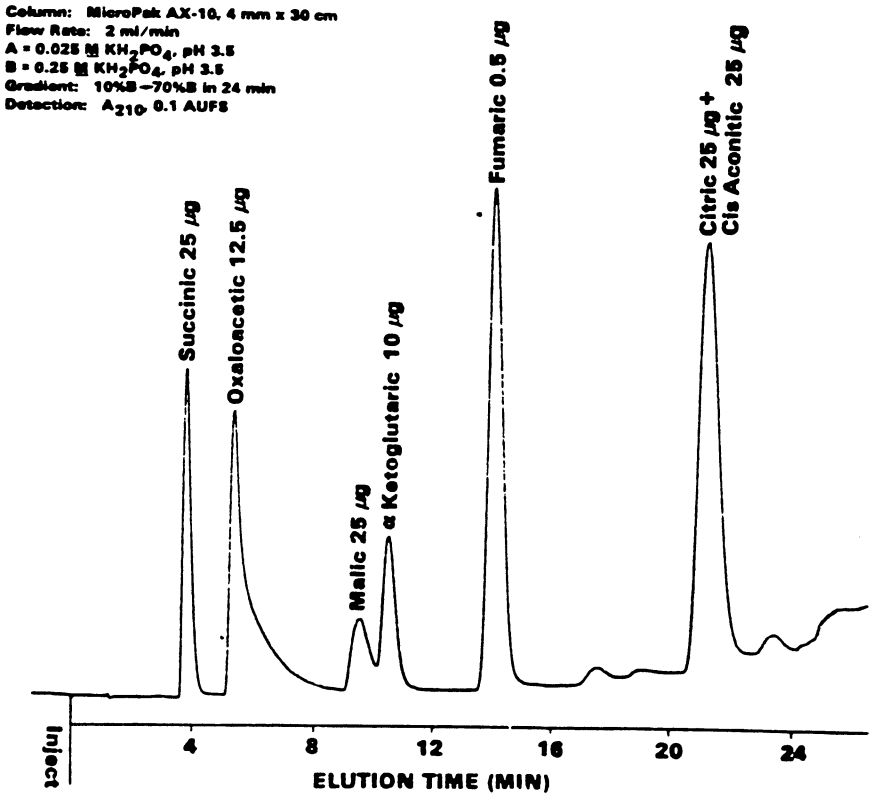


FIGURE 6

These acids have a pk value above 2.8, so at lower pH's their ionization is suppressed, increasing their retention times. At pH 2.5 we can do an isocratic separation, demonstrating the use of pH to control column interaction.

ISOCRATIC SEPARATION OF TRICARBOXYLIC ACIDS

Column: MicroPak AX-10, 4 mm x 30 cm
Solvent: 0.14 M KH_2PO_4, pH 2.5
Flow Rate: 2.0 ml/min
Detection: A_{210}, 0.2 AUFS

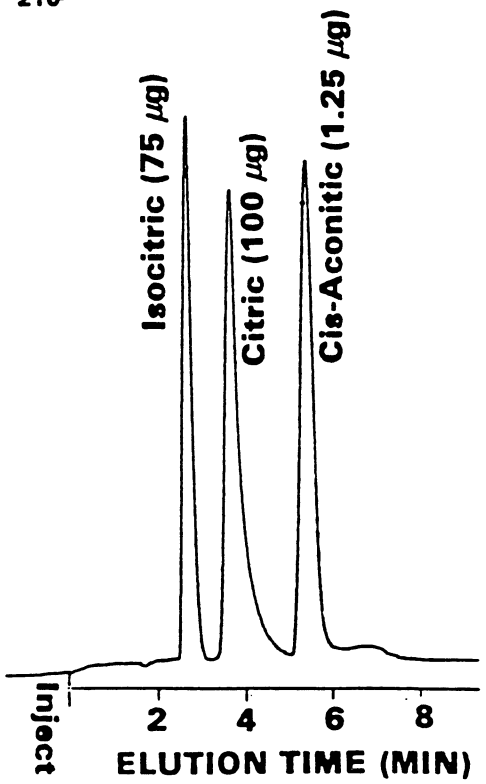


FIGURE 7

Very ionic species are ideal to separate by ion exchange. Figure 8 shows the separation of wine acids on the AX-10 ion exchanger. Again, gradient elution is used with increasing concentration of the counter ion. This type of compound is difficult to separate by other techniques because of its strong ionic character.

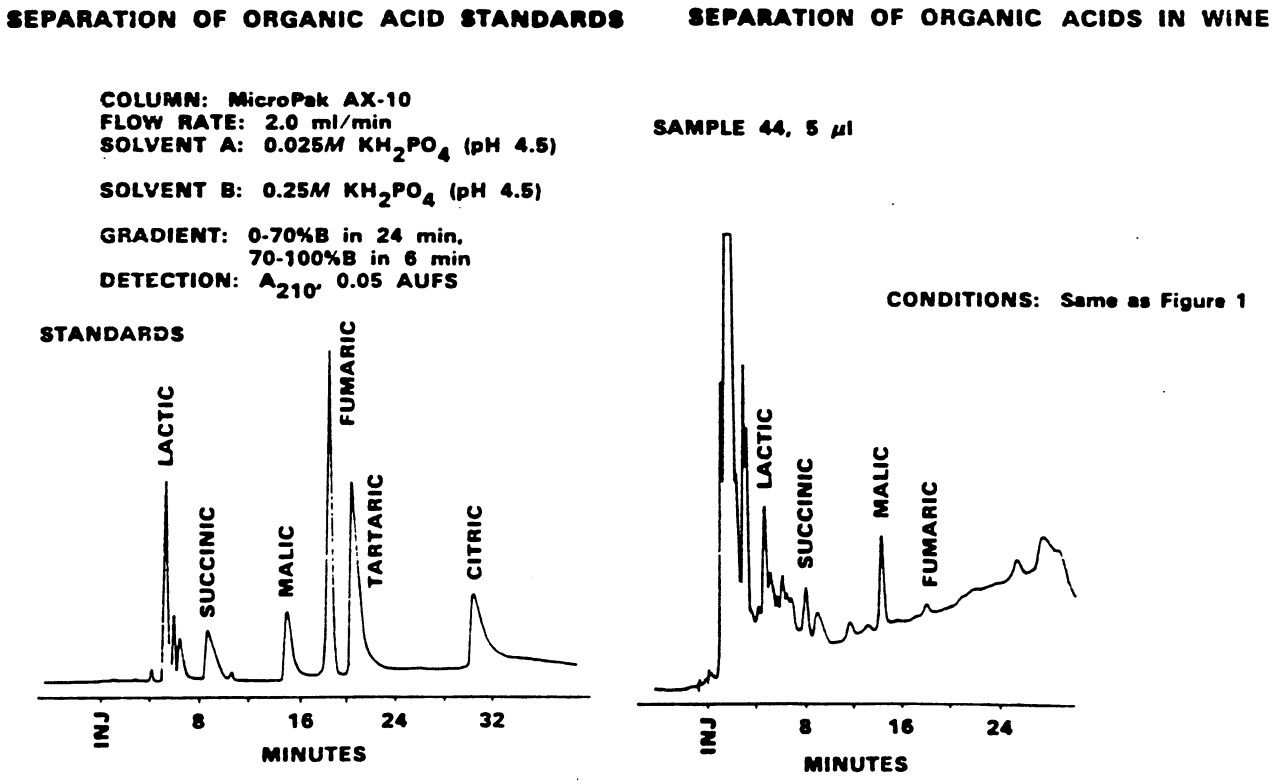
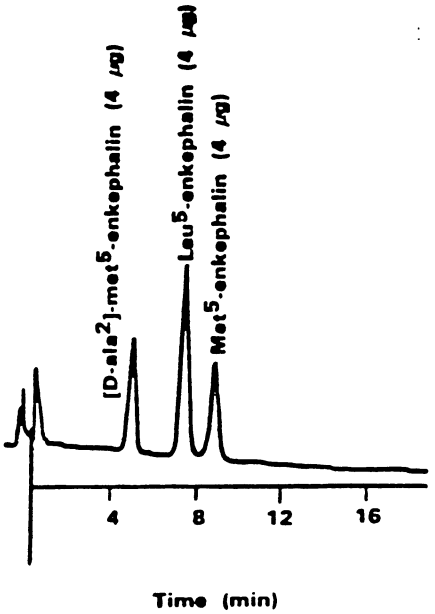


FIGURE 8

Enkephalins are pentapeptides used as regulators in the body. They can be separated by their hydrophobic nature in a reverse phase column by suppressing their ionic character using a high pH (pH=4.5). They can also be separated on anion exchangers taking advantage of their ionic character and suppressing their hydrophobic nature. This is an example of using multiple physical properties of a compound to obtain a separation by different modes.



Separation of peptides by anion-exchange chromatography. Column: MicroPak AX-5; eluant: 50% 0.0125 M KH_2PO_4, pH 4.5 and 50% acetonitrile; temperature: 30°C; detection: 254 nm, 0.02 AUFS.

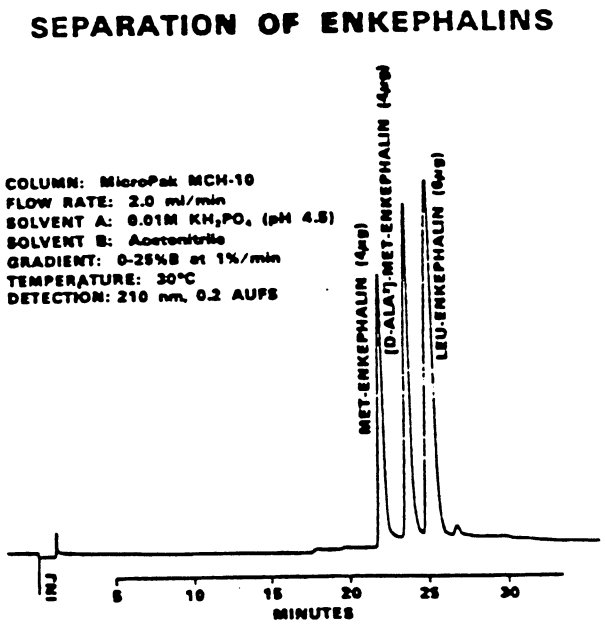
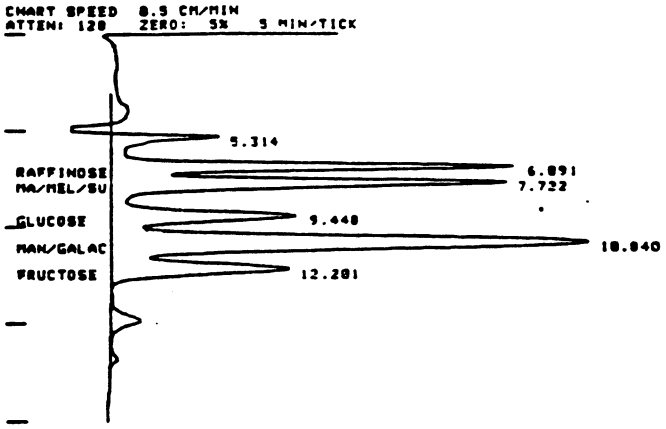


FIGURE 9

Carbohydrates are polyhydroxy aldehydes and ketones that have the empirical formula $(CH_2O)_n$ where n 3. These compounds can be separated on a strong cation exchange column which is packed with sulfonated polystyrene-divinyl-benzene resin, an example being Aminex HPX-87. The resin has 8% crosslinking and is the Ca++ form. The separation mode is a combination of partition and exclusion. These compounds can also be separated by normal phase chromatography on an NH_2-10 column.

SEPARATION OF SUGAR STANDARDS ON AMINEX HPX-87



RECALC
TITLE: 8 SUGAR STANDARDS 10:35 19 DEC 88

CHANNEL NO: 1 SAMPLE: 8 SUGAR STDS METHOD: SACC.

PEAK NO	PEAK NAME	RESULT	TIME (MIN)	TIME OFFSET	AREA COUNTS	SEP CODE		WID/2 (SEC)
1	RAFFINOSE	17.8710	6.891	0.881	2878588	VV		26.85
2	MA/MEL/SU	21.1466	7.722	0.882	2459578	VV	?	32.45
3	GLUCOSE	11.5638	9.448	-0.882	1344988	VV	?	42.25
4	MAN/GALAC	37.5957	10.848	0.888	4372778	VV	?	47.48
5	FRUCTOSE	11.8238	12.281	0.881	1375238	VV		47.18
TOTALS:		100.0888		0.882	11631188			

DETECTED PKS: 9 REJECTED PKS: 4

MULTIPLIER: 1.88888

NOISE: 28.4 OFFSET: 1[illegible]

FIGURE 10

Traditionally, ion exchange chromatography of proteins has been performed using ion exchange functional groups bonded to carbohydrate gels, such as DEAE-cellulose or DEAE Sephadex. The low mechanical strength has restricted flow rates, resulting in long analysis times. Special microparticulate ion exchangers have been developed such as the AX-300. This column consists of 10 m macroporous spherical silica with a polymeric layer of amine. The silica provides a rigid background compatible with high mobile phase velocities and has pores of sufficient diameter (330 A) to allow permeation of macromolecules. The amine phase has selectivity similar to DEAE. The polymeric coating minimizes exposure of proteins to residual silanols.

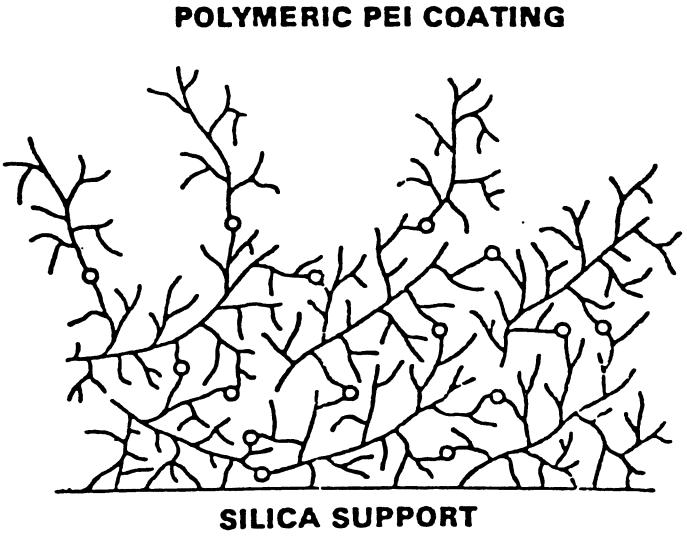


FIGURE 11

The separation of hemoglobin variants shows the selectivity of AX-300 to resolve proteins of similar size and amino acid sequence.

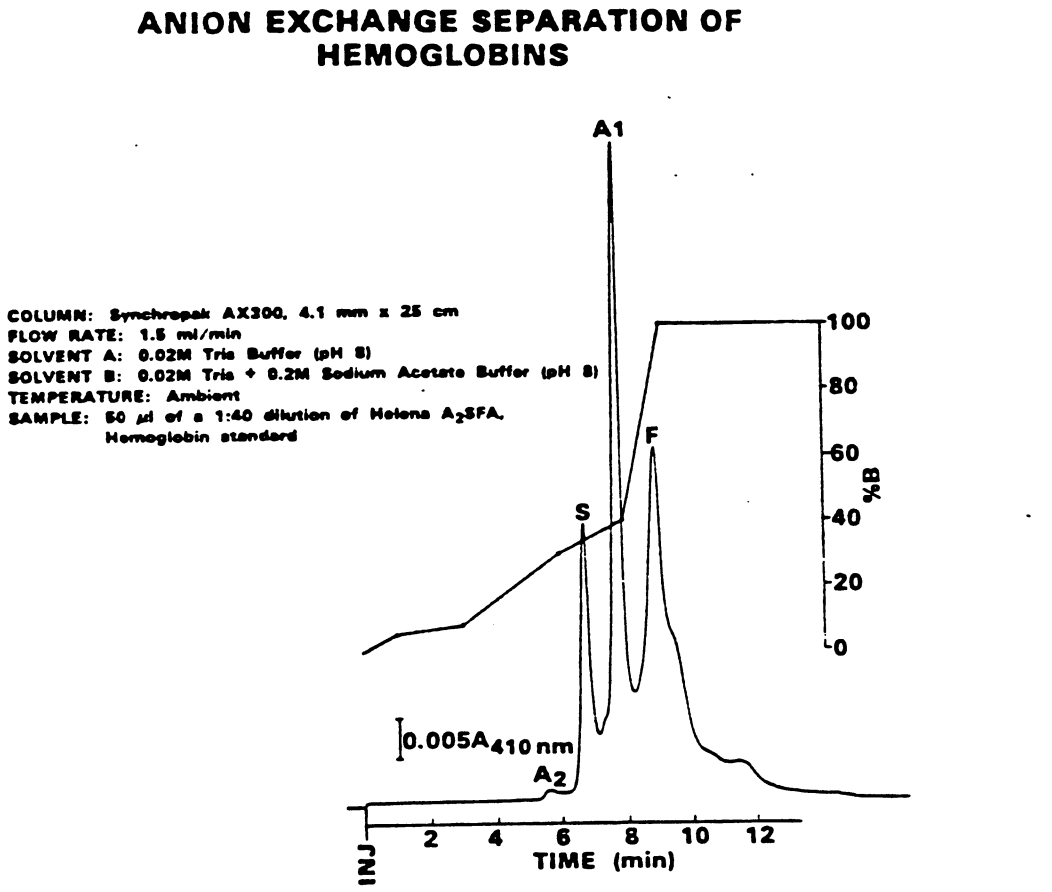


FIGURE 12

Proteins can also be separated on a weak cation exchanger. The TSK-IEX 535 CM contains carboxyls bonded to a silica base. Proteins are eluted using an increasing salt (NaCl) gradient.

SEPARATION OF PROTEIN STANDARDS ON MICROPAK TSK-IEX 535CM

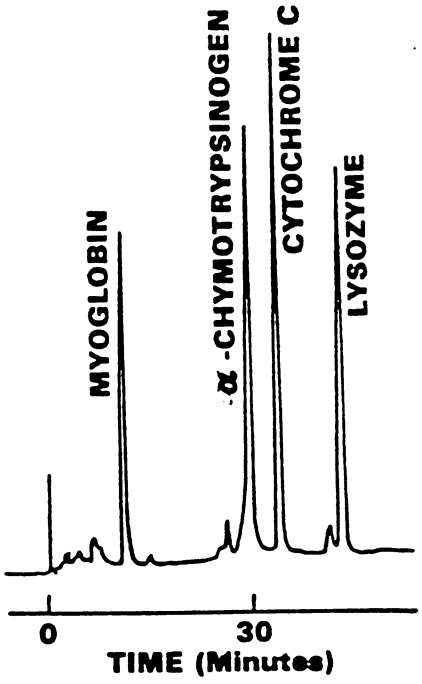


FIGURE 13

Eluent linear Gradient elution from A to B: 25mM Na_2HOP_4 pH 6.4,B, A + 0.5 M NaCl (8.33mM/min), Detection UV at 280nm. Reference; Komiya etal., Introduction and Evaluation of TSK-Gel IEX-500 Series Column for High Speed Ion-Exchange Chromatography, Toya Soda Company, Japan.

SIZE EXCLUSION CHROMATOGRAPHY

Size exclusion chromatography (SEC) is the separation of components according to their effective molecular size in solution. There are two types of SEC, Gel Filtration Chromatography (GFC) and Gel Permeation Chromatography (GPC). Gel Filtration Chromatography (GFC) is used for the separation of water soluble polymers and biopolymers using solvents such as water, buffers, and organic modifiers to control "non-exclusion effects" i.e. adsorption, and ion exchange effects. GFC was originated by Porath and Flodin in 1959 using soft, hydrophilic gels of crosslinked dextrans (Sephadex). GFC is used for the separation of proteins, polysaccharides, poly vinyl alcohols, and polyethylene glycols.

Gel Permeation Chromatography (GPC) is used for the separation of organic soluble polymers using solvents such as THF, chloroform and toluene usually without significant problems with "non-exclusion effects". J. C. Moore first originated the technique in 1964 on crosslinked polystyrene/divinyl benzene (PS/DVB) for organic polymer separations. Examples of organic polymers separated by this technique included polystrene, polyvinyl chloride, polyesters, polycarbonates, and nylons.

Size exclusion columns contain small porous particles with varying sizes of pores (see Figure 1). The solutes of different sizes permeate into the pores in varying degrees depending on their size in solution (hydro-dynamic volume). For example, we might have three sizes of molecules. The largest molecules are larger than the pores and are completely excluded from the packing material. Hence, they move with the solvent at the **void volume** or **exclusion volume.** The medium size molecules permeate partially into the packing and therefore are retained for medium periods of time. The small molecules permeate deeply into the pores and are retained for a long time. Therefore the elution order for molecules is: the largest come out first; if they are larger than the pores they come out at the exclusion volume. The medium size molecules come out after the exclusion volume, in the selective permeation range. The small molecules travel through all the pores and therefore come out at the **permeation volume.**

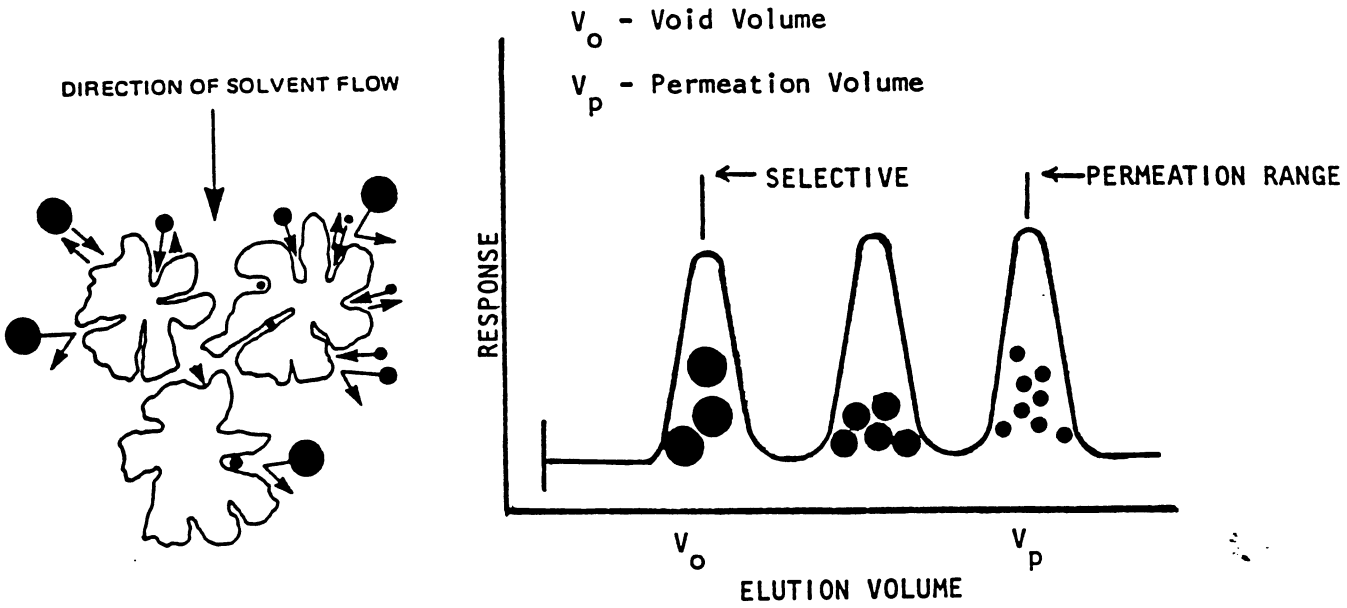


FIGURE 1

The criterion of separation in SEC is the size of the molecule in solution. Figure 2 shows that the spatial arrangement of the molecule is important. The effective size can change, depending on the solvent or the pH. This is very critical with protein separations. Proteins can be gobular or straight chained (denatured) depending on the solvent.

THE ROLE OF MOLECULAR SIZE IN SEC

Size as a Function of Molecular Structure

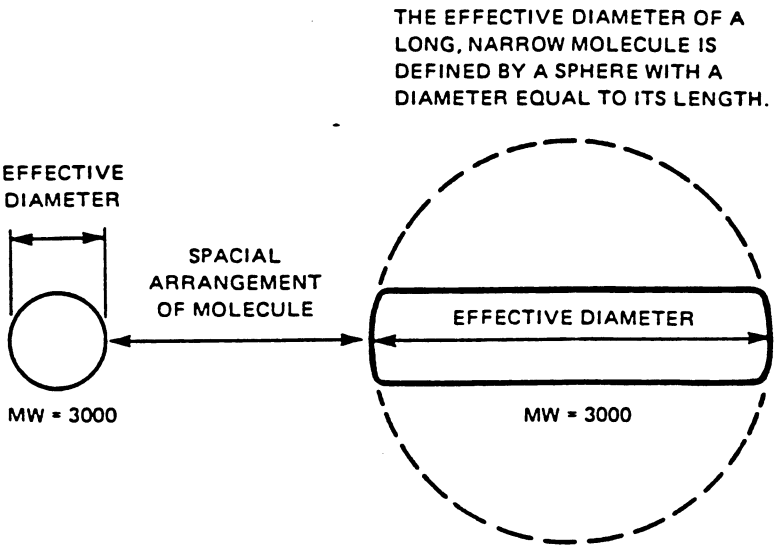


FIGURE 2

For industrial polymers, the type of bonding and structure of the polymer backbone greatly affects the hydrodynamic diameter. For example: polyethylene and polystyrene have different backbone structures (Figure 3) and therefore different lengths between repeating units and different sizes in solution. The Q factor is the ratio of the molecular weight divided by the molecular length, which indicates weight per unit length.

Molecular Size is measured by **Hydrodynamic Diameter:**

the length of the molecular backbone.

$$\text{Q-factor} = \frac{\text{Molecular Weight}}{\text{Molecular Length (Å)}}$$

Polyethylene: -c-c-c-c- Mol. Weight = 11 x (length, Å)

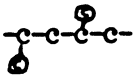
Polystyrene:  Mol. Weight = 41 x (length, Å)

FIGURE 3

Packing Materials:
There are three types of SEC packings: soft gels, semi-rigid, and rigid-packings. Soft gels are used for biopolymer separations using gravity feed; these packings cannot use HPLC pumps because of compression. Separation can take many hours to days. The most commonly used soft gel is Sephadex (cross-linked dextrans).

For HPLC systems, semi-rigid gels (cross-linked organic gels) were developed for their higher mechanical strength. Examples include Polystyrene-divinyl benzene (TSK gel type H) used for organic soluble compounds and Polyethers (hydroxylated) (TSK Gel Type PW) used for aqueous soluble compounds. The basic structure of these packings is shown below. The H type has hydrophobic groups and the PW type has hydrophilic groups.

CROSS-LINKED PS-DVB COPOLYMER

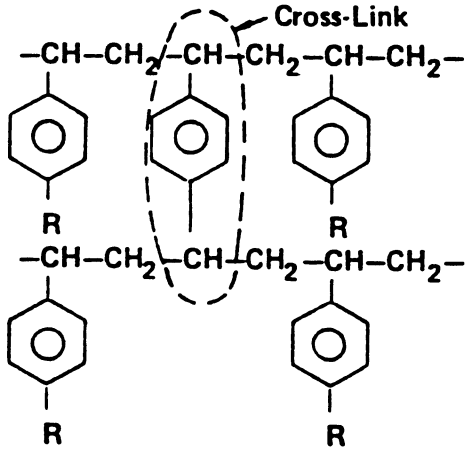
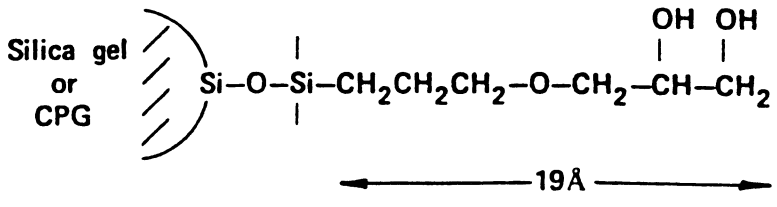


FIGURE 4

The second type uses rigid gels (silica and silica-based bonded phases). The diol-like bonded phases are used mainly for biopolymer separations (TSK type SW). The basic structure of these packings is shown in Figure 5 below.

GLYCOPHASE G (DIOL) BONDED PHASE



monomeric, ~40% coverage of silanol sites

FIGURE 5

In size exclusion chromatography we choose a column based on the solubility, the size of the compound and the purpose of the separation (e.g., molecular weight vs. fractionation). We first choose a column type based on solubility. Figure 6 shows an Application Chart for TSK columns. TSK stands for Toya Soda Company of Japan, a primary producer of SEC packings. We choose silica based hydrophilic packings for water soluble biopolymers. For water soluble synthetic polymers we choose hydroxylated polyether semi-rigid gels. For organic polymers we use polystyrene-divinyl benzene semi-rigid gel.

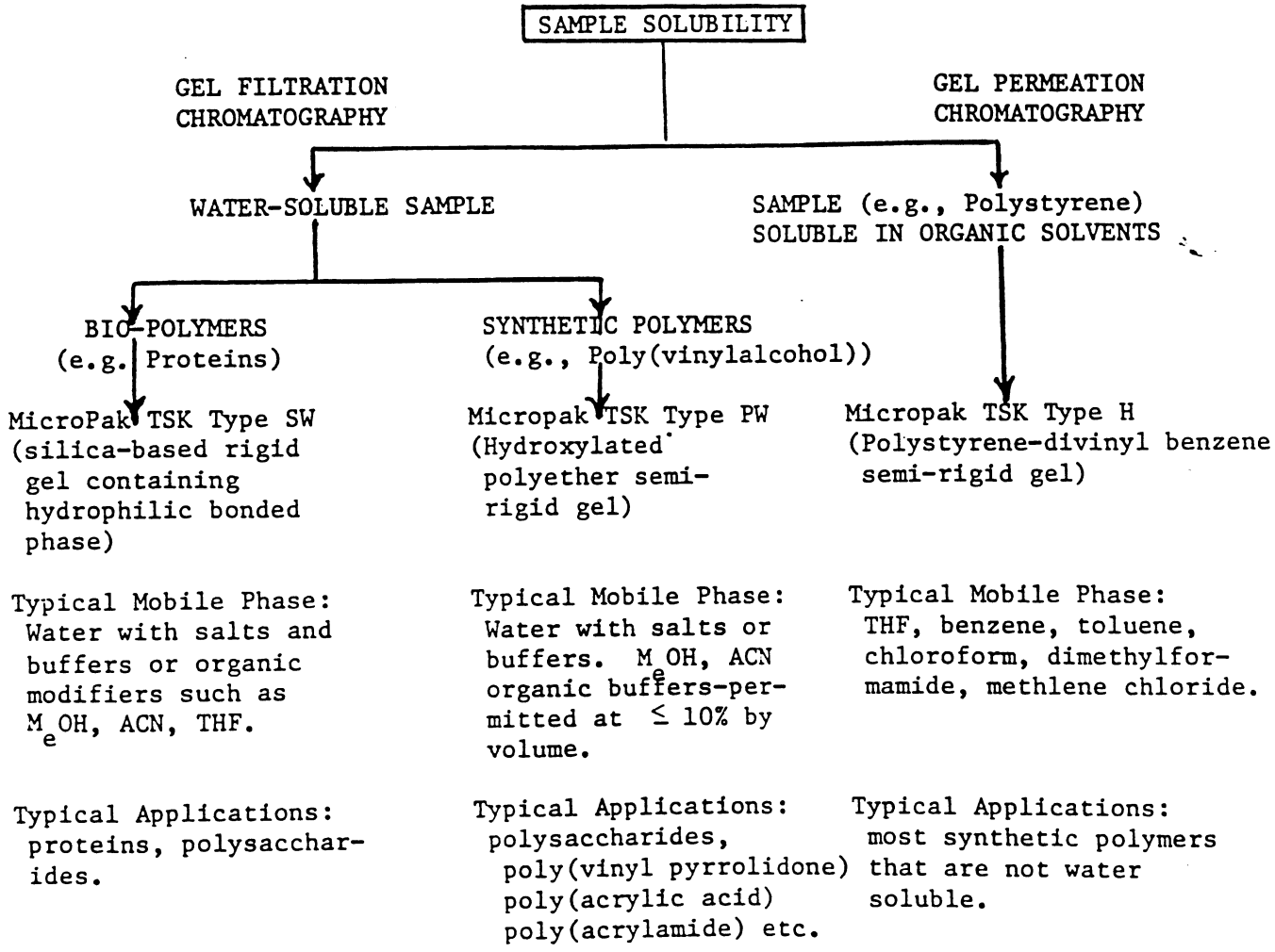


FIGURE 6

Applications Chart for TSK Columns

The second most important criterion for choosing a column is the relationship between pore size and molecular size of the components being separated. Figure 7 shows the structure of a porous-SEC packing material.

REPRESENTATION OF STRUCTURE OF POROUS SEC PACKINGS

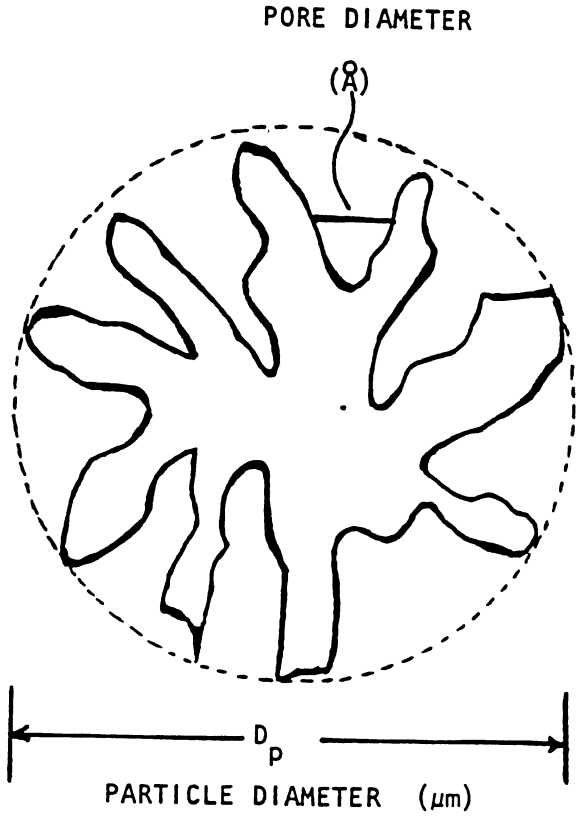


FIGURE 7

The pore size will govern the separation capability of a packing material.

TKS COLUMN SEPARATION RANGES AND EXCLUSION LIMITS

Packing Type	Column Type	Pore Size (Å)	Mol. Wt. Separation Range	Exclusion Limit
PS/DVB gel	Micropak TSK 1000H	40	100- 700	1,000 (polystyrene)
PS/DVB gel	2000H	250	100- 8,000	10,000
PS/DVB gel	2500H	500	500- 15,000	20,000
PS/DVB gel	3000H	1500	500- 50,000	60,000
PS/DBC gel	4000H	10^4	1,000- 300,000	400,000
PS/DVB gel	5000H	10^5	5,000- 1,000,000	4,000,000
PS/DVB gel	6000H	10^6	50,000- >1,000,000	40,000,000 (est.)
PS/DVB gel	7000H	10^7	100,000- >5,000,000	400,000,000 (est.)
PS/DVB gel	GMH6	1500→10^7	500- >5,000,000	400,000,000 (est.)
Polyether	Micropak TSK 1000PW	50	100- 1,000	1,000 (PEG)
(hydroxylated)	2000PW	50	100- 5,000	5,000
gel	3000PW	200	1,000- 50,000	50,000
"	4000PW	500 (est.)	2,000- 300,000	300,000
"	5000PW	1,000 (est.)	4,000- 800,000	1,000,000
"	6000PW	>1,000 (est.)	40,000- 8,000,000	10,000,000 (est.)
Silica-based	Micropak TSK 2000SW	130	500- 20,000	30,000 (PEG)
bonded phase	3000SW	240	1,000- 30,000	50,000
"	4000SW	250	2,000- 250,000	300,000

FIGURE 8

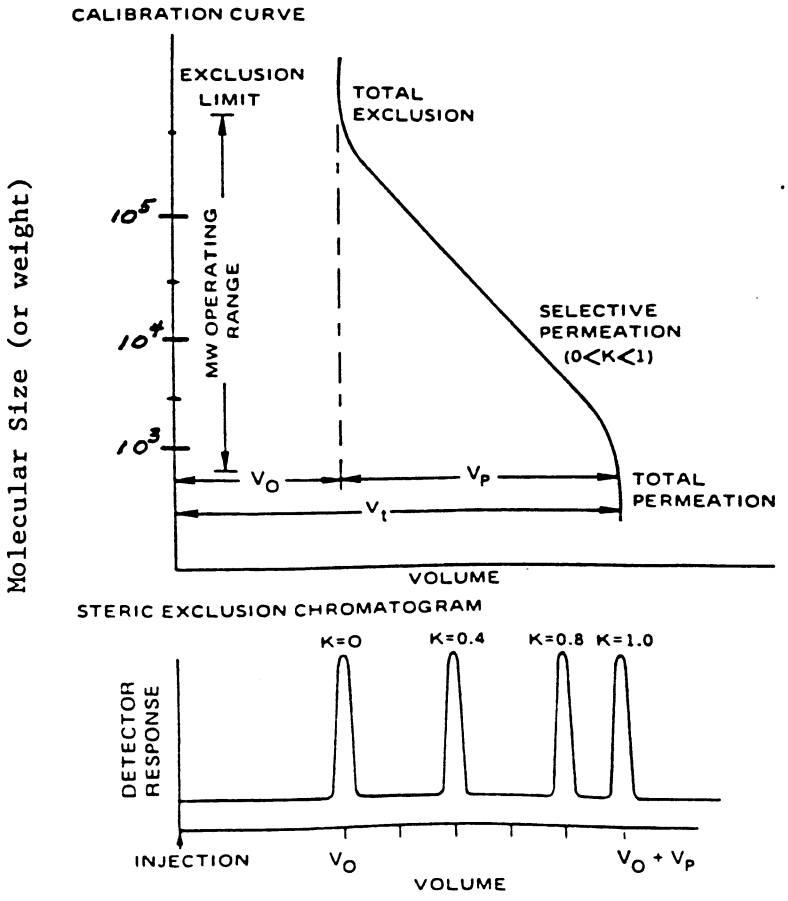
Table 8 lists column separation ranges and exclusion limits of columns for GPC (H type) and GFC (PW and SW types). As we can see, the larger the pore size, the larger the molecular weight separation range. If we wanted to study an organic soluble compound of MW 500 we would choose a 1000H column. If we want to separate components in the 25,000 MW range we would choose a 3000H. If we have a large molecular weight range we commonly use columns of differing pore sizes connected in series. Scientists doing research into polymer M.W. distributions use a set of columns (e.g., 400DH, 3000H, 2000H and 1000H for organic polymers).

To increase resolution in SEC we can use a number of columns of the same pore size in series. A rough rule of thumb is: to get baseline resolution between two compounds, we have to have a molecular weight difference of a factor of two.

Polymers can be divided into two major categories, mono-disperse (single molecular weight), and polydisperse (multiple molecular weights). Biopolymers often have specific sizes and molecular weights, so they are simply chromatographed and their elution volume is compared with the elution volume of marker biopolymers of known molecular weight. On the other hand, industrial polymers are often polydisperse, and their molecular weight can vary greatly depending on the synthesis conditions. The molecular weight and width of the M.W. distribution greatly affects their physical properties such as elasticity, tensile strength, hardness and heat resistance properties. Therefore, manufacturers of polymers and products which contain polymers want to know something about these compounds' molecular weight distributions. On the other hand, Life Scientists can often identify the molecular weight of proteins and enzymes using calibration curves.

Figure 9 shows a calibration curve for size exclusion columns. A series of standards of known molecular weights, which have been determined by alternative techniques such as light scattering, are used to calibrate a column. The plot consists of the log of molecular size or weight versus elution volume. Components of high molecular weight, which are totally excluded from the column packing, are found at the exclusion volume (elute first) and components which are very small and permeate through every pore are found at the permeation volume. In order to get separation you should choose a column with a pore size which separates components in the selective permeation range. If more resolution is needed, use a series of columns. A series of columns is also needed to separate wide ranges of molecular weight.

Once a column is calibrated using standards, the molecular weight range of a series of components can be determined.



Typical Calibration Curve and Corresponding Chromatogram for Exclusion Chromatography

FIGURE 9

Applications Types:
Size exclusion chromatography is applied to a number of analysis problems. They include:

1. **Molecular Weight Distribution Studies:**
   A column is calibrated with standard M.W. compounds. The unknown M.W. distribution can then be determined. This M.W. distribution is then related to the physical properties of the polymer for synthetic polymers. Biochemists calibrate with protein standards and are able to define approximate M.W. of unknown proteins.

2. **Finger-Printing:**
   A quick quality control test can be done with SEC separations of industrial mixtures. Bad products can thus be quickly determined.

3. **Fractionations:**
   Complex industrial mixtures such as paint, ink and packaging materials can be fractioned into the polymer and its additives, which can then be studied by other techniques.

4. **Sample Clean-Up:**
   When studying a complex mixture with matrix problems, small molecules can be separated from large molecules using size exclusion. For example, antioxidants in vegetable oils can thus be determined.

5. **Process Control:**
   SEC can be used to study and control an industrial polymerization process by taking samples at different times.

Gel Permeation Chromatography:
Gel permeation chromatography is used for the separation of organic soluble polymers. Figure 10 (next page) shows the calibration curve for the TSSK H type column using polystyrene molecular weight standards.

CALIBRATION CURVE

TSK TYPE H COLUMNS (7.5mm X 60 cm)

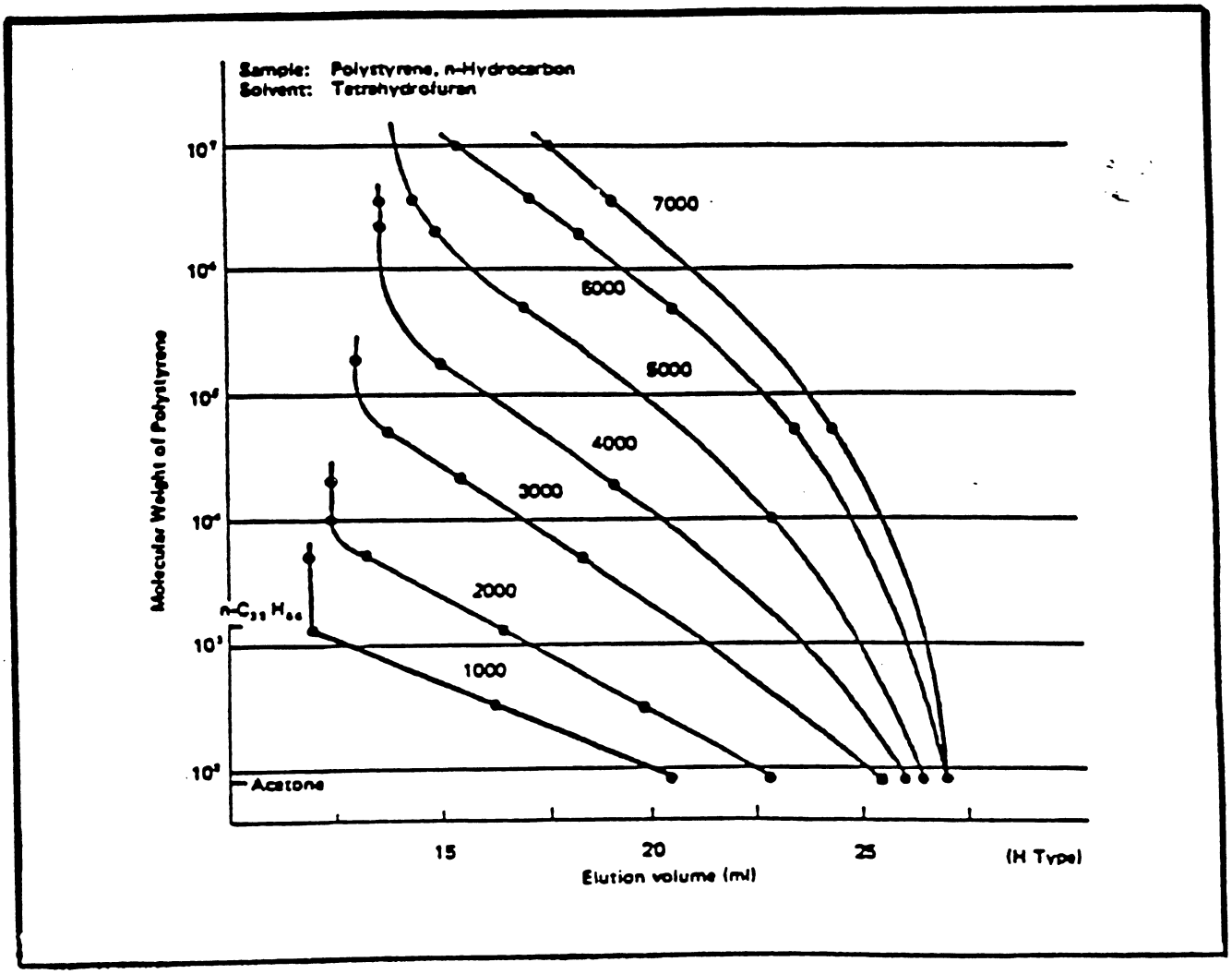


FIGURE 10

These curves are useful for choosing GPC columns. Choose the molecular weight range you want to study and draw a line over to the calibration curve, then draw a line down to the elution volume. For two components you can look at the elution volume difference.

Using a series of columns we can get baseline separation of large molecular weight ranges. Figure 11 is the calibration curve for 2000H, 3000H, and 4000H in series.

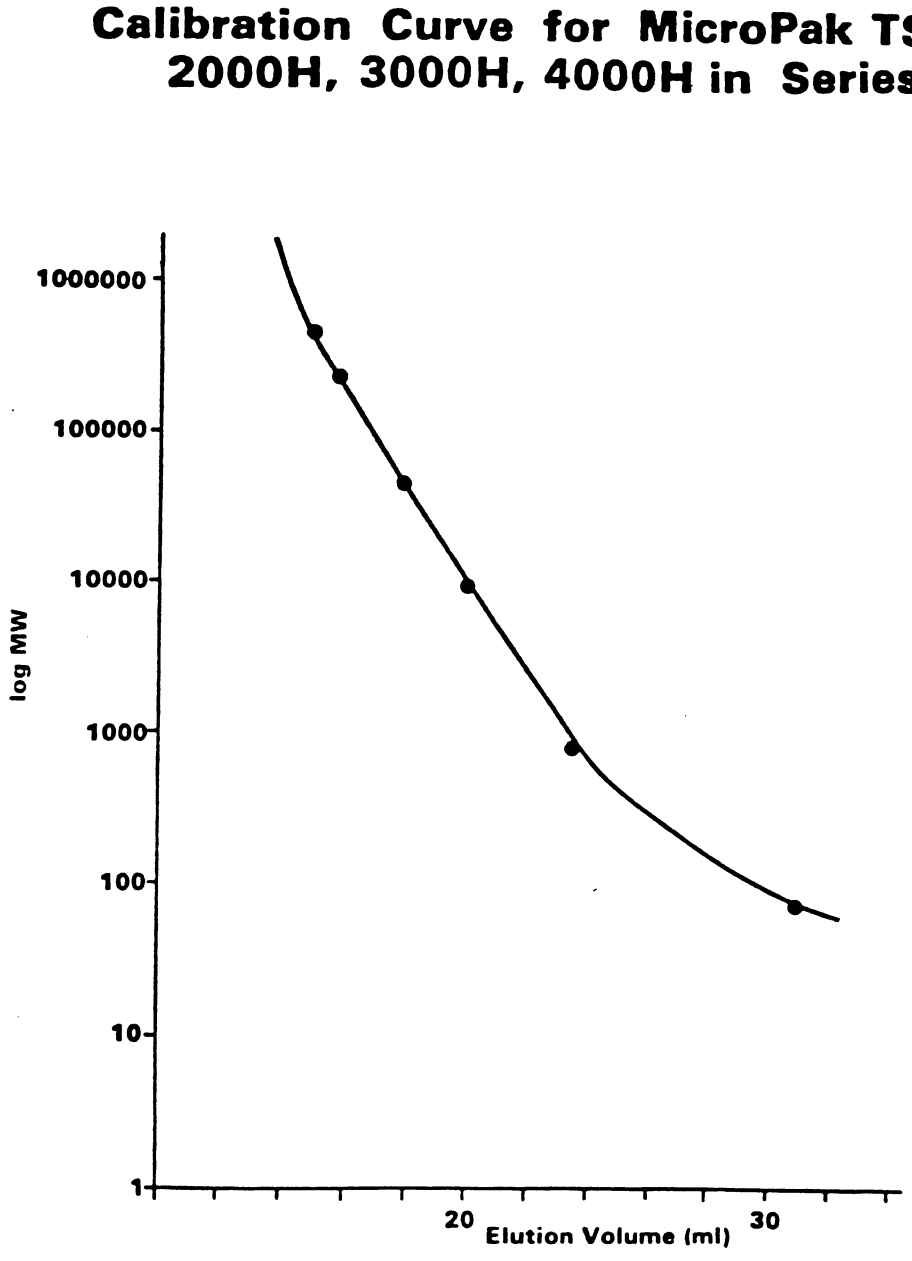


FIGURE 11

GPC is the most commonly applied to molecular weight distribution studies. Figure 12 shows the chromatogram of poly (styrene acrylonitrile) Copolymer with an MW of 100,000. Note: two detectors are used, UV (254 mm) and refractive index to study the polymer. The proportion of polystyrene monomer in the polymer can be determined.

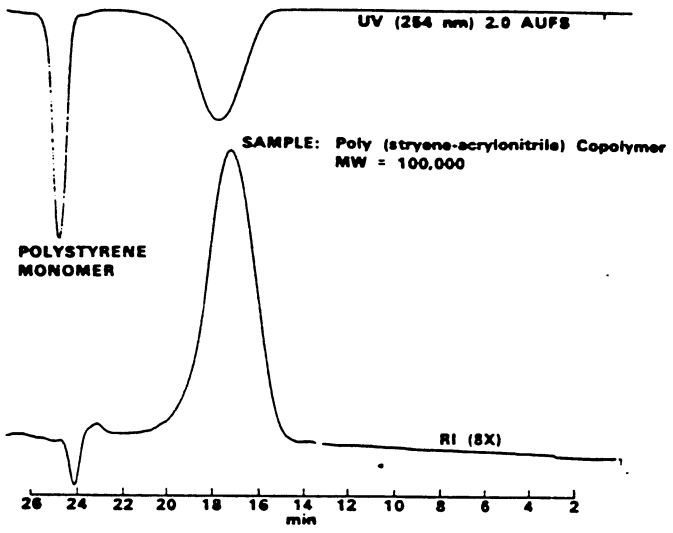


FIGURE 12

INSTRUMENT:	Varian Model 5010 LC
COLUMN:	MicroPak TSK Gel Type GMH6 (7.5 mm x 60 cm)
MOBILE PHASE:	THF
FLOW RATE:	1 ml/min
LOADING:	100 µl - 1/2% m/v solution

The GMH6 column contains a mixed bed of particles with different pore sizes allowing a broad molecular weight separation.

Aromatic polyisocyanates can be studied using GPC. In Figure 13 we see the large molecular weight polymers eluting first and baseline separations of trimer, dimer and monomer. Two small pore size columns are used, allowing molecular weight separations from 200 to 3000.

SAMPLE: Aromatic Polyisocyanate (p, p' diphenylmethylene diisocyanate)
plus higher oligomer isocyanates
APPROXIMATE MOLECULAR WEIGHT: 200 to 3,000
INSTRUMENT: Varian Model 5020 LC
COLUMN: Varian MicroPak TSK Gel Type H 2000 + 1000 (7.5 mm x 60 cm)
MOBILE PHASE: THF
FLOW: 1 ml/min
CHART SPEED: 1 cm/min
LOADING: 10 µl ~9% w/v solution
DETECTOR: Refractive Index Detector
Attn 16X

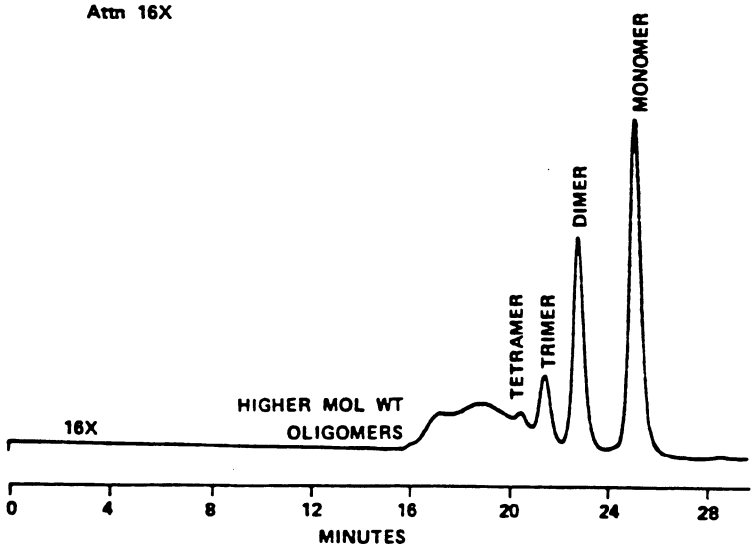
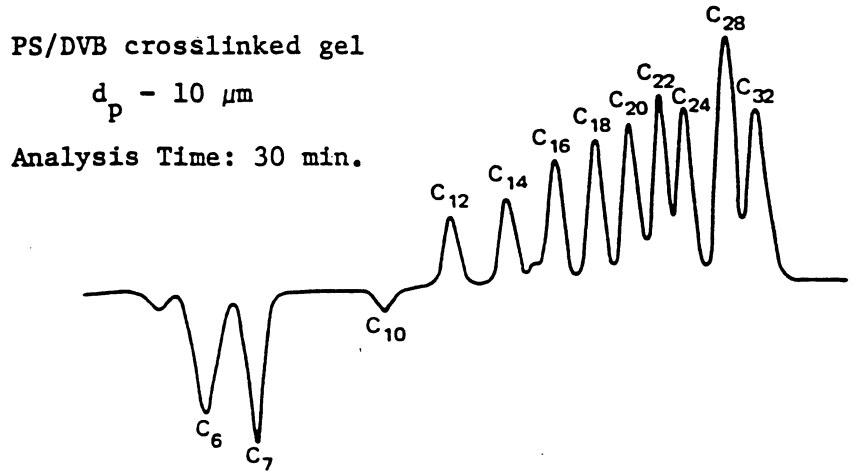


FIGURE 13

Another example of the use of SEC for small molecules (M.W. <2000) is shown in Figure 14. Here we see the separation of small normal paraffins on a TSK 1000H column (2 x 60 cm). We are able to separate the paraffins from C_6 to C_{32}. Note that C_6 and C_7 are negative peaks because they have a lower refractive index than the THF solvent.



Exclusion Separation of Normal Paraffins on TSK Gel. Column: TSK Gel G-1000H; dimensions: 2 x (610 mm x 8 mm); mobile phase: tetrahydrofuran; column temp.: room temp.; separation time: 30 min.[13] *(Reprinted with permission of Toyo Soda Mfg. Ltd., Japan.)*

FIGURE 14

GPC can be used for the fractionation of complex mixtures, especially coatings, paint, varnishes, lacquers, water based paints and hot applied polymers. Shown in Figure 15 is an adhesive polymer formulation of sytrene butadiene rubber (SBR) and polyterpene resin along with several additives, such as antioxidants and solvents. Identification can be obtained by collecting fractions during separation. Alternative techniques such as spectroscopic techniques, etc., can then be used.

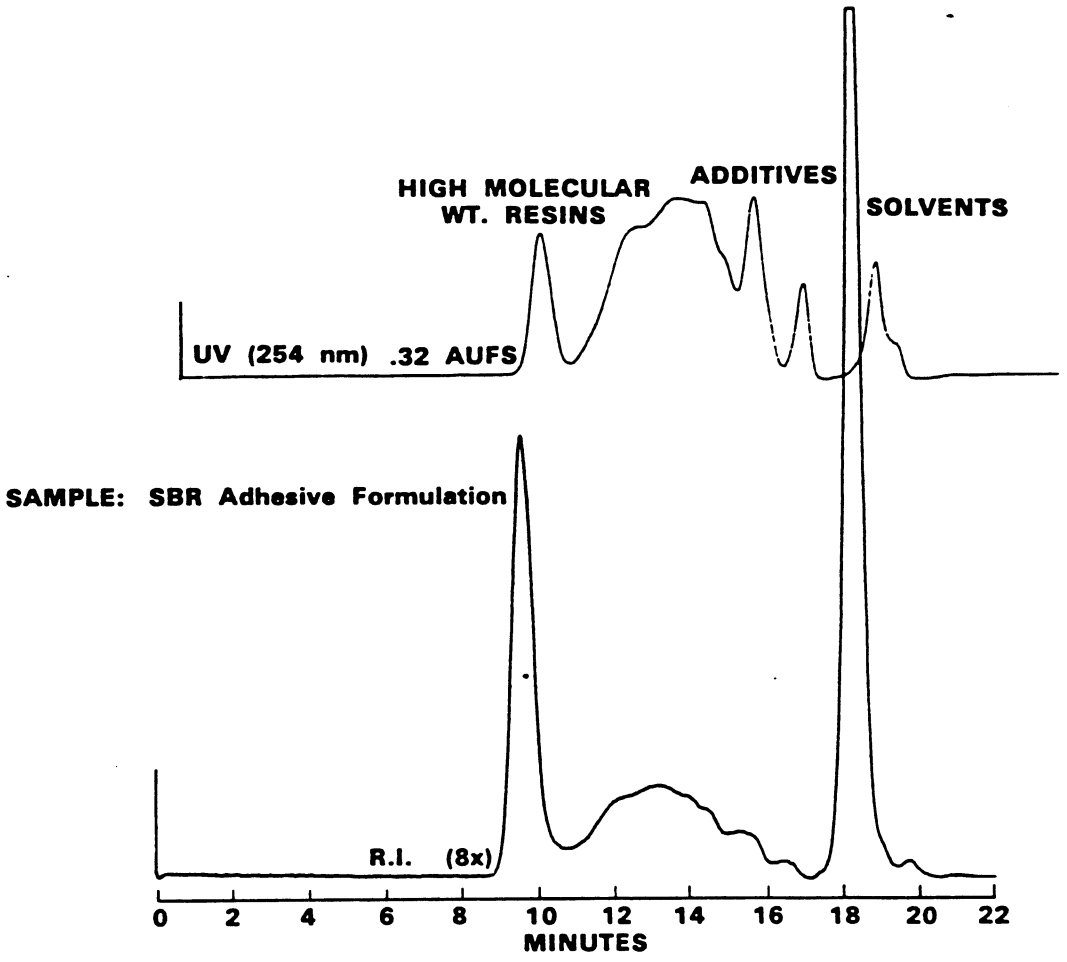


FIGURE 15

COLUMNS:	MicroPak TSK Gel, 3000H + 2000H + 1000H (7.5 mm x 30 cm)
MOBILE PHASE:	THF
FLOW:	1.5 ml/min
LOADING:	10 µl - 5% w/v solution

A simple application of GPC is to determine a good versus a bad final product. Figure 16 shows the analysis of a polystyrene packing material showing the absence of plastizers in the bad sample.

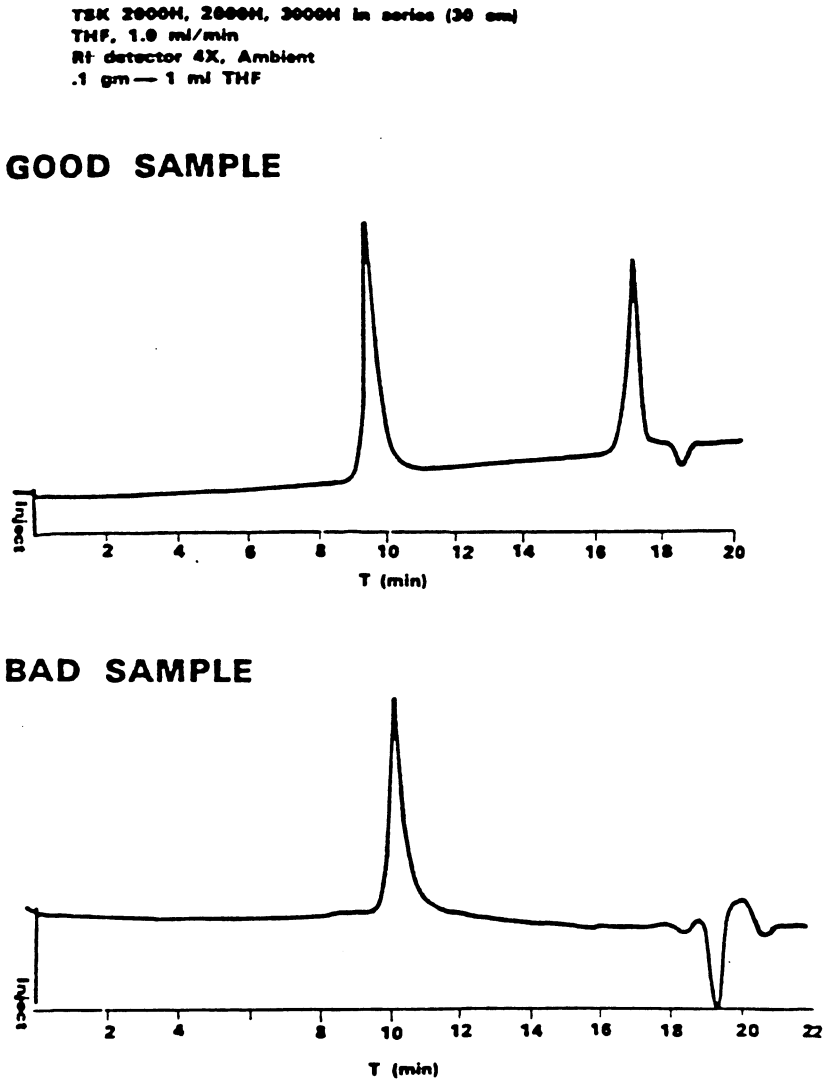


FIGURE 16

GPC can be used to look at additives in complex mixtures and to overcome matrix problems. In Figure 17 is seen the separation of BHT, BHA antioxidants from soybean oil. The BHT and BHA come out at the permeation volume, while larger triglycerides come out earlier.

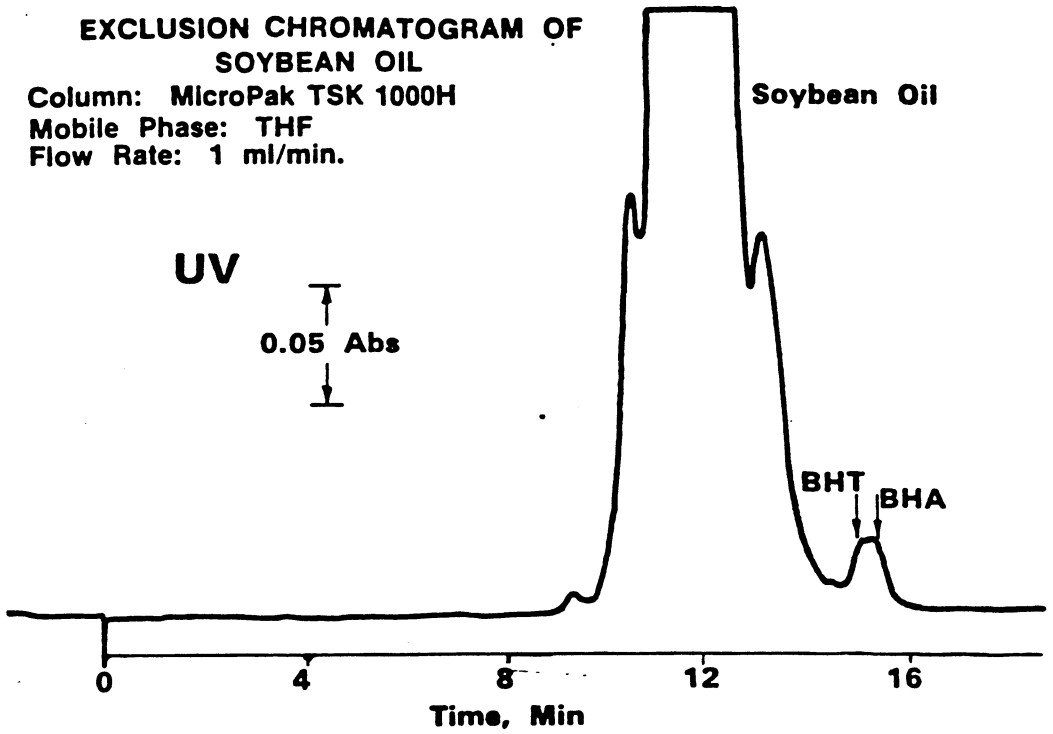


FIGURE 17

Gel Filtration Chromatography:
Gel filtration chromatography (GFC) is used for separation of aqueous polymers. There are two types of GFC columns, those for synthetic polymers (TSK PW type) and those for biopolymers (TSK SW type).

The PW type uses packings with polyether groups bonded to polystyrene divinyl benzene. These columns are used for the separation of a wide range of water soluble synthetic polymers (poly (acrylic acid) polymers, polyvinyl, alcohols and poly (vinyl pyrollidones, etc.).

Shown in Figure 18 are calibration curves using polyethylene glycol standards (dextrans are used for higher molecular weights).

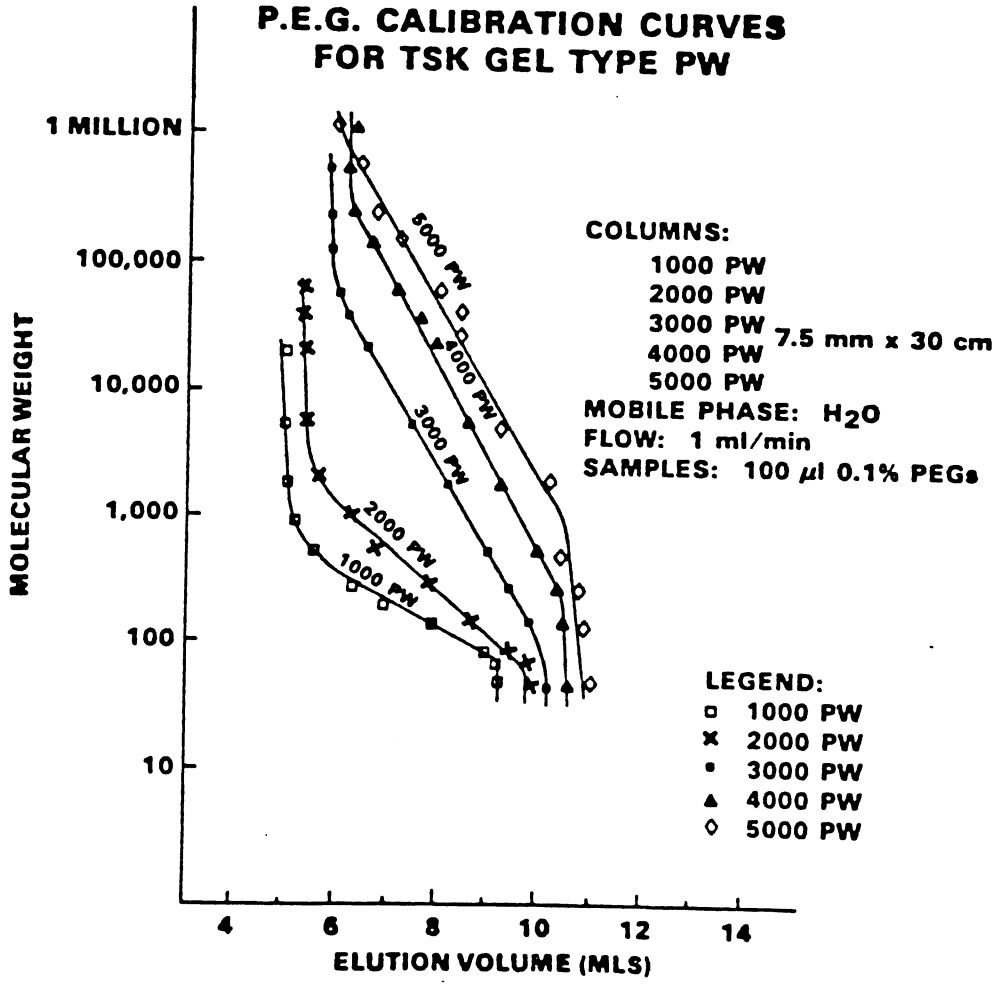


FIGURE 18

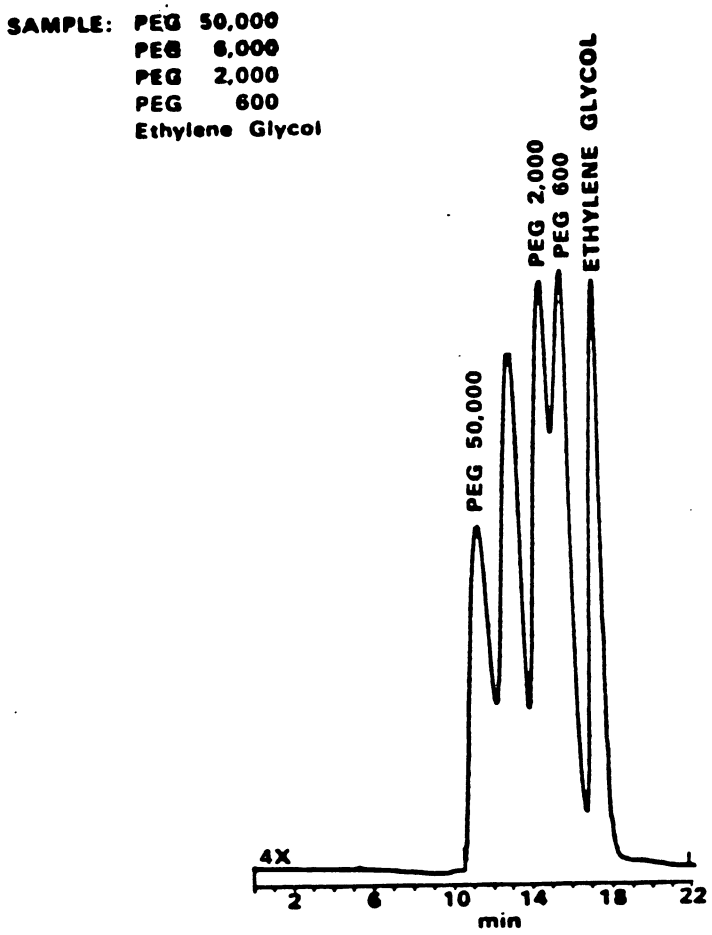


FIGURE 19

COLUMN:	3000PW (7.5 mm x 60 cm)
MOBILE PHASE:	H_2O
FLOW:	1.1 ml/min
DETECTOR:	RI
TEMPERATURE:	25°C
LOADING:	100 µl 1% w/v solution
PRESSURE:	10 Atm
CHART SPEED:	0.5 cm/min

Figure 19 shows a separation of polyethylene glycol standards on 3000PW (7.8 mm x 60 cm). Note how baseline separation is not achieved between differences of factors of four between 2000 PEG and 600 PEG. The PEG 50,000 comes out at the excluded volume.

SAMPLE: POLY (ACRYLIC ACID) POLYMERS

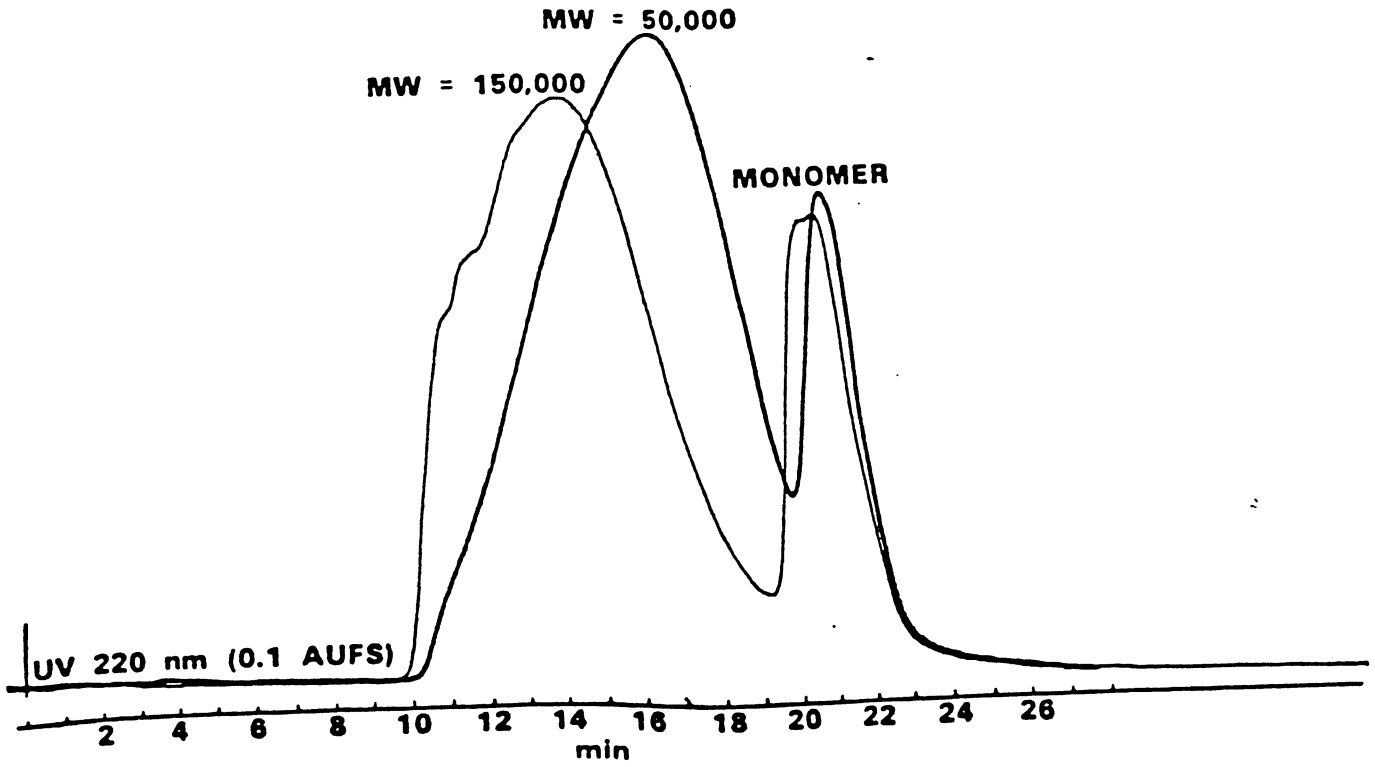


FIGURE 20

SAMPLE:	Poly (acrylic acid) polymers
COLUMN:	TSK Gel Type 5000PW (7.5 mm x 60 cm)
MOBILE PHASE:	0.1 M KH_2PO_4 0.3 M NaCl (pH 7.0)
FLOW:	1 ml/min
LOADING:	100 µl~1/2% w/v solution

Shown in Figure 20 is a poly (acrylic acid) polymer separation. Molecular weight distribution calculations can be applied to these polymers and related back to physical properties of the polymer. The mobile phase of 0.1M KH_2PO_4 and 0.3M NaCl pH (7.0) is needed to overcome secondary size exclusion effects such as ion inclusion, ion exclusion, ion exchange effects and in some cases even reverse phase effects. Because of these secondary column effects, aqueous GFC can be very difficult for polymers which are polar in nature and interact with the polyether type packing materials.

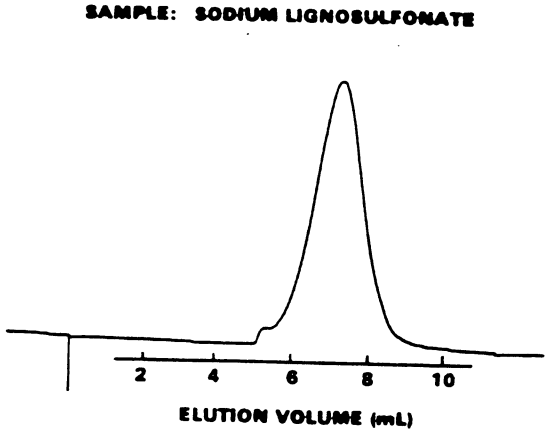


FIGURE 21

SAMPLE:	Sodium Lignosulfonate
COLUMN:	TSK Gel Type 4000PW (7.5 mm x 30 cm)
MOBILE PHASE:	0.05 N NaOH
FLOW:	1.0 ml/min
DETECTION:	A_{280}, 0.5 AUFS
LOADING:	10 μl
CHART SPEED:	1 cm/min

TSK PW packings are resin based and can be operated over a pH range of 2-13 unlike silica gel based columns with a pH range of 2-7.6. Shown in Figure 21 is the analysis of sodium lignosulfonate on a 4000PW column. The mobile phase contains 0.05N NaOH to keep sodium lignosulfonate in solution without destroying the column.

A second type of column used for gel filtration chromatography is the SW type with spherical microparticle silica with a hydrophilic bonded phase such as a diol or triol. These types of columns are used primarily for biopolymer separations. Figure 22 shows calibration curves of proteins of known molecular weight versus elution volume for the three types of SW columns, 2000SW, 3000SW and the 4000SW.

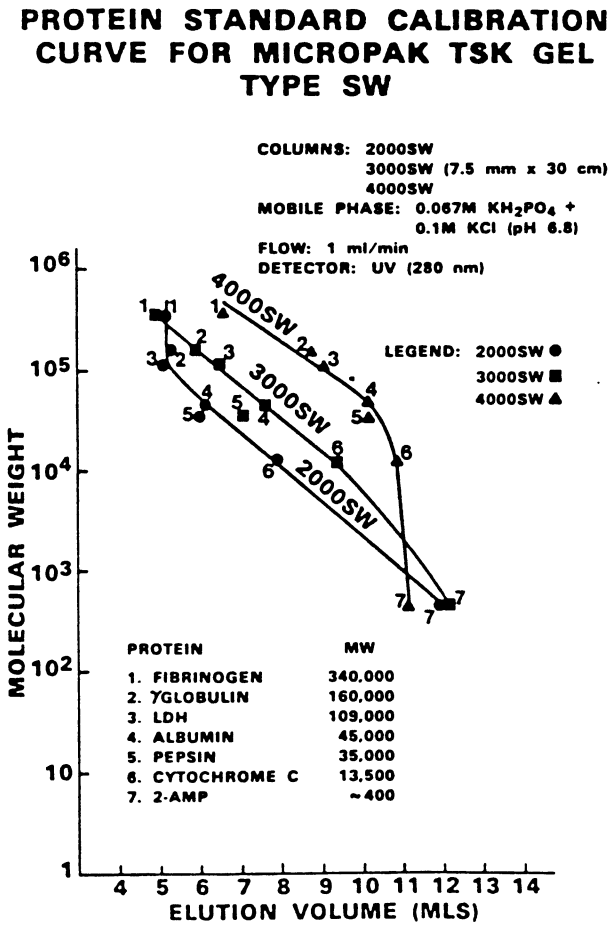
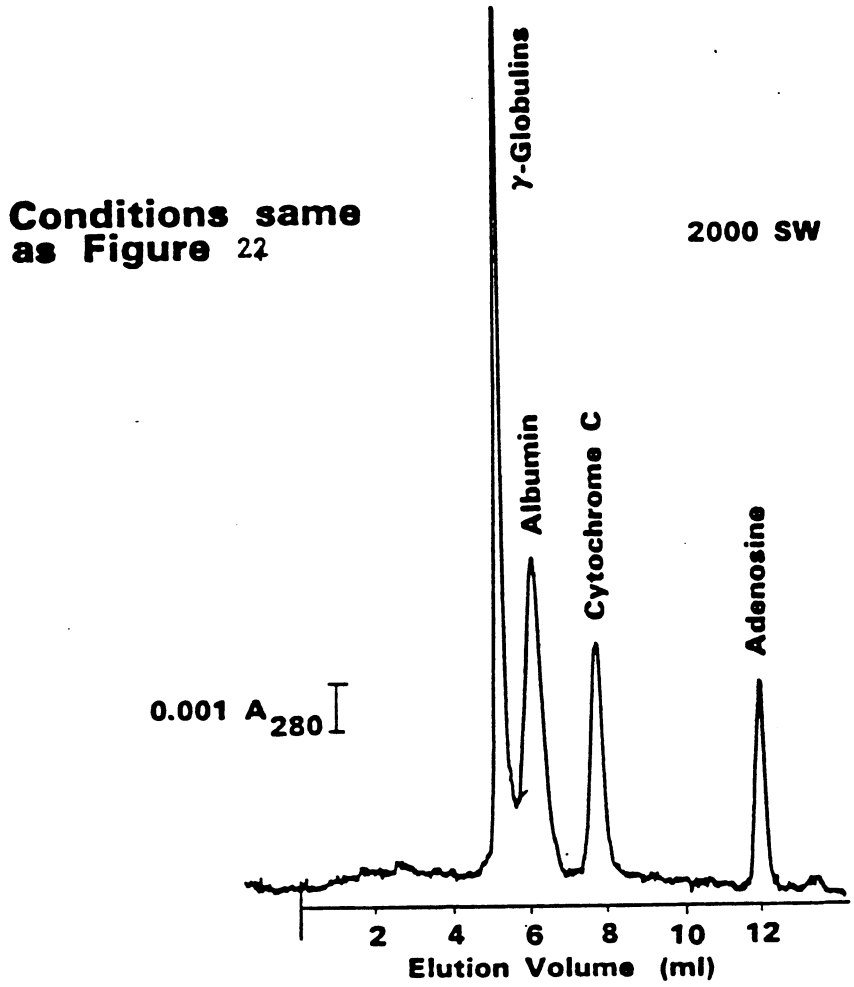


FIGURE 22

SEPARATION OF PROTEIN STANDARD MIXTURES



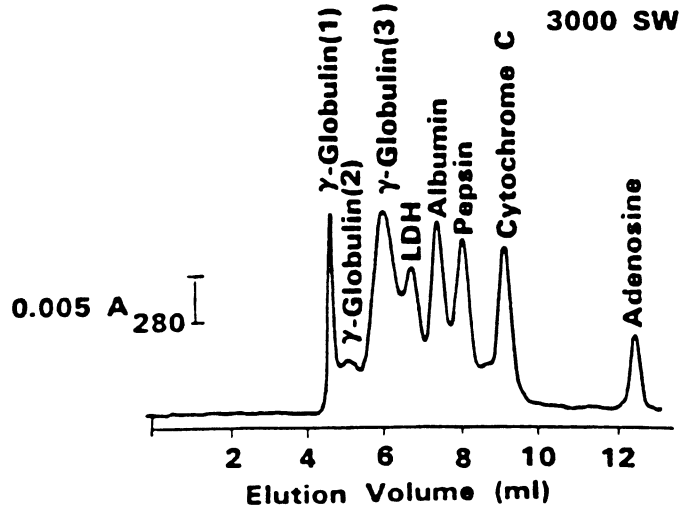


FIGURE 23

Shown in Figure 23 is the separation of these standards: proteins in 2000SW and 3000SW. Note that the 3000SW column provides higher resolution for the alpha globulins, being able to separate monomers, dimers, and trimer forms. On the 3000SW it should be noted the pepsin seems to have a higher molecular weight than albumin. This is due to denaturing of pepsin at pH 7.0 (active at pH 2-3 in globular form); thus it has a larger hydrodynamic volume.

The mobile phase used is 0.067M KH_2PO_4 and 0.1M KCl (pH 6.8). Mobile phases are chosen to keep the protein or enzyme on an active form for assays after separation. Figure 24 shows the different elution volumes of the same protein mixture on 2000SW, 3000SW and 4000SW. Note the shift of elution time and selective permeation range as the pore size increases. Choosing the column should be based on the proteins' molecular size.

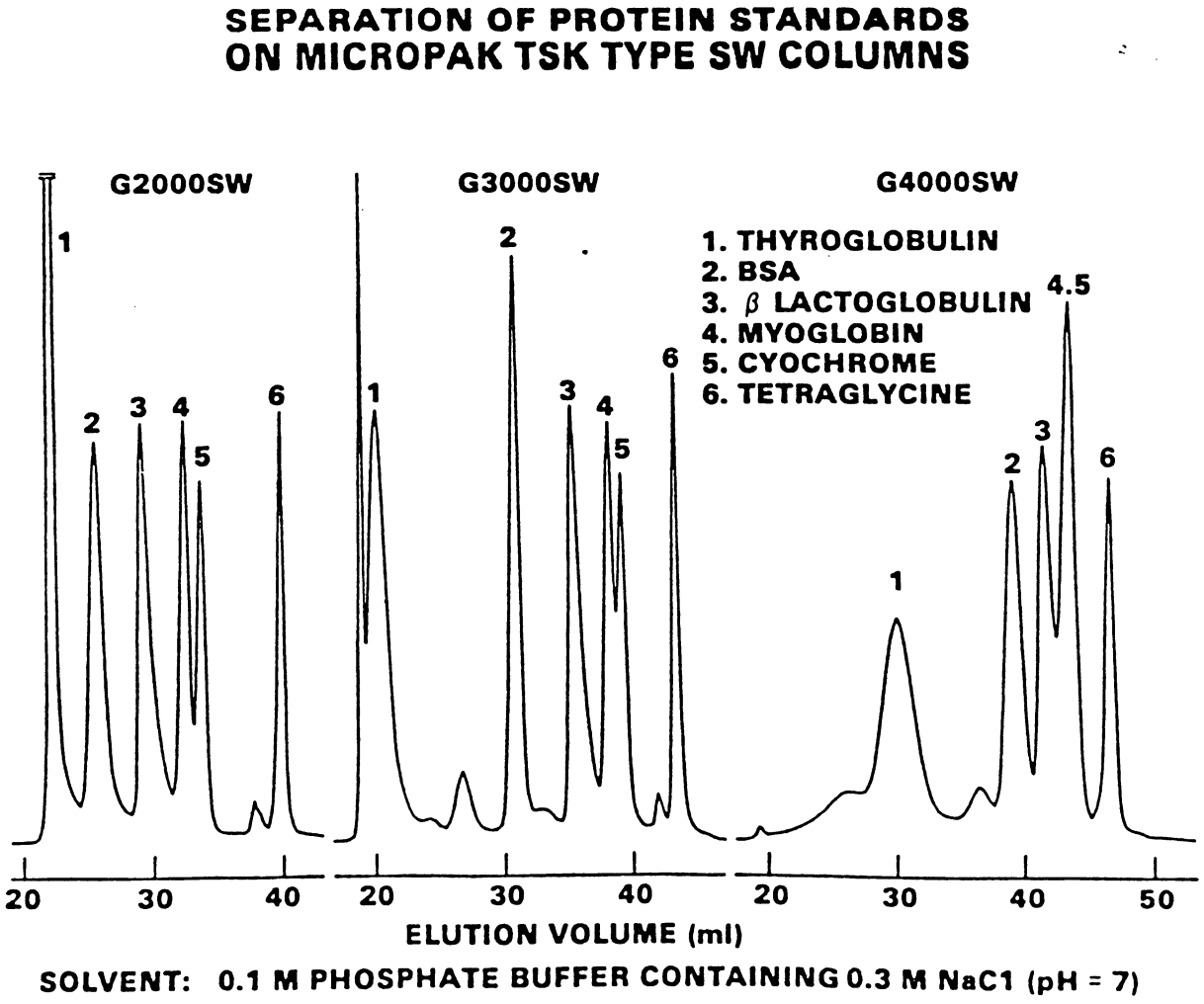


FIGURE 24

PREPARATIVE CHROMATOGRAPHY

- HPLC is an excellent way to purify a compound for secondary analysis such as IR, NMR, etc. Preparative chromatography is defined by the size of the sample. Table 1 shows the column diameter versus sample loading. As the diameter of the column increases, the capacity (loading) increases and the resolution decreases. Normally we do analytical chromatography with sample sizes of 5 ng to 100 µg. Our choice of column is based on sample size and resolution needed. We can choose a column ranging from 1 mm to 4.6 mm I.D. For a preparative separation we are interested in increased capacity, since we want to collect the maximum quantity of material. Using conventional chromatographs we are able to do what we call semi-preparative chromatography (up to 100 mg) with column diameters of up to 10 mm I.D. A special instrument is required to do true preparative work (quantities more than a gram), but these types of instruments cannot do conventional chromatography.

Typical Sample Loads for Various HPLC Columns

Separation mode	Recommended column inside diameter (mm) by sample load				
	5 ng-0.2 µg (analyt)	1 µg-100 µg (analyt)	100 µg-5 mg (semiprep)	100 mg (semiprep)	1 g (prep)
Reversed or normal phase and ion exchange GPC, GFC	1.0 —	2.0 —	4.0-4.6 7.5	8 21	21 Large

FIGURE 1

In developing a preparative analysis we first develop an analytical separation to find the solvent conditions for the separation. In Figure 2 we see an analytical separation of Capsaicins. Capsaicins are chemical compounds which produce the characteristic pungent (hot) taste of pepper. Using NMR and MS we can identify these compounds. In order to get enough compound to do NMR we need to do a semi-preparative separation by scaling the separation to an 8 mm semi-preparative column, as shown in Figure 3 (next page). This also requires increasing the flow rate.

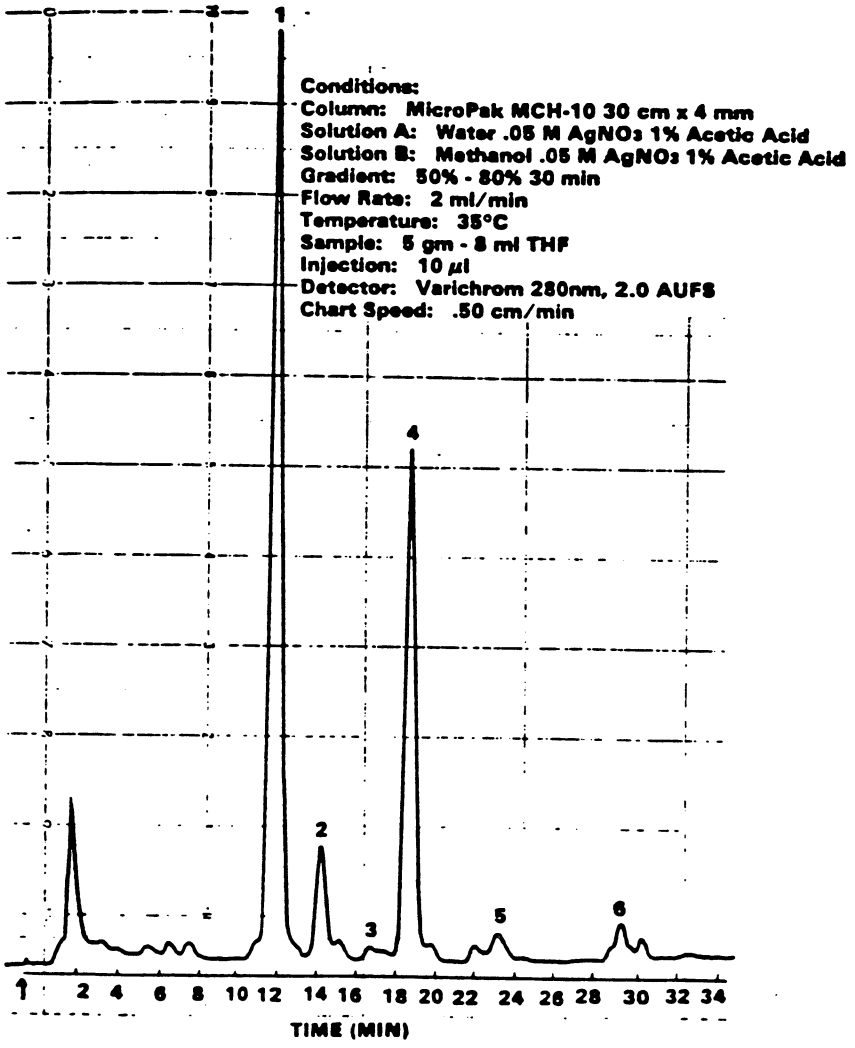


FIGURE 2

MANNHEIMER OLEO RESINS

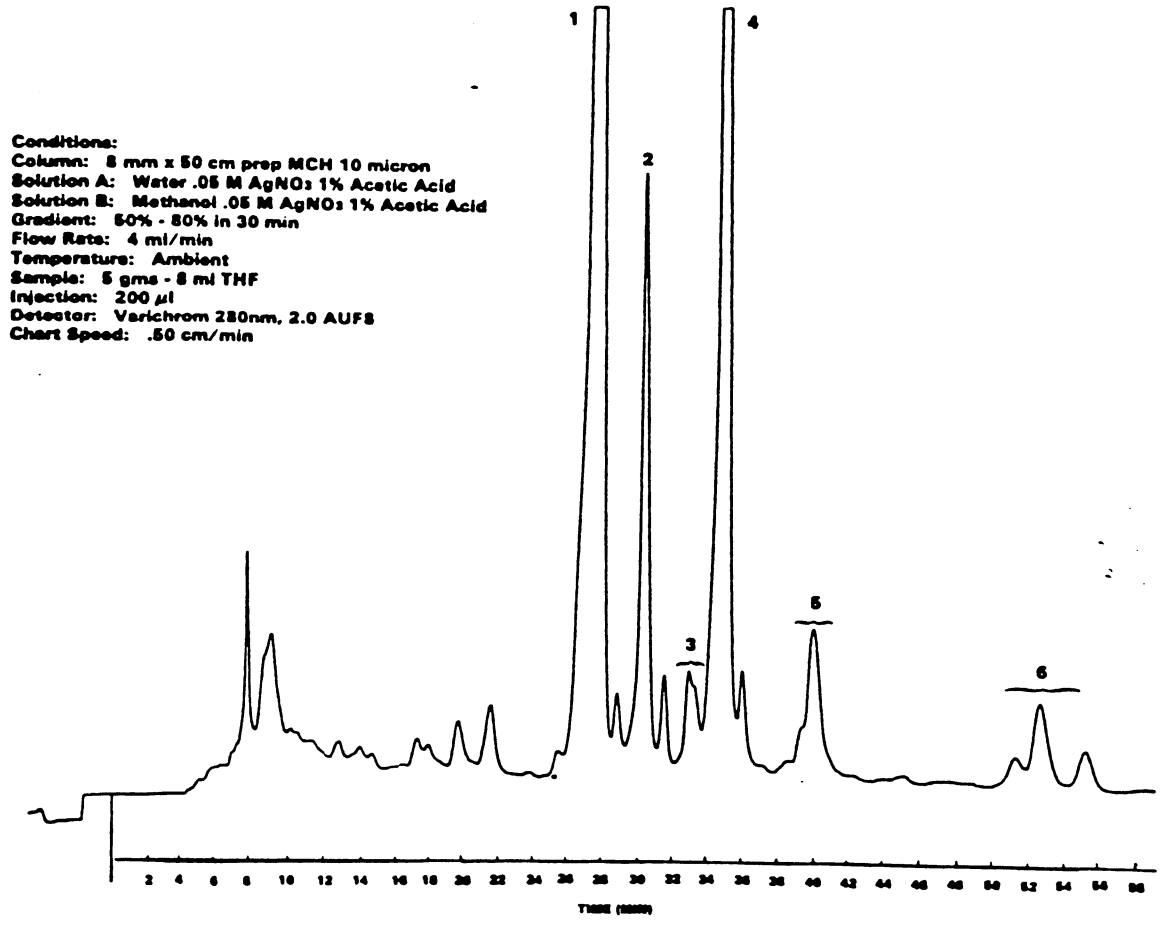


FIGURE 3

MANNHEIMER

Size exclusion columns have a very high capacity and can be used for the collection of mg quantities of proteins and enzymes. A fraction collector is usually used to collect the fractions, but a 6-position rotary value can be made to collect a fraction from repetitive injections, as shown in Figure 4.

SCHEMATIC PRESENTATION OF CHROMATOGRAPHY SYSTEM FOR PREPARATIVE CHROMATOGRAPHY USING REPETITIVE INJECTIONS

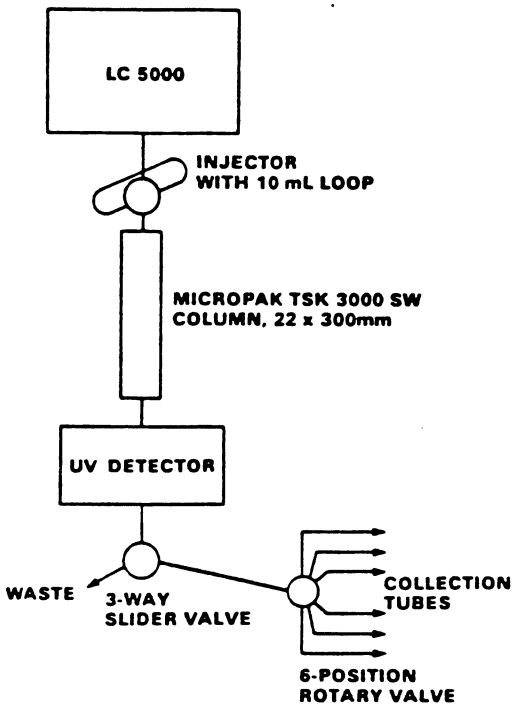


FIGURE 4

A large volume loop is used to inject a concentrated sample onto the column. The fraction is collected at a specified point in the run using a valving system.

Figure 5 shows the collection of fractions of each peak during a protein separation. These protein fractions can then be studied individually for their structures and activities.

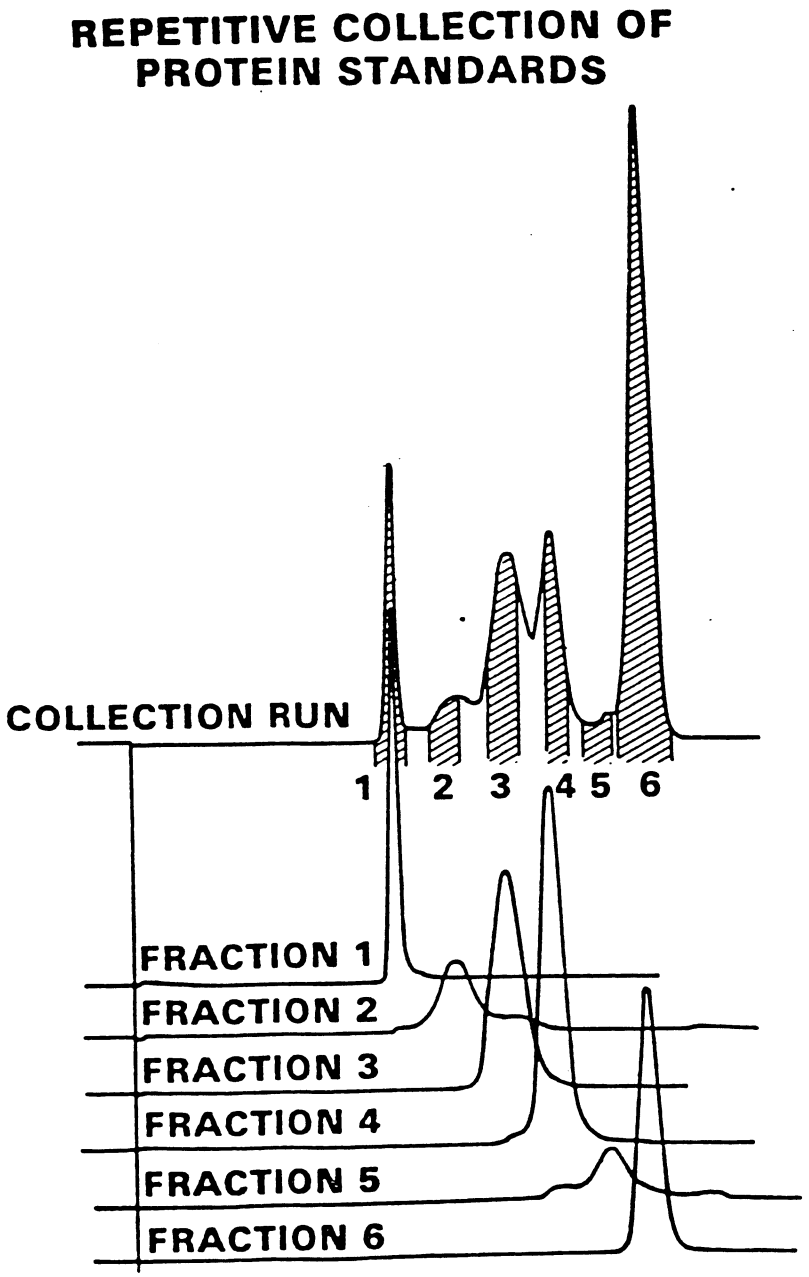


FIGURE 5

Some general considerations when doing semi-preparative HPLC are as following:

1. RI detectors are usually used due to their insensitivity, but variable wavelength UV detectors can be used if they are detuned.

2. The solvent may require degassing because it is recommended that you remove the back pressure restrictor to reduce volume. However, three or four feet of 0.009" 1.0. tubing will generate pressure on the flow cell without adding significant volume to the transfer line between the detector and fraction collector.

3. Inject the sample in the same solvent as the mobile phase to minimize secondary solvent effects.

4. Buffers for the mobile phase should be chosen carefully for ease in removal (e.g., ammonium carbonate, ammonium acetate).

5. Automated sample introduction is recommended, especially when using automated sample collection such as valving or a fraction collector.

CHROMATOGRAPHY

CALCULATIONS

AREA %:

Area % is not quantitative and is used primarily to obtain retention times of the peaks of interest prior to setting up another method of quantitation.

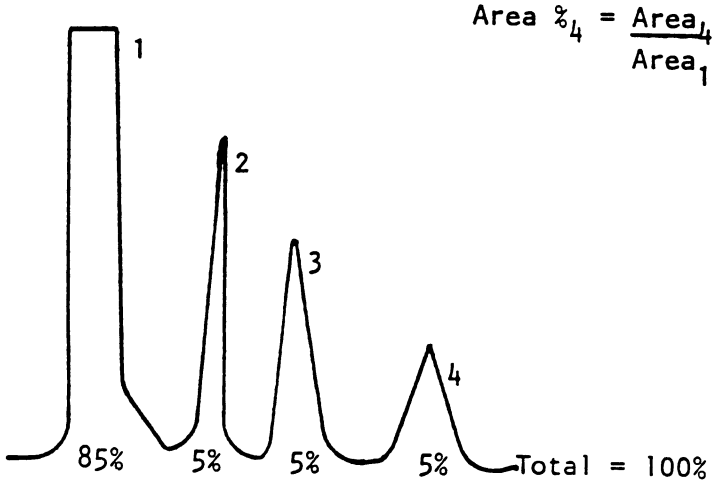
Poor quantitation is primarily due to the fact that detector response factors are not applied to the peak areas.

In addition, every peak in the chromatogram is used in the calculation, and an increase in the area of one peak leads to a decrease in the Area %'s reported for the other peaks, even if they have not changed in size. This is particularly bad if a large solvent peak is present.

AREA %

BASIC FORMULA

$$\text{Area } \%_4 = \frac{\text{Area}_4}{\text{Area}_{1+2+3+4}} \times 100$$



EXTERNAL STANDARD METHOD

Of the two primary methods of absolute quantitation (results expressed in actual amounts of analyte, not relative amounts) the external standard method is probably the easiest to understand.

The analyst first chromatographs a mixture containing known amounts of each of his components of interest, and determines the detector response for each component. The ratio of the amount of standard to the response (usually peak area) is known as the **calibration factor** for that particular component.

When analyzing an unknown, the amount of each component is obtained by multiplying the response (peak area) for that component by its calibration factor.

The simplicity of this method conceals certain experimental difficulties, the foremost of which is the need to know sample size accurately. If one injects less sample than he thinks he is injecting (8 $\mu\ell$ instead of 10 $\mu\ell$), then his results will err on the low side.

Secondly, instrument and detector response must be absolutely stable to avoid frequent recalibrations.

However, since careful work can result in reproducibilities on the order of 1-5%, which is all that is required for many analyses, the external standard method can be very useful, particularly if a suitable internal standard cannot be found.

In gas analysis, the external standard method is essential because internal standards cannot easily be introduced. However, gas sampling valves usually allow highly reproducible sample volumes. The external standard method is also used when a large variety of samples are analyzed and internal standards are not easily found.

EXTERNAL STANDARD

Advantages

1. Results can be relative or absolute.
2. Results are independent of all other peaks.
3. Detector sensitivity can be changed during a run.
4. Entire sample need not elute or be detected.
5. No internal standard is added.

Disadvantages

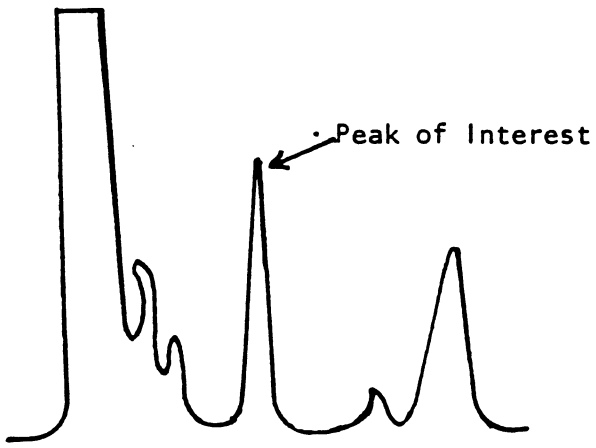
1. Results are directly dependent on detector sensitivity. Requires long term detector stability, unless frequent recalibrations are done.
2. Results are directly dependent on sample size injected.
3. Results are dependent on the care of transferring volumes and the reproducibility of extractions and derivatizations.
4. Instrument calibration is frequently required.
5. Accuracy and precision are limited by the above items.

Applications

1. Where absolute amounts or concentrations are required and an internal standard cannot be used.
2. Where the entire sample does not elute or is not detected and an internal standard cannot be used.
3. Where sample size and detector sensitivity are constant.
4. Where gas or liquid sampling valves are used. These have constant injection volumes.

EXAMPLE

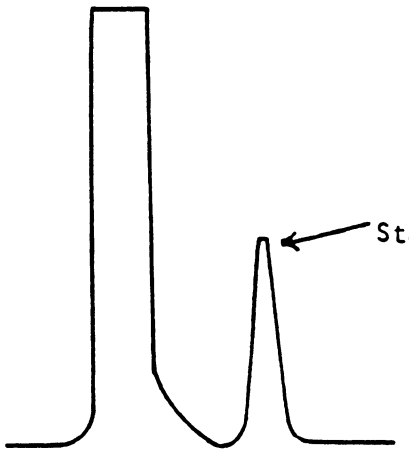
SAMPLE ANALYSIS



A calibration blend is prepared for the purpose of generating a Calibration factor (CF). For example, assume that 100 mg. of the standard is dissolved in the solvent.

Inject a **known volume** of the calibration blend.

CALIBRATION FACTOR

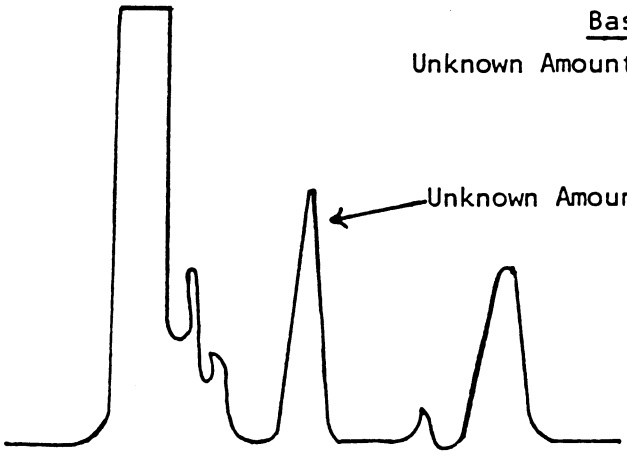


Basic Formula

$$CF = \frac{\text{Amount of Known}}{\text{Area of Known}}$$

Inject a **known volume** of the sample.

EXTERNAL STANDARD



Basic Formula

$$\text{Unknown Amount} = \text{Area} \times CF$$

$$= \frac{\text{Area of Unknown} \times \text{Amount of known}}{\text{Area of Known}}$$

If the area of unknown is the same as the known in the calibration mixture, then the amount of unknown would also be 100 mg if the volumes injected in both runs are the same. If the area is not the same, the following formula is used:

BASIC FORMULA EXTERNAL STANDARD

$$\text{Amount} = \text{Area} \times \text{CF}$$

$$\text{Amount} = \text{Area Unknown} \times \text{CF Known} = \frac{\text{Area Unknown} \times \text{Amount Known}}{\text{Area Known}}$$

External standard calculation then is the area ratio between Unknown and Known, times amount of Known.

Since the accuracy of the external standard method is directly dependent on the accuracy of the volume of sample injected, a correction factor must be applied to the result if different volumes are injected:

$$F = \frac{\text{Volume of sample injected}}{\text{Volume of standard injected}}$$

$$\text{Amount} = \frac{\text{Area unknown} \times \text{amount known}}{F \times \text{Area Known}}$$

INTERNAL STANDARD METHOD

RELATIVE RESPONSE FACTORS

To achieve accurate quantitation of different compounds in the same analysis, the difference in response of these compounds to a particular detector must be taken into account. In the external standards method, this was done with pure standards, resulting in calibration factors which could then be used to quantitate unknown samples.

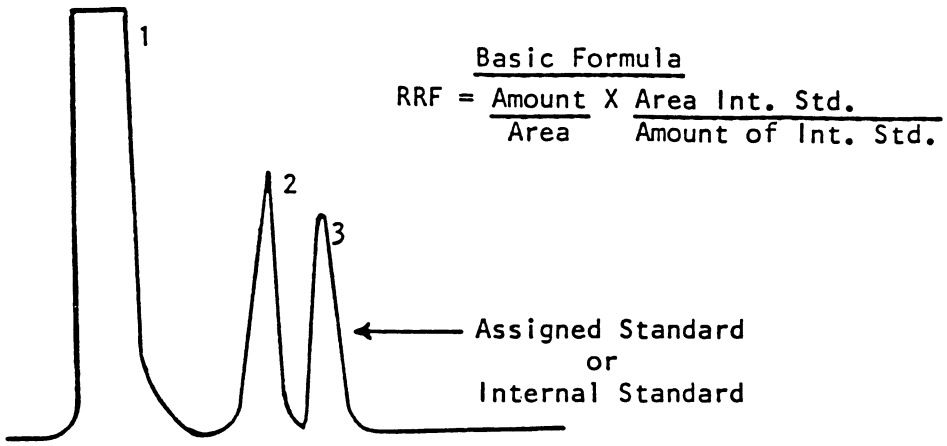
In the internal standard method and the normalized % method, this type of calibration leads to **relative response factors** (RRF). In the internal standard method, the RRF for a particular peak is calculated **relative to** the internal standard peak, which arises from a compound that has been added to the mixture.

In the normalized % method, a peak of interest already in the mixture is chosen as the peak to which all the other peaks of interest will be related. This is called the assigned standard. In either case, the RRF for the internal standard or the assigned standard is, by definition, 1.0. In both cases, the calculation for RRF's is the same, and uses the formula shown.

EXAMPLE

A calibration blend must be prepared which contains known amounts of all the peaks of interest plus either a reference peak or an internal standard peak. This leads to a chromatogram from which RRF's are calculated:

RELATIVE RESPONSE FACTOR



If the amount of Peak 2 is twice that of the internal standard (Peak 3) but the areas are the same, then the $RRF_2 = 2.0$.

INTERNAL STANDARD METHOD

The internal standard method, when possible to use, is generally the most accurate method of quantitation available to the chromatographer.

To use this method, one must find a compound that has the following properties:

a) It acts chemically like the compound of interest.

b) It must elute in an empty area of the chromatogram and be well resolved in the sample as well as the calibration mixture. It elutes near the components of interest (not absolutely necessary).

Preferably one adds the same amount of internal standard to both the calibration mixture and the unknown sample. When the calibration mixture is chromatographed, the component of interest will have a certain peak area, as will the internal standard. The ratio of these peak areas for **equal amounts** of the component of interest and the internal standard is called the **relative response factor.**

When an unknown sample is run, this ratio of areas multiplied by the relative response factor will give the amount of the component of interest. For example, if the ratio were the same as in the calibration sample, then the amount of unknown component would be the same as in the calibration sample. If the ratio were higher or lower than that found for the calibration sample, then the amount of unknown component would be correspondingly more or less.

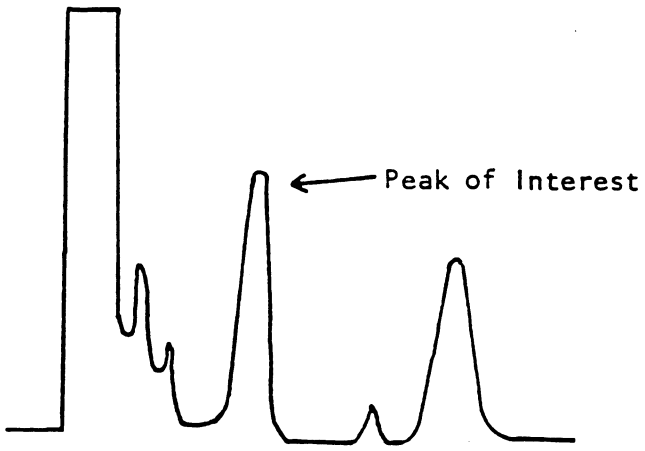
This means that the actual amounts of sample injected is not important; only the ratio of the peak areas matters.

Another important advantage of the internal standard method is that it may be added before a sample workup procedure, such as an extraction. Any losses of sample would be paralleled by the internal standard; the ratio of the peak areas would be unaffected.

INTERNAL STANDARD

Advantages	1.	Results can be extremely accurate.
	2.	Results can be relative or absolute.
	3.	Results are independent of detector sensitivity, i.e. long term variations.
	4.	Results are independent of sample size injected.
	5.	Results are independent of all other peaks (except IS).
	6.	Detector sensitivity can be changed during a run.
	7.	Entire sample need not elute or be detected.
Disadvantages	1.	An internal standard compound must be added to the sample.
	2.	Instrument calibration is required.
Applications	1.	Where highest accuracy and precision are required.
	2.	Where entire sample is not eluted or detected.
	3.	Where absolute amounts or concentrations are needed.
	4.	Where extraction efficiencies are not constant.

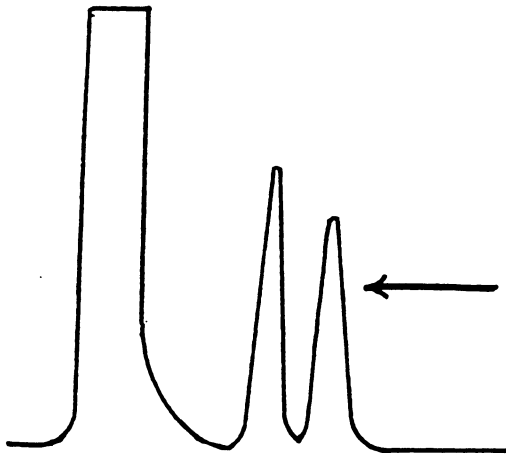
SAMPLE ANALYSIS



An internal standard component must be found to add to this mixture of peaks. The internal standard should meet the following criteria.

1. Resolved from the other sample components.
2. Not expected to occur in the unknown sample.
3. Have similar concentration to peaks to be quantitated.
4. Have a reasonable retention time.
5. Have a known purity, unless the same batch is used all the time.
6. Have a similar chemical structure if possible (not critical since RRF's will compensate for differences in detector response). This is important if extractions are done.

A binary calibration blend is prepared for the purpose of generating an RRF. For example, assume that 100 mg of each component was dissolved in the solvent.



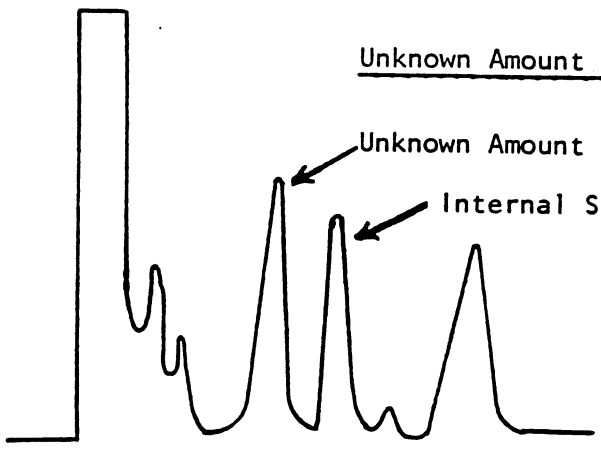
Basic Formula

$$RRF = \frac{\text{Amount}}{\text{Area}} \times \frac{\text{Area Int. Std.}}{\text{Amount Int. Std.}}$$

Assigned Standard
or
Internal Standard

Inject sample together with internal standard added.

INTERNAL STANDARD



Basic Formula

$$\text{Unknown Amount} = \frac{\text{Area of Unknown} \times \text{RRT Unknown \& Amount Int Std.}}{\text{Area Int. Std.}}$$

Unknown Amount

Internal Standard

Assume the RRF calculated to be 1.0, then if Unknown has the same area as internal standard, the Unknown is also 100 mg.

BASIC FORMULA

$$\text{Amount Unknown} = \frac{\text{Area Unknown X RRF Unknown X Amt. Int. Std.}}{\text{Area Int. Std.}}$$

Note below that this formula is similar to the external standard formula i.e. the amount of unknown is the area ratio of unknown versus known times the amount of the standard. RRF has been introduced to compensate for response differences. However, the difference between the external and internal standard methods is the fact that the standard and unknown runs are two separate runs in external standard, but together in same run in internal standard after RRF's are once found. Since internal standard is an area ratio between peaks internally within the same run, sample size variations will not affect result. Therefore, a relative standard deivation of $\pm$ 1% can be expected.

Comparison External, Internal Standard:

$$\text{Internal Standard} = \frac{\text{Area Unknown x Amt. Known (Int. Std.) x RRF Unknown}}{\text{Area Known (Int. Std.)}}$$

$$\text{External Standard} = \frac{\text{Area unknown x Amt. Known}}{\text{Area Known}}$$

SPECIAL DETECTORS

Derivatization:
In HPLC not all compounds have native absorption or fluorescence or have concentrations which can be detected using refractive index. Therefore, it is necessary to derivatize in order to detect them. The two major reasons for derivatizing are to:

1. Increase sensitivity (lower MDQ)
2. Increase selectivity of detection (increase response of components of interest over unwanted interferences and matrices).

There are two major types of derivatization in HPLC:

	ADVANTAGES	DISADVANTAGES
1.	Pre-Column Derivatization	
	-inexpensive and easy to set-up	-reproducibility is operator dependent when not automated
	-increased sensitivity and selectivity	-less chromatographic selectivity
		-interferences from side reactions
2.	Post-Column Derivatization	
	-increases sensitivity and selectivity	-requires additional hardware
	-more chromatographic selectivity because we are separating on the basis of small differences in structure of the molecule itself	
	-easily automatable and more reproducible	

Pre-Column Derivatization:
Pre-column derivatization reactions have become standard techniques for a number of analyses. Though they are decreasing in importance due to direct detection using low wavelength measurement with variable wavelength UV detectors, they cannot be totally replaced.

Figure 1 shows the separation of Phenylthiohydantoin (PTH) amino acids formed by an Edman degradation reaction used in sequencing analysis. These PTH amino acids are separated using a reverse phase column. Reverse phase is often used after derivatization due to the hydrophobic nature of the derivative.

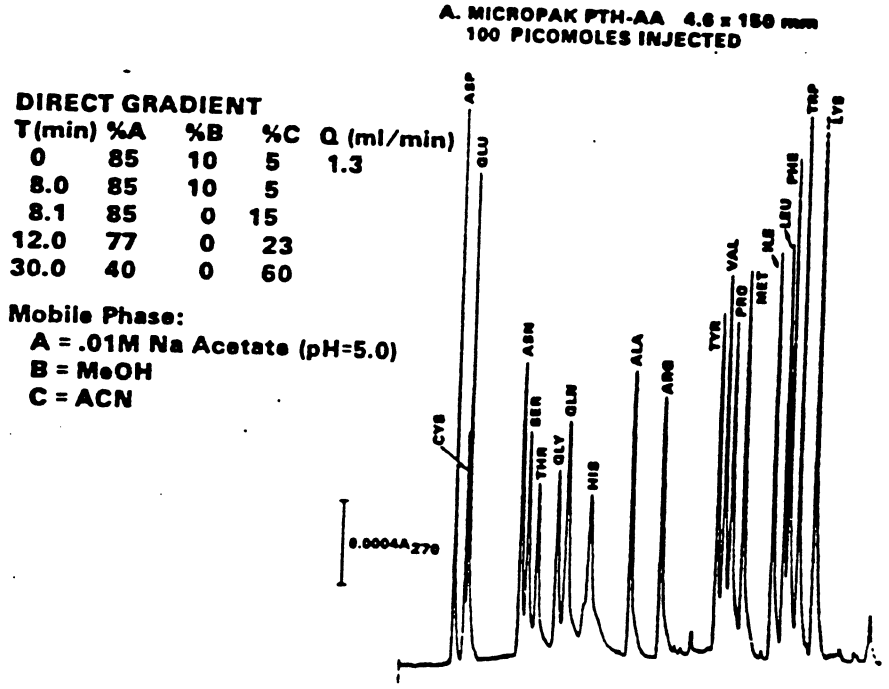


FIGURE 1

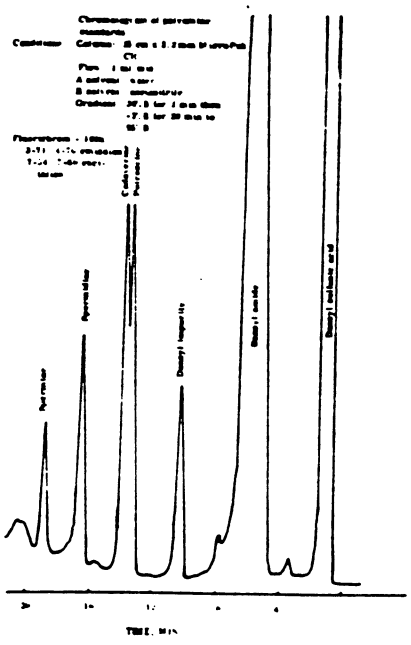


FIGURE 2

Figure 2 shows a separation of Dansyl derivatives of polyamines. The reagent dansyl chloride, is a common reagent for amines.

Fatty acids can be derivatized using 4-bromomethyl-7-methoxycoumarin to improve their detectability as shown in Figure 3.

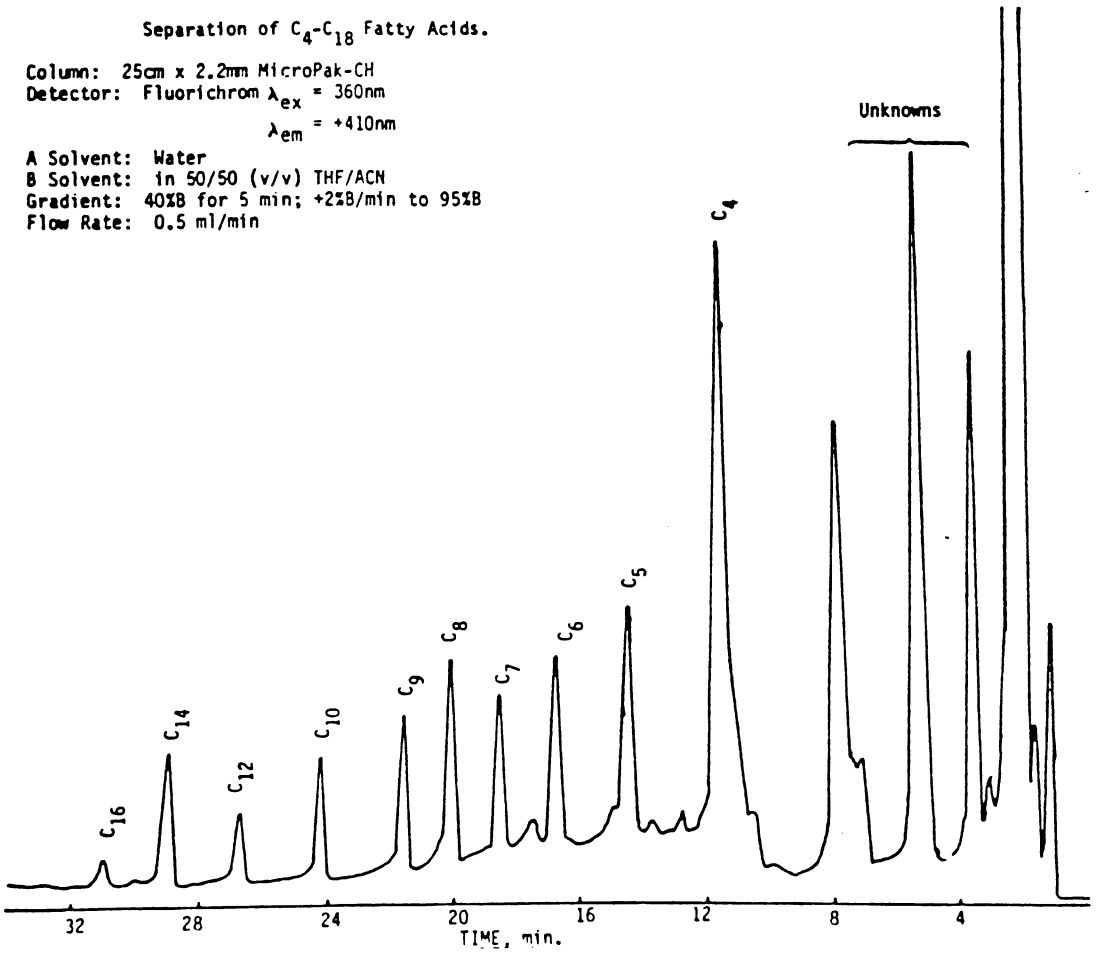


FIGURE 3

One of the major disadvantages of precolumn derivatization is that it is not easily automatable, but recently a system has been designed to increase the detectability of primary amino acids. Figure 4 shows a diagram of the system where the O-phthaladehyde is mixed with the sample prior to injection, developed through a reaction coil, and injected into a reverse phase column automatically.

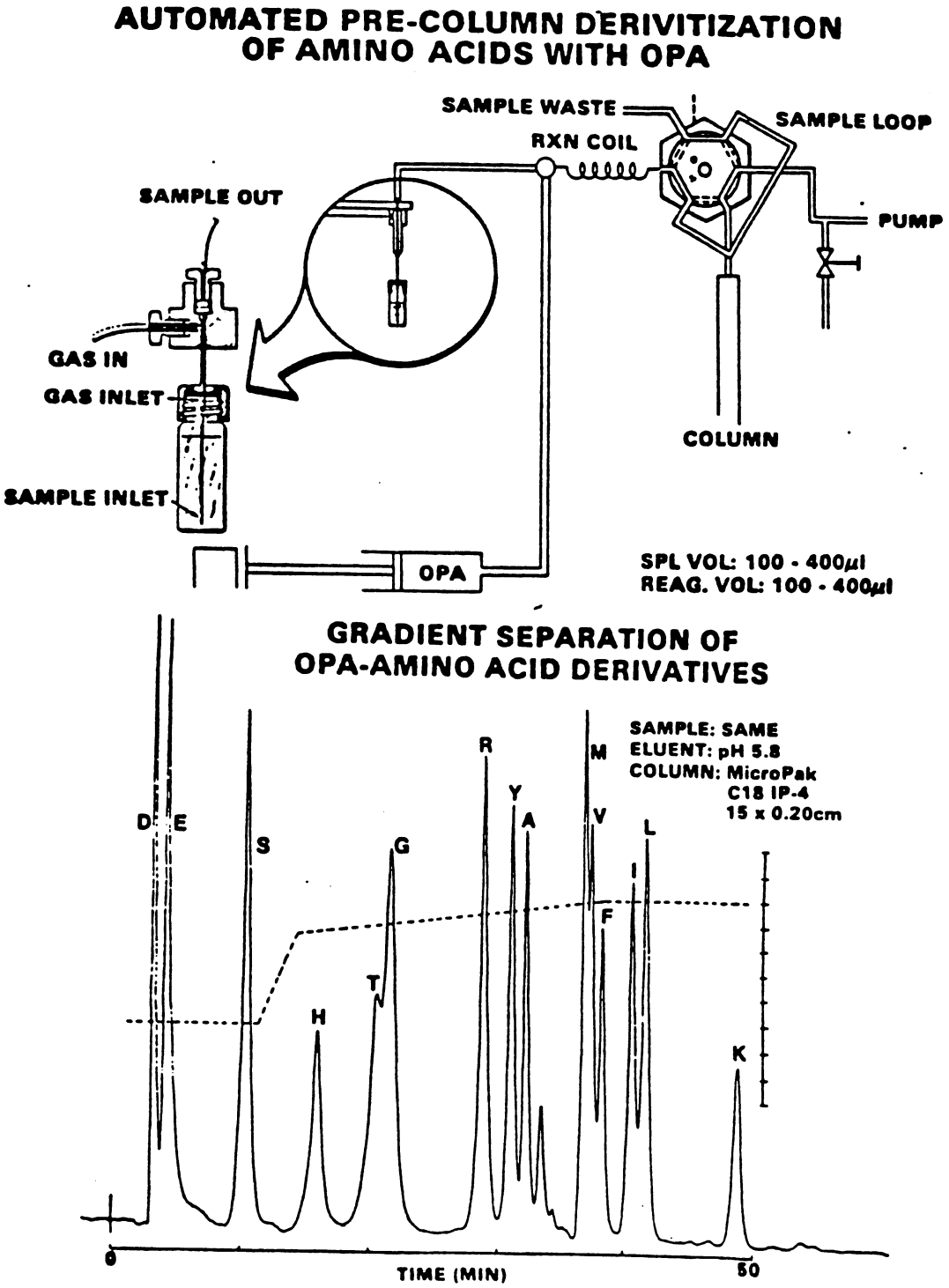


FIGURE 4

Post-Column Derivatization:
Post-column derivatization occurs after separation on the HPLC column. Figure 5 shows a diagram of a post column derivatization system. The post column hardware consists of reagent delivery hardware, reaction coil or coils, and a detector to measure the derivative. We separate components on the column according to the best mode of chromatography, then add in the reagent using a small reciprocating pump or syringe pump. The derivatization reaction takes place in a reaction coil for a specified period of time and at a specified temperature. In some cases, this can consist of a two step reaction (example: carbamate pesticides). Once the derivative is formed, it is detected in an appropriate detector (UV, fluorescence, or electrochemical).

POST COLUMN SYSTEM DIAGRAM

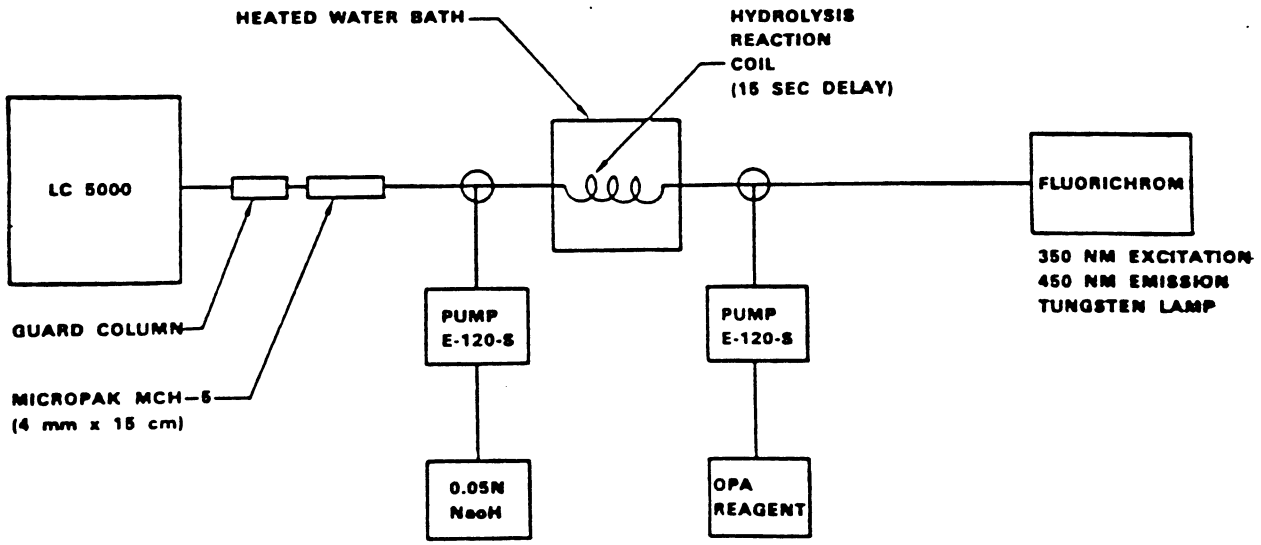


FIGURE 5

Two reagent pump post column reaction system. (This system is used for carbamate analysis).

One of the most widely used post column systems is the amino acid analysis system using the chemistry and column technology developed approximately twenty years ago. Figure 6 shows amino acid hydrolysis separation using OPA derivatization of primary amines and fluorescence detection. There are a number of reagents including OPA, ninhydrin, and fluorescamine derivatization reagents used for amino acids analysis. Thus, the Life Scientist can do these analyses on his HPLC.

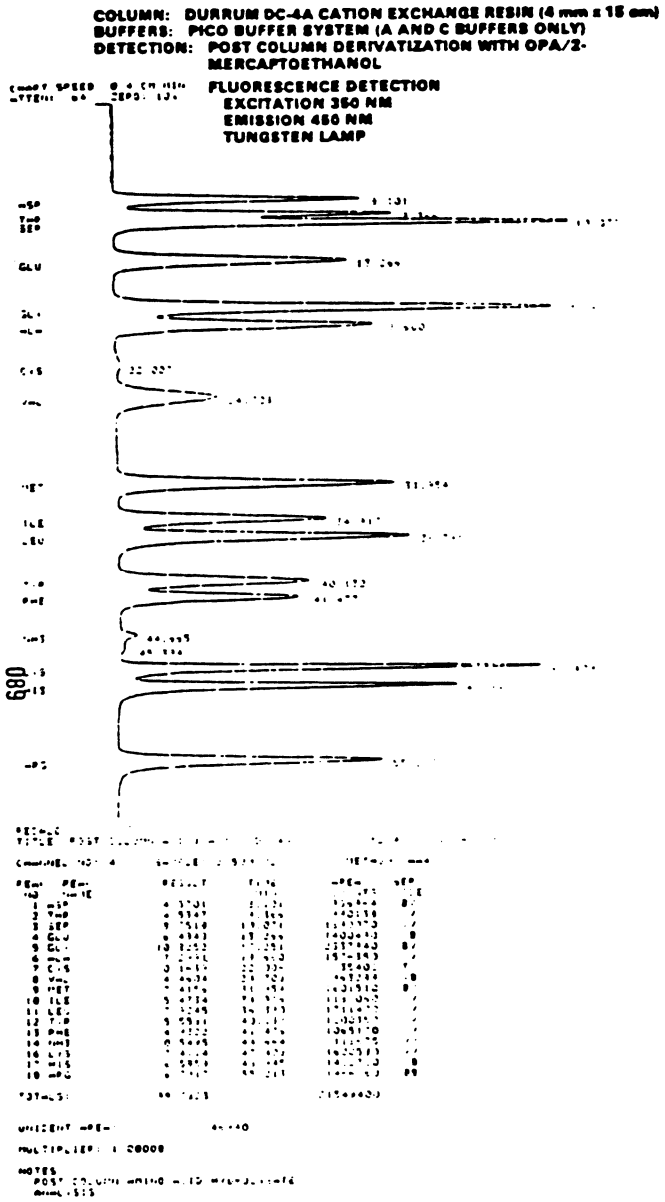
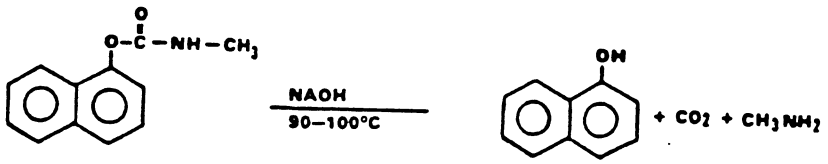


FIGURE 6

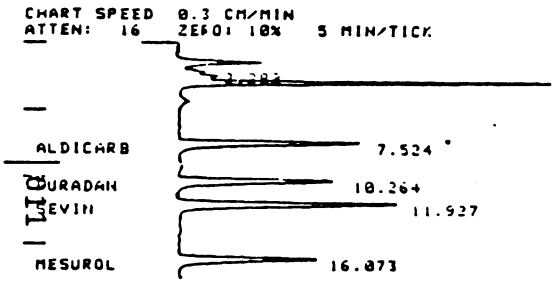
Carbamate pesticides are used for pest control in agriculture because they are readily biodegradable. They are hard to detect by UV detection because as a class they vary greatly in extinction coeffients. They are also hard to analyze by GC because they break down in the injector. Using a PCR we easily hydrolyze them with base to methyl amine, then react with OPA and detect using fluorescence. Figure 7 shows the hydrolysis of sevin, using NaOH to form the methyl amine, which then reacts with OPA and is detected. The chromatogram shows detection of aldicarb, furadan, sevin and mesurol.

CARBAMATE PESTICIDES



SEVIN, CARBARYL

SPIKED VEGETABLE EXTRACT



TITLE: CARBAMATE PESTICIDES BY PCR 16:18 30 JAN 81

CHANNEL NO: 4 SAMPLE: SPIKED VEGIE METHOD: CARB

PEAK NO	PEAK NAME	RESULT	TIME (MIN)	TIME OFFSET	AREA COUNTS	SEP CODE		WI 2 (SEC)
1		1.4070	2.288		6125	BV	?	11.40
2		2.7928	2.569		12114	VV	?	13.18
3	CARBARYL	23.4349	3.019	-0.011	102017	VB		11.78
4	ALDICARB	17.9089	7.524	-0.106	77961	BB		19.15
5	FURADAN	16.2888	10.264	-0.076	70905	BB		20.60
6	SEVIN	23.5944	11.927	-0.033	102711	BB		21.15
7	MESUROL	14.5840	16.072	0.002	63467	BB		20.95
TOTALS:		100.0000		-0.224	435320			

DETECTED PKS: 7 REJECTED PKS: 0

MULTIPLIER: 1.00000

NOISE: 4.9 OFFSET: -76

SAVED FILE: CARB049

NOTES:
CARBAMATE PESTICIDES BY POST COLUMN REACTION AND FLUORESCENCE DETECTION

FIGURE 7

Polyamines are determined in physiological fluids as markers for diseases. They can be easily analyzed using a PCR. Figure 8 shows a separation of polyamine standards with derivatizations with OPA after separation on a reverse phase column. Amines show strong interaction with residual silanols, so TMS is added to the mobile phase. The separation is done by ion pairing using heptane sulfonic acid as the ion pairing reagent.

POLYAMINES

- **PUTRESCINE (1, 4 — DIAMINOBUTANE)**

  $H_2N - (CH_2) - NH_2$

- **CADAVERINE (1, 5 — DIAMINOPENTANE)**

  $H_2N - (CH_2)_5 - NH_2$

- **SPERMIDINE**

  $H_2N - (CH_2)_4 - NH - (CH_2)_3 - NH_2$

- **SPERMINE**

  $H_2N - (CH_2)_3 - NH - (CH_2)_4 - NH - (CH_2)_3 - NH_2$

- **POLYAMINES ARE DETERMINED IN PHYSIOLOGICAL FLUIDS AS MARKERS FOR DISEASED STATES.**

DETECTION BY POST COLUMN DERIVATIZATION WITH OPA

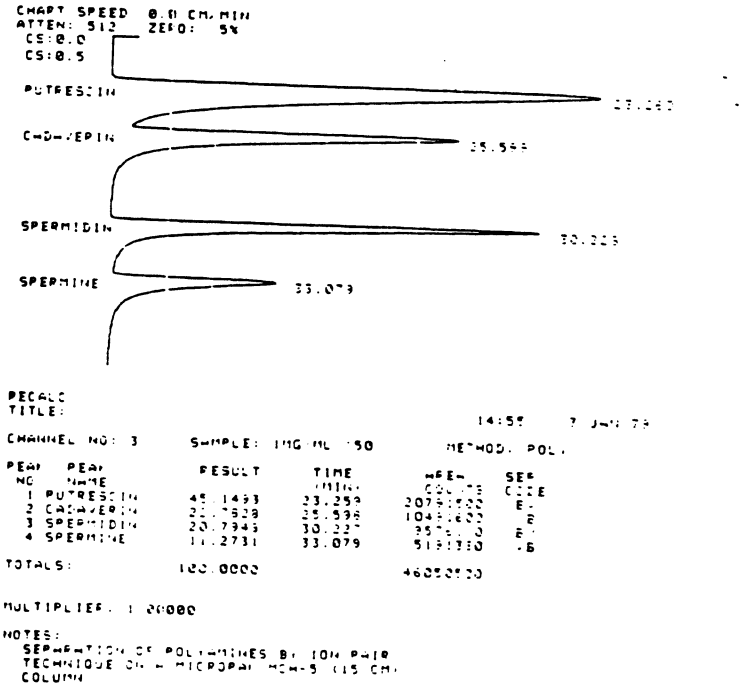


FIGURE 8

Carbohydrates have poor UV response in the far-UV (192 nm) and the RI is not very sensitive. Post column reaction systems can be used to overcome this problem. Figure 9 shows the quantitation of sugars in Riesling wine using an cyano acetamide system.

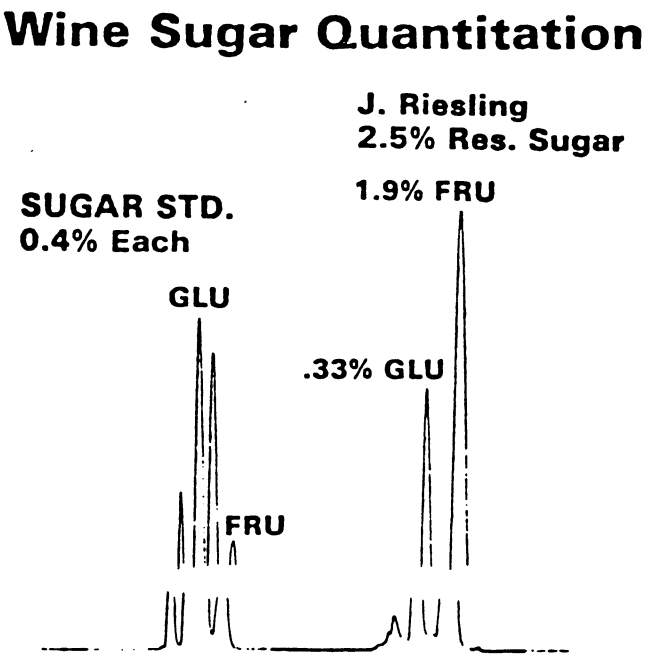
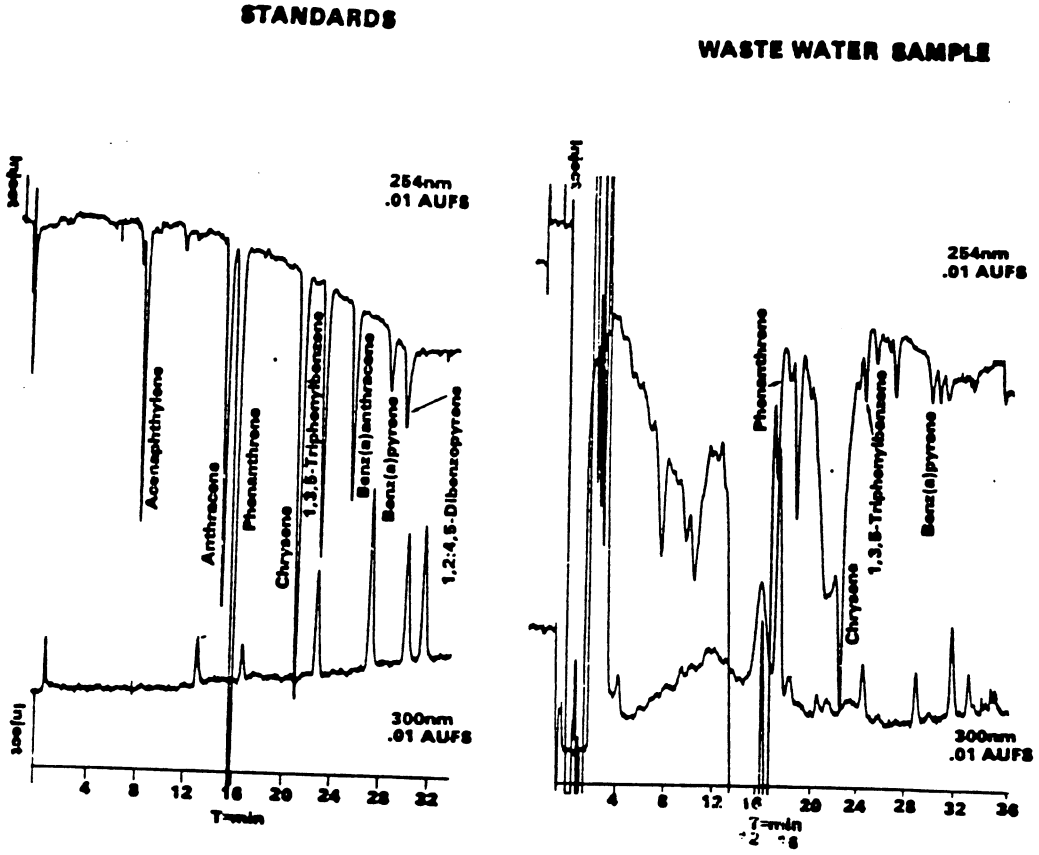


FIGURE 9

Wavelength Ratio:
The absorbance at a specific wavelength and fluorescence at specific excitation and emission wavelengths are physical properties of a compound. When working with standards it is easy to quantitate with a single wavelength or specific fluorescence condition, but this is not the case when doing sample analysis of an unknown in the presence of matrix impurities. These impurities could co-elute with the peak of interest and interfere with quantitation. To confirm peak purity, two wavelengths of fluorescence vs ultraviolet can be used because the ratio of response of two detectors under defined conditions is a physical property of each compound. These detector response ratios can be used to help validate peak purity in the presence of complex matrices.

This technique has been applied to the analysis of polynuclear aromatic hydrocarbons, some of which are considered to be chemical carcinogens. Figure 10 shows a chromatogram of standards and samples using two wavelengths, 254 nm and 300 nm. If the ratio of wavelengths in the sample is different than that from the standard run, the peak being evaluated is probably impure and contaminated with a co-eluting peak. In this waste water sample we can conclude the possibility of the presence of chrysene, benz (a)anthracene, and benz(a)pyrene, since the 300 nm/254 nm ratio is close to standards.



300nm/254nm RATIOS

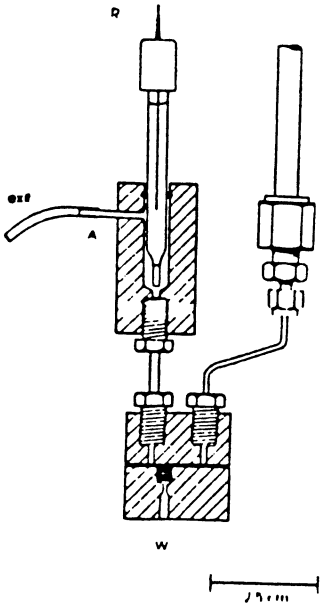
Compound	Standard	Sample
Acenaphthylene	0	0
Phenanthrene	.10	0
Anthracene	0	0
Chrysene	.26	.30
1,3,5-Triphenylbenzene	0	0
Benz(a)anthracene	.93	.83
Benz(a)pyrene	2.23	2.25
1,2:4,5-Dibenzopyrene	1.77	0

FIGURE 10

Electrochemical Detection:
Electrochemical detectors cause a sample to be oxidized or reduced at the electrode surface under controlled potential conditions. The electron current at the electrode is monitored as a function of time to produce a signal, which is then integrated by a data system.

An example of an electrochemical detector is the Bioanalytical Systems Detector, as shown in Figure 11. The electrochemical detector is very selective, monitoring only electroactive compounds under specific sample and solvent conditions. It is the most sensitive detector for compounds such as catecholamines, aromatic amines, and phenols, having sensitivities in the 1-10 ppt range.

CROSS-SECTION OF ELECTROCHEMICAL DETECTOR (BIOANALYTICAL SYSTEMS, INC.)

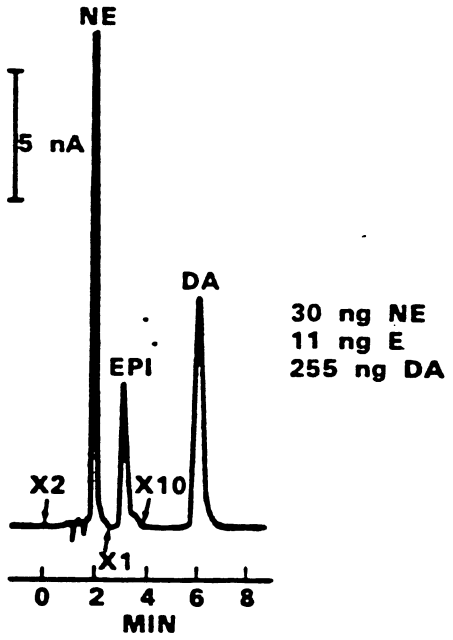


W = working
A = auxiliary
R = reference electrodes

FIGURE 11

ION PAIR HPLC – ELECTROCHEMICAL DETECTION OF CATECHOLAMINES IN URINE

C_{18} column, 4 mm x 30 cm
Carbon paste electrode, +0.7V
100 µL urine extract (500 µL urine)
0.7 mL/min 0.065M citric acid,
0.035M sodium phosphate,
10^{-4}M sodium octyl sulfate



Urine levels: 60 ppb NE, 22 ppb E, 510 ppb DA

FIGURE 12

Some considerations in using an electrochemical detector are:

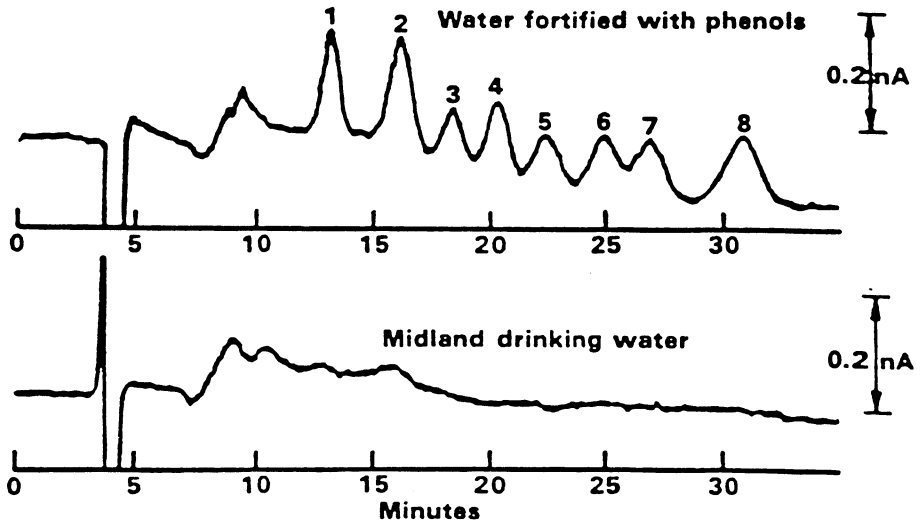
1. The solvent system must be relatively polar, such as water, or water mixed with alcohols (ion exchange and reverse phase systems).

2. The electrode must be compatible with the solvents. Electrode fouling is a problem and the detector takes a long time to stabilize.

Figure 12 shows a reverse phase separation of catecholamines using an electrochemical detector, which is the most sensitive detector for this type of analysis. The detector can also be applied to the trace analysis of phenols in waste water, as shown in Figure 13.

HPLC-ELECTROCHEMICAL DETECTION-PHENOLS IN DRINKING WATER

Carbon-polythylene tubular anode +1.10 volts
10μ Cation-exchange resin 12.7 mm ID x 12 cm
235 μL waste water injected
1 mL/min 31% CH_3CN in 0.04N H_2SO_4



1. 0.83 ppb phenol
2. 1.6 ppb chlorophenol
3. 1.2 ppb 2,6-dichlorophenol
4. 1.5 ppb 2,4-dichlorophenol
5. 1.4 ppb 2,4,6-trichlorophenol
6. 1.6 ppb 2,4,5-trichlorophenol
7. 2.1 ppb 2,3,4,6-tetrachlorophenol
8. 2.2 ppb pentachlorophenol

Armentrout, et al, Anal. Chem, 51, 1039 (1979)

FIGURE 13

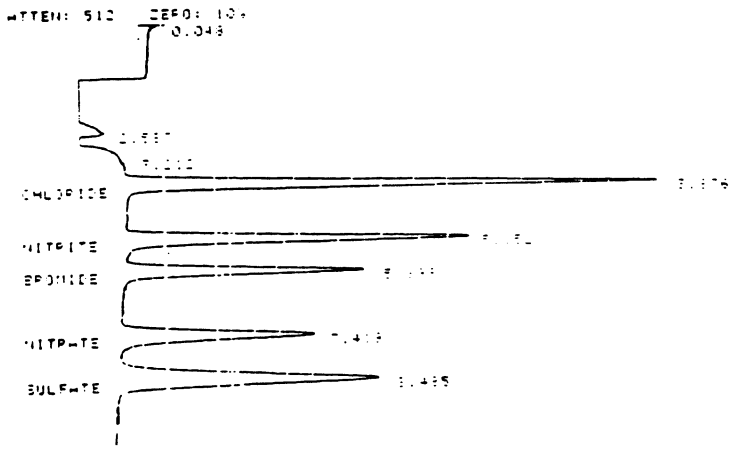
Ion Chromatography and Conductivity Detection:
The determination of ions in different aqueous environments is very important environmentally to monitor rivers, lakes, waste water, drinking water, and rain. There are two types of ion chromatography systems:

1. Determination of anions and cations using an ion exchange column for the separation, a "stripper column" to remove the background electrolyte, and conductivity detection. (The stripper column has to be regenerated regularly).

2. Separation of anion with low conductivity buffers such as 5 x 10–4 M potassium phthalate or 5 x 10-4M 0-sulfonylbenzoic acid ammonium salts. The background electrolytes can be suppressed electronically in the detector, and no suppressor column is needed.

The detector commonly employed for this type of analysis is a Wescan Model 213A conductivity detector. This detector has the ability to measure differences in conductivity. Using the non-suppressed system we can measure quantities down to the sub-ppm range of ions including chloride, nitrate, nitrite, and sulfate. The suppressed system does have some lower detection limits but requires regeneration of column and the purchase of a separate HPLC system.

Figure 1 **HPLC/IC Separation of Anion Standards**



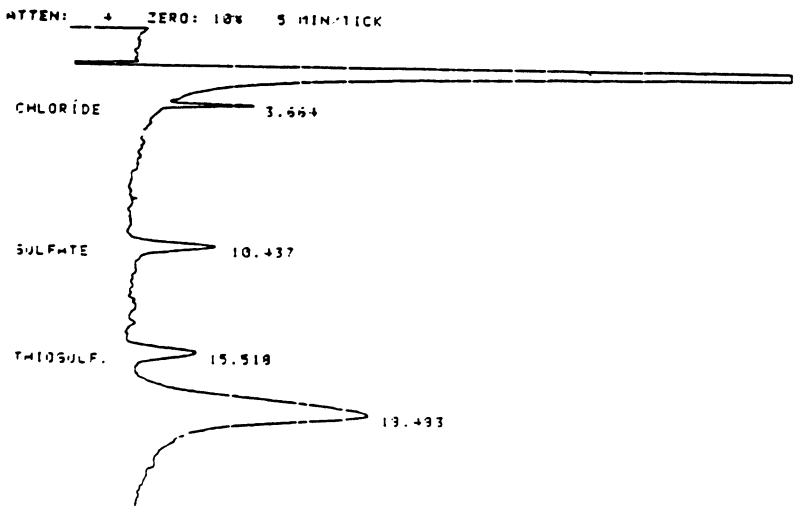
Varian IC-100 Ion Chromatography System

Eluent:	4 mM potassium hydrogen phthalate (KHP) pH 4.5
Flow:	2 ml/min
Sample:	5 PPM mixture of five anion standards
Injection Volume:	500 µl
Detector:	CM-1 conductivity detector

FIGURE 14

Figure 15 shows the separation of Anion standards using the non-suppressed system to determine chloride, nitrite, bromide, nitrate and sulfate. This method can also be applied to wine, waste water, cola drink and pulp processing liquor to quantitate anions.

Figure 2 **Analysis of Pulp Processing Liquor**



Eluent: 2.5 mM Phthalic acid pH 5.0
Flow: 2 ml/min
Sample: Pulp Processing Liquor 1:200 dilution
Injection Volume: 50 µl
Detector: CM-1 conductivity detector

FIGURE 15

Solvents:

TYPICAL SOLVENT SYSTEMS

SEPARATION MODE	SOLVENT SYSTEM	
	WEAK	STRONG
NORMAL PHASE SILICA	HEXANE PENTANE	METHYLENE CHLORIDE ETHYL ACETATE
REVERSE PHASE C_{18}	WATER OR BUFFER	METHANOL ACETONITRILE
ION EXCHANGE	LOW IONIC STRENGTH BUFFER	HIGH IONIC STRENGTH BUFFER
SIZE EXCLUSION ORGANIC SOLUBLE POLYMER	TETRAHYDRO-FURAN	SAME
SIZE EXCLUSION WATER SOLUBLE POLYMER	WATER OR BUFFER	SAME

FIGURE 1

The different modes of liquid chromatography require specific solvent systems for separation. Each of these solvent systems requires some special care. All organic solvents used for HPLC should be HPLC grade and the UV cut-off should be condidered if a UV-detector is used.

The physical properties of HPLC solvents should be considered depending on the mode of chromatography used. We discussed the UV cut-off earlier, but when using a refractive index monitor we have to consider the RI value. Shown on the next page is a table giving RI and UV properties of common solvents.

PROPERTIES OF COMMON HPLC SOLVENTS

Solvent (Increasing polarity)	Viscosity (cP, 20°C)	RI	UV Cut Off (nm)**
Iso-octane	0.51	1.404	210
Heptane	0.41	1.388	210
Hexane	0.33	1.375	210
Cyclohexane	1.00	1.427	210
Carbon Tetrachloride	0.97	1.466	265
Isopropyl ether	0.37	1.368	220
Isopropyl chloride	0.33	1.378	225
Toluene	0.59	1.496	285
n-propyl chloride	0.35	1.389	225
Chlorobenzene	0.80	1.525	—
Benzene	0.65	1.501	280
Ethyl ether	0.23	1.353	220
Chloroform	0.57	1.443	245
Methylene chloride	0.44	1.424	245
Tetrahydrofuran	—	1.408	220
Ethylene dichloride	0.79	1.445	230
Methyl ethyl ketone	—	1.381	330
Acetone	0.32	1.359	330
Dioxan	1.54	1.422	220
Ethylacetate	0.47	1.370	260
Methylacetate	0.37	1.362	260
Di-ethylamine	0.38	1.387	275
Acetonitrile	0.37	1.344	210
Isopropanol	2.30	1.380	210
Ethanol	1.20	1.361	210
Methanol	0.60	1.329	210
Acetic acid	1.26	1.372	—

**UV cut off is defined as the wavelength at which transmittance drops to 10%, i.e., that point at which the mobile phase has an absorbance of 1.0 A.U.

FIGURE 2

Oil and water don't mix. This rule applies in chromatography. From a chemist's point of view polar and non-polar often do not mix. For example, water and hexane are non-miscible and could freeze the pump. When changing from an absorption mode using solvents like hexane to a reverse phase mode using aqueous solvents use an intermediate solvent such as isopropanol which is miscible with both modes to prevent pump blockage and detector upsets.

LC SOLVENT MISCIBILITIES

Solvent B \ Solvent A	1	2	3	4	5	6	7	8	9	10	11	12	13
1. Acetic Acid													
2. Acetone	m												
3 Acetonitrile	m	m											
4. Carbon tetrachloride	m	m	m										
5. Di-ethyl ether	m	m	m	m									
6. Ethanol	m	m	m	m	m								
7. Ethylacetate	m	m	m	m	m	m							
8 Hexane	m	m	p	m	m	m	m						
9 Methanol	m	m	m	m	m	m	m	p					
10. Methylene chloride	m	m	m	m	m	m	m	m	m				
11 Tetrahydrofuran	m	m	m	m	m	m	m	m	m	m			
12. Toluene	m	m	m	m	m	m	m	m	m	m	m		
13 Water	m	m	m	i	p	m	p	i	m	i	m	i	

FIGURE 3

Normal phase chromatography, especially silica, requires dry solvents. In absorption chromatography using solvents like hexane, small amounts of water will cause deactivation of the column and changes in retention. To obtain reproducible chromatography, drying on 4A molecular sieve may be required.

In reverse phase chromatography, water, methanol, and acetonitrile are the common solvents. The water is a major problem in HPLC. Distilled water can be used but only after it has been further purified by using a commercial ultrafiltration system. Water quality can be checked in a reverse phase by first running 100% acetonitrile to wash off all contaminates on the column, then by programming to 100% water for 20 minutes followed by a gradient up to 100% acetonitrile over 10 minutes and holding for 10 minutes. If you have a large number of peaks eluting from the column, then your water is contaminated. HPLC grade water can be used as a standard to compare against, but usually you will find you have water close to the cleanliness of HPLC grade using a conventional ultrafiltration system. For far-UV work such as carbohydrate analysis, you may require the HPLC grade water.

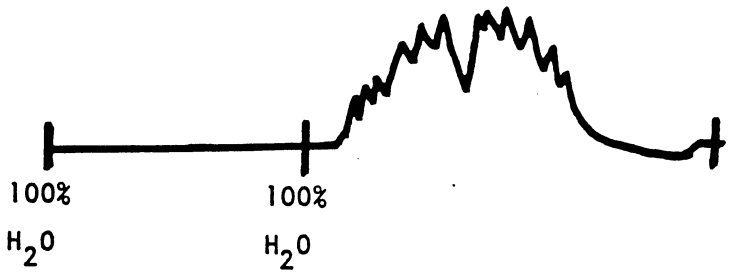


FIGURE 4

Ion exchange requires the use of buffers (also RP-ion suppression). A general rule of thumb is to filter all buffers, ion pairing solvents, or any solvent containing dissolved solids. A solvent clarification kit is a must for all HPLC users. There are two types of clarification kits:

1. For organic solvents using 0.5 mm PTFE filters (includes aqueous solvents containing organic solvents such as acetonitrile).

2. For aqueous solvents using 0.45 mm mixed cellulose ester membrane filters.

The aqueous filters should be used daily on buffers to remove bacterial growth. Sodium azide could also be used to prevent bacterial growth. Never use an aqueous filter with an organic solvent because the filter will dissolve. Filtration of solvent with an aspirator can also remove dissolved gases. Always rinse the filter system first with the solvent being filtered and discard. Most commercial HPLC grade solvents are prefiltered and do not require filtering again. The best quality buffers should be used for HPLC. J. T. Baker and other companies offer pre-purified buffer salts for use with HPLC solvent systems.

A second important consideration is that solvents should be premixed to prevent programming the solvent composition to the point where the organic solvent causes the precipitation of a buffer. (The expensive HPLC killer)

Some solvents such as tetrahydrofuran can form explosive peroxides. Therefore, they should be collected in separate containers and disposed of with due regard to their potential hazard. The THF is used mainly for GPC work and as a modifier in RPC.

GFC solvents should be handled the same as those for reverse phase and ion exchange as discussed above, to avoid precipitation of buffers in the presence of organic solvent.

Toxicity of HPLC solvents should be considered and appropriate cautions should be exercised. Some HPLC solvents such as dichloronapthalene, used for GPC, require putting the HPLC in a vented cabinet or connecting ducting to the vent fan on the back of the liquid chromatograph.

Solvent systems should not dissolve the HPLC. Shown on next page is a list of corrosive solutions. The use of solvents like LiCl buffers (used for physiological amino acids) can dissolve the HPLC if left in the system. The moral to the story: Always wash your system with distilled water after use and be cautious when using strong salts in overnight automations or else the result could be pump replacement for corrosion if the pump stops and is left with the corrosive solvent in it.

Compound	Maximum Concentration in Water (%)	Maximum Temperature (°C)
Acetic acid	60	50
Acetic anhydride	10	25
Ammonium bromide	20	25
Ammonium chloride	Will corrode at all concentrations	
Ammonium citrate	40	100
Ammonium diphosphate	10	100
Ammonium fluoride	Will corrode under all conditions	
Ammonium formate	20	50
Ammonium iodide	20	25
Ammonium nitrate	60	100
Ammonium oxalate	30	100
Ammonium perchloriate	10	100
Ammonium monophosphate	40	25
Ammonium sulphate	40	25
Amly chloride	Will corrode at all concentrations	
Aniline hydrochloride	Will corrode at all concentrations	
Hexachlorobenzene	60	50
Benzoyl chloride	Will corrode at all concentrations	
Boric acid	10	100
Bromoform		100
Formaldehyde		75
Formic acid	Acidity must be lower than pH3	
Hydrazine	50	25
Hydrochloric acid	Will corrode at all concentrations	
Hydrofluoric acid	Will corrode at all concentrations	
Hydroxyacetic acid		50
Iodoform		25
Lactic acid	60	50
Oxalic acid		25
Perchlorethylene		55
Phosphoric acid		50
Potassium bicarbonate	30	100
Potassium bromide	Will corrode at all concentrations	
Potassium carbonates	50	100
Potassium chlorate	10	100
Potassium chloride	Will corrode at all concentrations	
Potassium hydroxide	10	25
Potassium hypochlorite	Will corrode at all concentrations	
Potassium idodide	Will corrode at all concentrations	
Potassium nitrate	20	50
Pyridine		100
Sodium bicarbonate	10	100
Sodium borate	40	50
Sodium bromide	Will corrode at all concentrations	
Sodium carbonate	10	50
Sodium chloride	Will corrode at all concentrations	
Sodium formate	50	50
Sodium hydroxide	40	25
Sodium nitrate	40	50
Sodium phosphate		<75
Sulfuric acid	25	25
Trichloroacetic acid	Will corrode at all concentrations	

FIGURE 5
TYPICAL CORROSIVE SOLUTIONS

Sample Preparation:

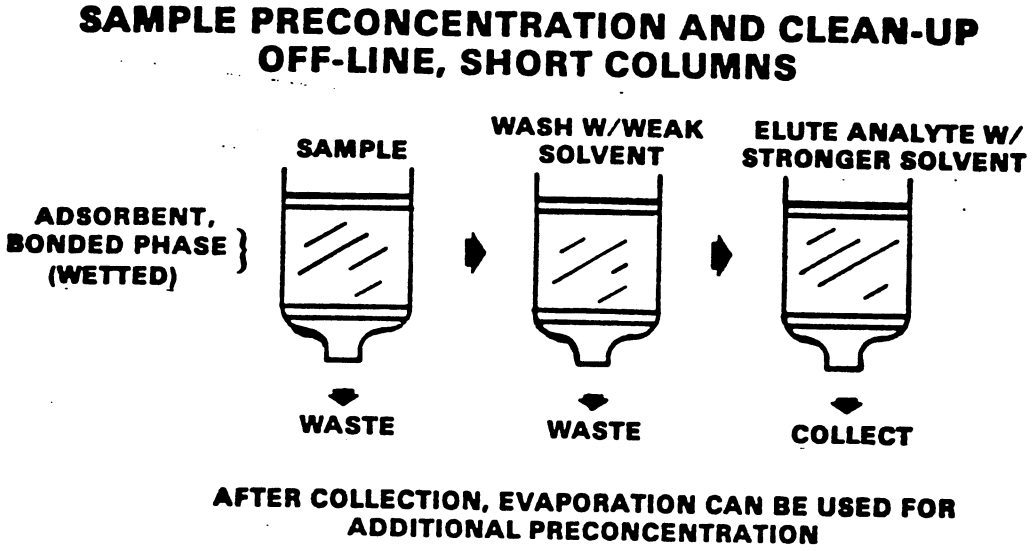


FIGURE 6

Sampling considerations are beyond the scope of this manual but sample source, storage, contamination and sampling procedure should all be considered. Good chromatography is useless if the sample is not representative or is contaminated.

Depending on the sample the degree of clean-up will vary. Some samples require liquid phase extraction where the efficiency of extraction should be evaluated. Samples from natural sources require filtration. A simple sample may only require a sample clarification kit using a filter syringe. The filter used will depend on the solvent used to dissolve the sample: for organic samples we use PTFE filters (0.5 mm) and for aqueous samples use methyl cellulose filters (0.45 mm).

There are small columns available as well as syringe cartridges for sample clean-up and pre-concentration off-line. Some of these columns contain reverse phase or ion exchange absorbents. They can do either adsorption of specific compounds while eluting undesired materials followed by elution of the analyte using a strong solvent, as shown in Figure 6, or they can absorb interfering materials while eluting the analyte.

HPLC Column Care:
There are two major types of columns: Resin based and Silica gel based. The resin based (ion exchange gel filtration, and some reverse phase) have a pH range of 2-12, but they have mechanical strength limitations and the upper pressure limit should be set as to prevent column compression. The second type of column, the silica based column, has pH limitations. Shown below is the aqueous solubility of silica gel at different pH's at 25°C.

AQUEOUS SOLUBILITY OF SILICA GEL, 25°C

pH	Equilibrium Solubility
1	140 PPM
3	150
4.2	130
7.7	100
10.3	490
10.6	1120

pH 2-8 recommended operational limits at ambient temperature

FIGURE 7

It should be noted that solubility increases greatly at pH's above 8.

FACTORS INCREASING SILICA DISSOLUTION RATE

- High pH - alkaline salts
- Strong ion-pairing bases in mobile phase (tetra-alkyl ammonium >> amines)
- Elevated temperature
- High surface area

FIGURE 8

FACTORS DECREASING SILICA DISSOLUTION RATE

- Low pH - acidic salts
- Acetonitrile in aqueous mobile phase
- High bonded phase coverage
- Hydrophobic character of bonded phase
- Bonded phase chain length (C18 > C8 >> C2)

FIGURE 9

COLUMN MAINTENANCE

SYMPTOM: HIGH BACKPRESSURE AND/OR LOW EFFICIENCY

SOURCE OF PROBLEM	MAINTENANCE
• LOW SOLUBILITY CONTAMINATION	• WASH W/VARIETY OF SOLVENTS
• TOP OF COLUMN VOIDED	• TOP OFF W/PELLICULAR PACKING
• ENTRANCE FRIT PLUGGED	• REPLACE OR BACKFLUSH FRIT
• TOP OF COLUMN BED PLUGGED	• REMOVE BAD PACKING AND TOP OFF W/PELLICULAR PACKING
• EXIT FRIT PLUGGED	• REPLACE OR BACKFLUSH FRIT
• VOID INTERNAL TO BED (UNLIKELY)	• TRY TO COMPACT BED W/HIGH FLOW/ HIGH PRESSURE

FIGURE 10

Column maintenance is a very important part of HPLC. Above is a table of possible sources of problems. The two major symptoms are high back pressure and/or low efficiency.

With the continued use of an HPLC column contaminants contained in both the sample and the eluent build up on the column, causing a decrease in efficiency and higher back pressure. Therefore, periodically, a column has to be regenerated.

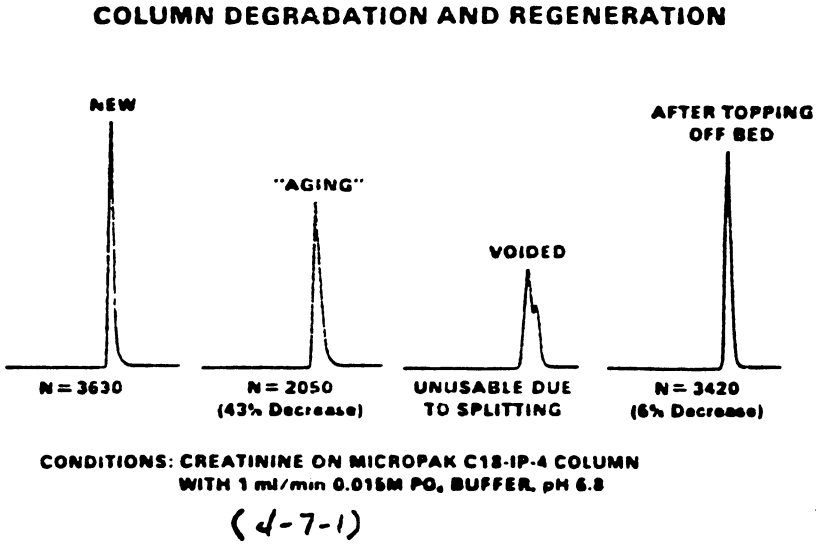


FIGURE 11

Figure 11 shows the effect of aging on a reverse phase column. In some cases column efficiency can be increased by washing the column with a number of different solvents, for example:

a) Adsorption -
- The following series of solvents can be used for regeneration: heptane, 1, 1, 1,-trichlorothane, ethyl acetate, acetone, ethanol and water. (use reagent grade solvents). Reverse the sequence to return to operating conditions.

b) Reverse Phase -
- Pump around 25 ml of acetonitrile or methanol through the column, then methanol/chloroform, then back to pure methanol, followed by pure water, then a strong buffer such as 0.5 M H_3PO_4 after the water. After rinsing off with water you can return to original conditions.

c) Ion Exchange -
- Pump around 25 ml of concentrated buffer, followed by 25 ml distilled water, then 25 ml of 0.5 M acetic acid, followed by distilled water, including, if necessary, a complexer (0.2 M sodium EDTA), followed by distilled water, followed by methanol to remove organics, followed by distilled water, then back to the buffer. (Avoid buffer precipitation with organic solvents.)

In Figure 11 we can also see peak splitting which is due to a void. If the void is at the top of the column, this can be solved by topping up the column with pellicular packing material to improve efficiency.

Insoluble material can built up on the frits at either end of the column. If at the inlet frit, the column can be reversed after disconnecting from the detector. (For specific column types, check with manufacturer.) In extreme cases you can replace small portions of packing from the front of the column.

Some general column considerations are:

1. Keep the column ends tightly capped. This is especially critical with ion exchange and GPC resins.
2. Store column in the original box at room temperature.
3. Check miscibility of solvents and buffer precipitation.
4. Use ion-pairing columns for ion pairing. They are permanently modified.
5. Maintain a slow flow rate when using buffers and ion pairing reagents.
6. Filter dirty samples and use dilute solutions when trying the sample for the first time.
7. Use column protection devices, such as guard columns.
8. Store column in a compatible solvent with the ends capped. See table below.

RECOMMENDED SOLVENTS FOR COLUMN STORAGE

Columns	Solvents
MicroPak Si-10, Si-5	Hexane
MicroPak Al-10, Al-5	Hexane
MicroPak CN-10	Hexane
MicroPak NH_2-10	Hexane or acetonitrile
MicroPak AX-10	Acetonitrile
MicroPak MCH-10, MCH-5	Acetonitrile or methanol
MicroPak CH-10	Acetonitrile or methanol
Aminex A-7, A-27, A-29	Aqueous buffer used by chromatographer with 3×10^{-4} M sodium azide to prevent microbial growth.

FIGURE 12

Specific column considerations include:

Silica Columns, Si-5, Si-10
Continuous loss of t_R which is due to adsorption of water onto Si, Solution is pass N_2 or He through column at 110°C (use GC oven). Also use dryer column and dry solvents.

Amino Propyl Column NH_2-10
-Nucleotides and nucleosides can cause stripping of bonded phase (loss of t_R). Replace NH_2-10 with AX-10 for these compounds.

-Ketone or aldehyde can react with NH_2-10 columns and can be irreversibly absorbed. It is recommended to use CN-10 or MCH-10 column for these compounds (loss of signals).

Specific Column considerations:
NH_2 and AX-columns.
Buildup of trace metals from buffers can change selectivity, cause tailing, lower t_R. Cure - wash with dilute EDTA, then buffer. Clean up buffer by passing through Chelex resin column.

AX-10 Column
-Ketone and aldehydes react with AX-10, thus avoid these compounds.
-Avoid precipitation of buffer in column with organics (wash with pure H_2O prior to storing with CH_3CN).

Reverse Phase Columns (MCH-10, MCH-5, CH-10)
-Low N, poor peak shape problem is often from interactions with residual silanol. Add 0.01 - 0.02 M tetramethylammonium chloride to mobile phase buffer and keep pH low.

NOTE FOR ALL SILICA BASED COLUMNS

Dissolution of silica substrate in aqueous buffer solvent system at pH 8

- increases with increase temperature

- can precondition solvent

Column Protection Devices

COLUMN PROTECTION DEVICES

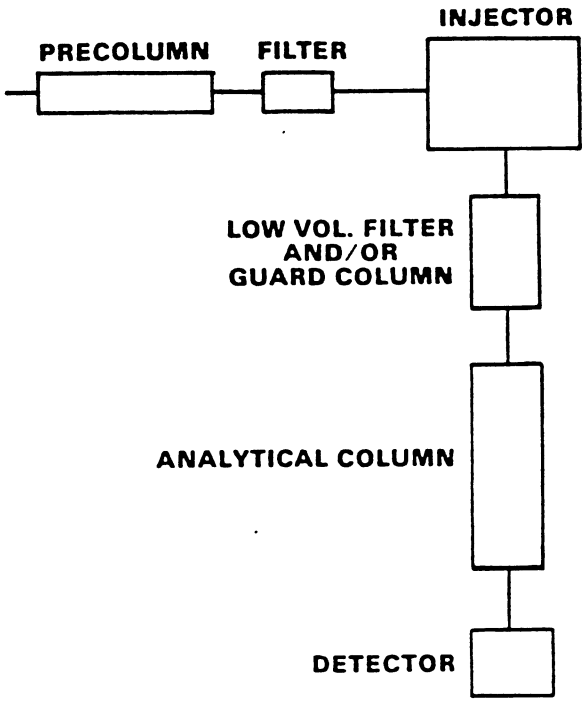


FIGURE 13

The most important column protection device is the guard column. The guard column protects the analytical column from the sample contaminants which are irreversibly absorbed. The guard column matches the stationary phase of the analytical column. For example, a reverse phase column would use a guard column filled with reverse phase packing. The guard column can be replaced periodically depending on the level of contamination in the sample.

There are a number of types of guard columns. Pellicular packing can be dry packed into a universal guard column (4.6 mm x 4 cm) to make a guard column which adsorbs contaminants that would otherwise damage the analytical column. The advantage of this type of guard column is the ease in obtaining matching packing. For most of the column types, prepacked guard columns (slurry packed), with exactly matching packing, are available. These columns increase efficiency by increasing column length. The Brownlee Co. also produces a cartridge guard column which has prepacked cartridges with 10 m microparticle packing. These are extremely convenient because one holder can be used when changing phases by replacing the cartridge (store used cartridge in appropriate mobile phases).

Shown below is the effect of guard columns on the efficiency and capacity of the chromatographic system. As expected, the prepacked guard column is the best and, surprisingly, the cartridge guard column is the worst. The convenience and cost saving of the cartridge guard column may be worth the loss in efficiency.

INFLUENCE OF GUARD COLUMNS AND FILTERS ON COLUMN EFFICIENCY AT MODERATE k'

	k'	N	ΔN vs. COLUMN
COLUMN (5 μm, 15 cm)	8.1	7793	—
W/CARTRIDGE GUARD (10 μm, 3 cm)	10.1	5342	-31%
W/PELLICULAR GUARD (40 μm, 4 cm)	8.4	6096	-22%
W/PREPACKED GUARD (5 μm, 4 cm)	9.8	8961	+15%
W/IN-LINE FILTER (0.5 μm)	8.1	7648	-2%

Based on methoxybenzamide at 0.7 ml/min, 0.01M KH_2PO_4: Me CN (87.13), ambient.

FIGURE 14

PRECOLUMN

1. Filters contaminants from mobile phase.
2. Saturates Mobile phase with silica or bonded phase.
3. Undesirable for gradient work; isocratic only.
4. Can use large porous particles.

IN LINE FILTER

LOW INTERNAL VOLUME

- Available with 0.5 or 2.0 μM replaceable frits
- Used before **OR** after injector
- Filters mobile phase **AND** sample
- Small frits replaced more often
- Minimal loss of column efficiency even with 5 μM, 15 CM columns

Will **NOT** protect analytical column from dissolved material that might irreversibly adsorb to packing

LARGE INTERNAL VOLUME

- Available with 0.5 or 2.0 μM replaceable frits
- Must be before injector
- Filters mobile phase only
- Large surface area for maximum lifetime
- Large volume slows gradient response

LIQUID CHROMATOGRAPHY TERMS AND RELATIONSHIPS

AMERICAN NATIONAL STANDARD ANSI/ASTM E 682 - 79

11/79

AMERICAN SOCIETY FOR TESTING AND MATERIALS
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Standard Practice for LIQUID CHROMATOGRAPHY TERMS AND RELATIONSHIPS[1]

This standard is issued under the fixed designation E 682; the number immediately following the designation indicates the year of original adoption or, in the case of revision, the year of last revision. A number in parentheses indicates the year of last reapproval.

1. Scope

1.1 This practice deals primarily with the terms and relationships used in liquid column chromatography. However, most of the terms should also apply to other kinds of liquid chromatography, notably planar chromatography such as paper or thin-layer chromatography.

NOTE 1—Although electrophoresis can also be considered a liquid chromatographic technique, it and its associated terms have not been included in this practice.

1.2 Since most of the basic terms and definitions also apply to gas chromatography, this practice is using whenever possible symbols identical to the ASTM Recommended Practice E 355 for Gas Chromatography Terms and Relationships.[2]

2. Names of Techniques

NOTE 2—In the chromatographic literature one may often find the term "high-performance (or high-pressure) liquid chromatography," abbreviated as HPLC. This term was introduced to distinguish the present-day column chromatographic techniques employing high inlet pressures and columns containing small diameter packing from the classical methods. The utilization of this term or any derivative term (for example, HPLSC for high-performance liquid-solid chromatography) is not recommended.

Similarly, the use of the term "high-performance thin-layer chromatography," abbreviated as HPTLC, describing newer variations of thin-layer chromatography, is also not recommended.

2.1 *Liquid Chromatography*, abbreviated as LC, comprises all chromatographic methods in which the mobile phase is liquid under the conditions of analysis. The stationary phase may be a solid or a liquid supported by or chemically bonded to a solid.

2.2 The stationary phase may be present on or as a plane (*Planar Chromatography*), or contained in a cylindrical tube (*Column Chromatography*).

2.3 Separation is achieved by differences in the distribution of the components of a sample between the mobile and stationary phases, causing them to move along the plane surface or through the column at different rates (differential migration).

2.3.1 In *Planar Chromatography*, the differential migration process will cause the sample components to separate as a series of spots behind the mobile phase front.

2.3.2 In *Column Chromatography*, the differential migration process will cause the sample components to elute from the column at different times.

2.3.3 In *Dry-Column Chromatography*, mobile phase flow is stopped as soon as the mobile phase has reached the end of the dry column. Solute visualization is from the extruded or sliced column packing.

2.4 The basic process of selective distribution during the chromatographic process can vary depending on the type of stationary phase and the nature of the mobile phase.

2.4.1 In *Liquid-Liquid Chromatography*, abbreviated LLC, the stationary phase is a liquid and the separation is based on selective partitioning between the mobile and stationary liquid phases.

2.4.2 In *Liquid-Solid Chromatography*, abbreviated as LSC, the stationary phase is an

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[1] This practice is under the jurisdiction of ASTM Committee E-19 on Chromatography.

[2] Current edition approved March 30, 1979. Published May 1979.

[3] *Annual Book of ASTM Standards*, Part 42.

interactive solid. Depending on the type of the solid, separation may be based on selective adsorption on an inorganic substrate such as silica gel, or an organic gel. In this definition, *Ion-Exchange Chromatography* is considered to be a special case of LSC in which the interactive solid has ionic sites and separation is due to ionic interaction.

2.4.3 In some cases such as with bonded stationary phases, the exact nature of the separation process is not fully established and it may be based on a combination of liquid-liquid and liquid-solid interactions.

2.4.4 In *Steric Exclusion Chromatography*, the stationary phase is a noninteractive porous solid, usually silica or an organic gel. In this case, separation is affected by the size of the sample molecules, where those which are small enough penetrate the porous matrix to varying extents and degrees while those that are largest are confined to the interstitial region of the particles. Thus, the larger molecules elute before the smaller molecules.

2.5 In liquid chromatography, the composition of the mobile phase may be constant or changing during a chromatographic separation.

2.5.1 The term *Isocratic* is used when the composition of the mobile phase is kept constant during a chromatographic separation.

2.5.2 The term *Gradient* is used to specify the technique when a deliberate change in the mobile phase operating condition is made during the chromatographic procedure. The change is usually in mobile phase composition, flow rate, pH, or temperature. The first-named change is called *Gradient Elution. Flow Programming* is a technique where the mobile phase linear velocity is changed during the chromatographic procedure. The changes are made to enhance separation or to speed elution of sample components, or both. Such changes in operating conditions may be continuous or step-wise.

2.6 In the standard modes of liquid chromatography, the stationary phase is more polar than the mobile phase. The opposite case is also possible, in which the mobile phase is more polar than the stationary phase. This version of the technique is called *Reversed-Phase Chromatography.*

2.7 *Planar Chromatography* comprises two versions: paper chromatography and thin-layer chromatography.

2.7.1 In *Paper Chromatography*, the process is carried out on a sheet or strip of paper. Separation is usually based on LLC in which water held on the cellulose fibers acts as the stationary phase. Separation based on LSC may also be utilized when the paper is impregnated or loaded with an interactive solid.

2.7.2 In *Thin-Layer Chromatography*, the solid stationary phase is utilized in the form of a relatively thin layer on an inactive plate or sheet.

2.7.3 In any version of planar chromatography, the mobile phase may be applied in a number of ways. In *Ascending Development* or *Horizontal Development* the mobile phase movement depends on capillary action. In *Horizontal Development* the mobile phase may move predominantly linearly or radially. In *Descending Development* the mobile phase movement is governed mainly by gravity. In *Radial Development* the mobile phase is applied as a point source.

2.7.4 The *Mobile Phase Front* is the leading edge of mobile phase as it traverses the planar media. In all forms of development, except radial, the Mobile Phase Front is essentially a straight line parallel to the mobile phase surface.

2.7.5 *Consecutive Developments* of planar media may be carried out after removal of the mobile phase from a previous development. If the consecutive development is accomplished in the same direction as previously, this is *Multiple Development.* If a second development is accomplished at a right angle to the first development, this is *Two-Dimensional Development.*

3. Apparatus

3.1 *Pumps*—The function of the pumps is to deliver the mobile phase at a controlled flow rate to the chromatographic column.

3.1.1 *Syringe Pumps* have a piston that advances at a controlled rate within a smooth cylinder to displace the mobile phase.

3.1.2 *Reciprocating Pumps* have a single or dual chamber from which mobile phase is displaced by reciprocating piston(s) or diaphragm(s). The chamber volume is relatively small compared to the volume of the column.

3.1.3 *Pneumatic Pumps* employ a gas to displace the mobile phase either directly or through a piston or collapsible container. The

volume within these pumps may be large or small as compared to the volume of the column.

3.2 *Sample Inlet Systems* represent the means for introducing samples into the column.

3.2.1 *Septum Injectors*—Sample contained in a syringe is introduced directly into the pressurized flowing mobile phase by piercing an elastomeric barrier. The syringe is exposed to pressure and defines the sample volume.

3.2.2 *Septumless Injectors*—Sample contained in a syringe is introduced into an ambient-pressure chamber, which chamber is subsequently mechanically displaced into the pressurized flowing mobile phase. The syringe is not exposed to pressure and defines the sample volume.

3.2.3 *Stopped Flow Injectors*—Sample contained in a syringe is introduced into a chamber that is in the flowing mobile phase path. However, flow is stopped during injection. The syringe is not exposed to pressure and defines the sample volume.

3.2.4 *Valve Injectors*—Sample contained in a syringe (or contained in a sample vial) is injected into (or drawn into) an ambient-pressure chamber which is subsequently displaced into the pressurized flowing mobile phase. The displacement is by means of rotary or sliding motion. The chamber is a section (loop) of tubing or an internal chamber. The chamber can be completely filled, in which case the chamber volume defines the sample volume, or it can be partially filled, in which case the syringe calibration marks define the sample volume.

3.3 *Columns* consist of tubes that contain the stationary phase and through which the mobile phase flows.

3.4 *Detectors* are devices that respond to the presence of eluted solutes in the mobile phase emerging from the column. Ideally, the response should be proportional to the mass or concentration of solute in the mobile phase. Detectors may be divided either according to the type of measurement or the principle of detection.

3.4.1 *Bulk Property Detectors* measure the change in a physical property of the mobile phase passing from the column. Thus a change in the refractive index, conductivity, or dielectric constant of a mobile phase can indicate the presence of eluting components.

3.4.2 *Solute Property Detectors* measure the physical or chemical characteristics of the component eluting from the column. Thus, light absorption (ultraviolet, visible, infrared), fluorescence, and polarography are examples of detectors capable of responding in such a manner.

3.4.3 *Differential Detectors* measure the instantaneous proportion of eluted sample components in the mobile phase passing through the detector or their instantaneous rate of arrival at the detector.

3.4.4 *Integral Detectors* measure the accumulated quantity of sample component(s) reaching the detector.

3.4.5 The detectors used in liquid chromatography may also be based on a variety of other physical or chemical phenomena.

3.5 *Fraction Collectors* are devices for recovering time-separated fractional volumes of the column effluent. The fraction collectors may be operated manually or automatically. Automatic fraction collectors consist of a series of test tubes or flasks. Column effluent is carried to one of the vessels and after a measured volume is collected or a set period of time has passed, the system automatically places the next vessel into position to receive a corresponding aliquot.

3.6 *Developing Chamber* is a closed container, customarily of relatively large internal volume, used to enclose the media used in paper or thin-layer chromatography and also the mobile phase. It may be lined with a porous paper (*Saturated Development*) or it may be unlined (*Unsaturated Development*). A *Sandwich Chamber* has walls that are a uniform distance apart giving a relatively small internal volume.

3.7 *Spotting Device* is a syringe or micropipet used to deliver a known volume of sample as a spot or streak to the paper or thin-layer media at the origin or near the beginning end of the planar media.

3.8 *Visualization Chamber* is a device in which the planar media may be viewed under ultraviolet light or sprayed with visualization reagents.

3.9 *Densitometer* is a device that allows portions of the developed paper or thin-layer media to be scanned with a beam of light of variable wavelength. The instrument in this manner is able to respond to differences in spot size and density in order to quantitate the

separated compounds. The device may work in a transmission or reflectance mode.

4. Reagents

NOTE 3—In liquid chromatographic techniques the term "solvent" has been widely used to describe the mobile phase (that is, developing solvent, eluting solvent, solvent front). Due to the ambiguity of this term, its use is not recommended.

In various liquid chromatographic techniques the term "carrier" has been used to describe the solid on which the stationary phase is distributed or certain active groups involved in the separation process are bonded. Due to the similarity to the term "carrier gas" used as a synonym for the mobile phase in gas chromatography, the use of this expression is not recommended.

4.1 The *Mobile Phase* is the liquid used to sweep or elute the sample components along the planar surface or through the column. It may consist of a single component or a mixture of components.

4.2 The *Stationary Phase* is the active immobile material on the planar surface or within the column that delays the passage of sample components by one of a number of processes or their combination. There are three types of stationary phase: *Liquid Phases*, *Interactive Solids*, and *Bonded Phases*. Inert materials that merely provide physical support for the stationary phase are not part of the stationary phase.

4.2.1 The *Liquid Phase* is a stationary phase which has been sorbed (but not covalently bonded) to a solid support, paper sheet, or thin layer. Differences in the solubilities of the sample components in the liquid and mobile phase constitute the basis for their separation. Examples of materials that can be used as liquid phases are β,β'-oxydipropionitrile, silicone oil, and water.

4.2.2 The *Interactive Solid* is a stationary phase that comprises a relatively homogeneous surface on which the sample components sorb or ion-exchange onto and off effecting a separation. Examples are silica, alumina, graphite, and ion exchangers.

4.2.3 The *Bonded Phase* is a stationary phase that comprises a chemical (or chemicals) that has been covalently attached to a solid support. The sample components sorb onto and off the bonded phase differentially to effect separation. Octadecylsilyl groups bonded to silica represent a typical example for a bonded phase.

4.3 The *Solid Support* is the inert material to which the stationary phase is sorbed (liquid phases) or covalently attached (bonded phases). It holds the stationary phase in contact with the mobile phase.

4.4 The *Column Packing* consists of all the material used to fill packed columns. There are two types: totally porous and pellicular.

4.4.1 *Totally Porous Packing* is one where the stationary phase is found throughout each porous particle.

4.4.2 *Pellicular Packing* is one where the stationary phase is found only on the porous outer shell of the otherwise impermeable particle.

4.5 *Solutes* are the sample components the separation of which is attempted on the column (column chromatography), paper sheet or thin-layer plate (planar chromatography) as they are swept or eluted by the mobile phase. These may be unretained (that is, not delayed) by the stationary phase in which case no separation is achieved, or they may be retained permanently. If partially retained, then separation to varying degrees may be accomplished.

4.6 *Binders* are the additives used to hold the stationary phase or solid support to the inactive plate or sheet in thin-layer chromatography. These may be calcium sulfate hemihydrate, starch, poly(vinyl alcohol), or others. Ideally, they play no part in the separation mechanism.

4.7 *Visualization* is that series of steps applied to planar media which may include evaporating off the mobile phase used for development, applying visualization reagents (one or a series, by spraying, vaporizing, or dipping), heating, and examination under visible or ultraviolet light to detect otherwise colorless solutes.

5. Readout

5.1 The *Chromatogram* is the result of the separation of solutes through the process of chromatography.

5.1.1 If the separation is by means of column chromatography, the chromatogram is the graphic representation of the detector response versus retention time or retention volume as the solutes elute from the column and through the detector. An idealized chromatogram obtained with differential and integral detectors of an unretained and a retained component from a column is shown in Fig. 1.

5.1.2 If the separation is by means of planar chromatography, the chromatogram is the paper or thin-layer media itself on which the

solute mixture has been placed and separated. An idealized chromatogram of a planar separation is shown in Fig. 2. The planar media may be passed under a densitometer in order to quantitate the separated compounds. The densitometer then produces a graphic representation of detector response versus distance traveled (retention time).

5.2 The definitions in 5.2.1 through 5.2.6 apply to chromatograms obtained directly by means of differential detectors or indirectly by differentiating the response of integral detectors.

5.2.1 A *Baseline* is the portion of a chromatogram recording the detector response when only the mobile phase emerges from the column.

5.2.2 A *Peak* is the portion of a chromatogram recording detector response when a single component, or two or more unresolved components, elute from the column.

5.2.3 The *Peak Base, CD* in Fig. 1, is the interpolation of the baseline between the extremities of a peak.

5.2.4 The *Peak Area, CHFEGJD* in Fig. 1, is the area enclosed between the peak and the peak base.

5.2.5 *Peak Height, EB* in Fig. 1, is the distance measured in the direction of detector response, from the peak base to peak maximum.

5.2.6 *Peak Widths* represent retention dimensions parallel to the baseline. *Peak Width at Base* or *Base Width, KL* in Fig. 1, is the retention dimension of the peak base intercepted by the tangents drawn to the inflexion points on both sides of the peak. *Peak Width of Half Height, HJ* in Fig. 1, is the retention dimension drawn at 50 % of peak height parallel to the peak base. The *Peak Width at Inflexion Points, FG* in Fig. 1, is the retention dimension drawn at the inflexion points (= 60.7 % of peak height) parallel to the peak base.

5.3 The definitions in 5.3.1 and 5.3.2 apply to chromatograms obtained with integral detectors, or by integration of the records obtained using differential detectors. In this mode of operation, as sample components pass through the detector, the baseline is displaced cumulatively.

5.3.1 A *Step* is the change in baseline position when a single component or two or more unresolved components elute.

5.3.2 The *Step Height, NM* in Fig. 1, is the distance, measured in the direction of detector response, between straight-line extensions of the baselines on both sides of a step.

5.4 The definitions in 5.4.1 through 5.4.3 apply to reading information from planar media.

5.4.1 The *Mobile Phase Distance, PQ* in Fig. 2, is the length of mobile phase traveling along the media from the center of the sample spot at the origin to the mobile phase front.

5.4.2 The *Solute Distance, PR* in Fig. 2, is the length of solute travel up the media from the center of the sample at the origin to the center of the solute spot. If the solute spot is other than circular, an imaginary circle is used whose diameter is the smallest diameter of the spot, and the center of this circle is taken as point *R*.

5.4.3 The *Spot Diameter, ST* in Fig. 2, which is equivalent to a peak width in chromatograms obtained by differential detectors, is the breadth of the solute spot after chromatography. As mentioned in 5.4.2, if the spot is not circular, the smallest diameter of the noncircular spot is used as the distance ST.

6. Retention Parameters, Symbols, and Units

6.1 Retention parameters, symbols, units, and their definitions or relationship to other parameters are listed in Table 1.

NOTE 4—From these the adjusted retention time, capacity ratio, number of theoretical plates, and relative retention are strictly speaking only meaningful in an isocratic, constant-flow system.

6.2 Figure 1 can be used to illustrate some of the most common parameters measured from chromatograms obtained with differential detectors:

Elution time of unretained component $= OA$

Retention time $= OB$

Adjusted retention time $= AB$

Capacity ratio $= (AB)/(OA)$

Peak width at base $= KL$

Peak width at half height $= HJ$

Number of theoretical plates

$$= 16[(OB)/(KL)]^2 = 5.54[(OB)/(HJ)]^2$$

Relative retention (Note 5) $= (AB)_i/(AB)_s$

Peak resolution (Notes 5 and 6)

$$= \frac{2(OB)_j - (OB)_i}{(KL)_i + (KL)_j} \simeq \frac{(OB)_j - (OB)_i}{(KL)_j}$$

NOTE 5—Subscripts *i*, *j*, and *s* refer to some peak, a following peak, and a reference peak (standard), respectively.

NOTE 6—The second fraction may be used if peak resolution of two closely spaced peaks is expressed: in such as case $(KL)_1 \simeq (KL)_2$.

6.3 Figure 2 can be used to illustrate some of the most common parameters measured from chromatograms obtained for planar media:

Mobile phase distance	$= PQ$
Solute distance	$= PR$
Spot diameter	$= ST$
Retention factor (relative to mobile phase front) R_f	$= (PR)/(PQ)$
Retention factor (relative to a standard) (Note 5) R_s	$= (PR)_1/(PR)_2$
Number of theoretical plates	$= 16[(PR)/(ST)]^2$

TABLE 1 Summary of Parameters, Symbols, Units and Useful Relationships in Liquid Chromatography

Parameter	Quantity Symbol	Unit	Definition or Relationship to Other Parameters[A]
Time	t	min	
Temperature of mobile phase	T	K	°C + 273.15 at the point where mobile phase flow is measured
Temperature of column	T_c	K	
Ambient temperature	T_a	K	
Column inlet pressure	P_i	Pa	
Column outlet pressure	P_o	Pa	
Pressure drop along the column	P	Pa	$P = P_i - P_o = Lu/B_o$
Relative column pressure	P		$P = P_i/P_o$
Ambient (atmospheric) pressure	P_a	Pa	
Column length	L	cm	
Column inside diameter	d_c	cm	
Average diameter of solid particles in the column	d_p	cm	
Pore radius	r_p	cm	
Interparticle porosity	ϵ		fraction of column cross section available for the moving phase
Column cross-sectional area	A_c	cm^2	$A_c = (d_c)^2\pi/4$
Volume of mobile phase in column + system	V_M	cm^3	$V_M = F_c t_M$
Interstitial volume of column	V_c	cm^3	In ideal case, assuming no extracolumn volume in system: $V_M = V_c$ In actual systems: $V_M = V_c + V_I + V_D$ where V_I is the volume between the effective injection point and the column inlet and V_D is the volume between the column outlet and the effective detection point
Molar volume	V_m	cm^3/mol	
Specific column permeability	B_o	cm^2	$B_o = \frac{d_p^2\epsilon^3}{180(1-\epsilon)^2} \approx \frac{d_p^2}{1000}$
Flow rate of the mobile phase from the column	F_a	cm^3/min	measured at ambient temperature and pressure
Flow rate of mobile phase from the column, corrected to column temperature	F_c	cm^3/min	$F_c = F_a\frac{T_c}{T_a}$
Linear velocity of mobile phase	u	cm/s	$u = \frac{L}{60t_M} = \frac{F_a}{60\epsilon A_c}$
Optimum linear velocity of mobile phase	u_{opt}	cm/s	the value of u at the minimum of the HETP versus u plot; the value of u where the measured HETP is the smallest.
Viscosity of mobile phase	η	P [g/(cm·s)]	expressed at column temperature
Reduced mobile phase velocity	ν	...	$\nu = \frac{ud_p}{D_M}$
Diffusion coefficient of solute in mobile phase	D_M	cm^2/s	
Diffusion coefficient of solute in stationary phase	D_S	cm^2/s	
Retention time (total retention time)	t_R	min	time from sample injection to maximum concentration (peak height) of eluted compound
Mobile phase holdup time	t_M	min	observed elution time of an unretained substance
Adjusted retention time	t_R'	min	$t_R' = t_R - t_M$
Retention volume (total retention volume)	V_R	cm^3	$V_R = t_R F_c$
Adjusted retention volume	V_R'	cm^3	$V_R' = t_R' F_c$
Peak width at inflection points	w_i	cm	retention dimension between the inflection points (representing 60.7 % of peak height) of any single-solute peak
Peak width at half height	w_h	cm	retention dimension between the front and rear sides of any single-solute peak at 50 % of its maximum height

TABLE 1 *Continued*

Parameter	Quantity Symbol	Unit	Definition or Relationship to Other Parameters[A]
Peak width at base	w_b	cm	retention dimension between intersections of baseline with tangents to the points of inflection on the front and rear sides of any single-solute peak
Peak area	A	cm^2	
Distribution constant (partition coefficient)[B]	K		$K = \frac{\text{solute concentration in the stationary phase}}{\text{solute concentration in the mobile phase}}$
Capacity ratio (partition ratio, capacity factor, mass distribution ratio)[B]	k		$k = t_R'/t_M = (t_R - t_M)/t_m$ $= V_R'/V_M = (V_R - V_M)/V_M$
Number of theoretical plates[C]	n		$n = 16(t_R/w_b)^2 = 5.54(t_R/w_h)^2 = 4(t_R/w_i)^2$
Number of effective plates[C]	N		$N = 16(t_R'/w_b)^2 = 5.54(t_R'/w_h)^2 = 4(t_R'/w_i)^2$ $= n\left(\frac{k}{k+1}\right)^2$
Height equivalent to one theoretical plate[C]	h, HETP	cm	$h = L/n$
Height equivalent to one effective plate[C]	H, HEETP	cm	$H = L/N$
Reduced plate height[C]	h_r		$h_r = h/d_p$
Retention factor	R_f		a term used in paper and thin-layer chromatography $R_f = \frac{\text{distance moved by solute}}{\text{distance moved by mobile phase}}$ Sometimes the values are multiplied by 100.
R_M value	R_M		$R_M = \log[(1/R_f) - 1]$
R_s value	R_s		$R_s = R_f/R_{f(s)}$
Peak resolution (see Note 6, 6.2)	R_s		$R_s = \frac{2(t_{Rj} - t_{Ri})}{w_{bi} + w_{bj}} \simeq \frac{t_{Rj} - t_{Ri}}{w_{bj}}$ where $t_{Rj} > t_{Ri}$
Relative retention	$r_{i,s}$		$r_{i,s} = t_{Ri}'/t_{Rs}' = K_i/K_s = k_i/k_s$
Relative retention (separation factor, separation ratio)	α		$\alpha = t_{R2}'/t_{R1}' = K_2/K_1 = k_2/k_1$ The symbol r is used to designate relative retention of a peak relative to the peak of a standard while the symbol α is used to designate the relative retention of two consecutive peaks. By agreement, $t_{R2}' > t_{R1}'$ and thus, the value of α is always larger than unity while the value of r can be either larger or smaller than unity, depending on the relative position of the standard peak.
Number of theoretical plates required for a given resolution of peaks 1 and 2	n_{req}		$n_{req} = 16R_s^2\left(\frac{\alpha}{\alpha - 1}\right)^2\left(\frac{k_2 + 1}{k_2}\right)^2$
Number of effective plates required for a given resolution of peaks 1 and 2	N_{req}		$N_{req} = 16R_s^2\left(\frac{\alpha}{\alpha - 1}\right)^2$
Weight-average molecular weight	M_W	g/mol	second moment of a polymer distribution
Number-average molecular weight	M_N	g/mol	first moment of a polymer distribution
Molecular weight distribution	MWD		weight (or number) fractions as a function of molecular weight
Integral molecular weight distribution	∫MWD		sum of weight fractions as a function of molecular weight
Differential molecular weight distribution	d(MWD)		relative abundance of a fraction as a function of molecular weight
Dispersity	d		a measure of the breadth of a molecular weight distribution
Hydrodynamic volume	V_h	cm^3/mol	a polymer molecular property proportional to M
Exclusion limit	V_h, max	cm^3/mol	maximum V_h that entered into pore
Solute designations (subscripts)	i		any solute
	j		a solute eluting after solute i
	s		a standard or reference solute
	1, 2		two consecutive solutes from which solute 2 elutes later than solute 1

Footnotes on next page.

TABLE 1 *Continued*

[A] Peak position and width parameters refer to any one sample component unless otherwise shown by multiple-solute subscripts.

[B] In the literature, the symbol k is sometimes also used for the partition coefficient with the consequent use of k' (or K') for the capacity ratio. These usages are the result of individuals' preferences and have never been officially endorsed by the IUPAC or ASTM.

[C] The symbols used here for the various plate numbers and plate heights correspond to the long-standing nomenclature of ASTM in gas chromatography and also to the nomenclatures recommended by other standardizing groups. One can also find in the literature other meanings of the symbols and, therefore, it is important to always ascertain the meaning attributed in the particular publication. The most important differences from the usage recommended here are: (*a*) using N for the number of theoretical plates and N_{eff} for the number of effective plates; (*b*) using H for the HETP, H_{eff} for the HEETP, and h for the reduced plate height.

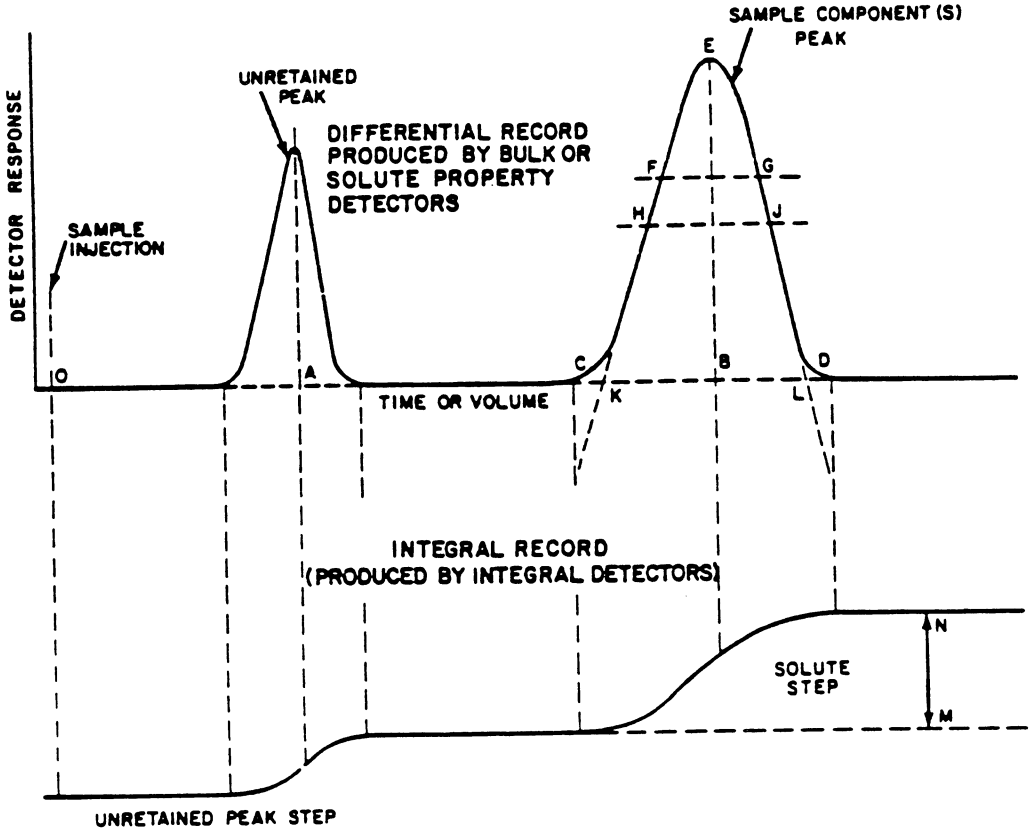

FIG. 1 Typical Chromatogram.

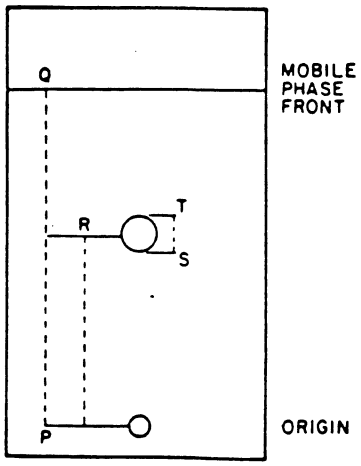

FIG. 2 Typical Planar Chromatogram.

2

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Introduction to HPLC

Basic Theory

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What is HPLC?

High Performance Liquid Chromatography

High Pressure Liquid Chromatography

High Priced Liquid Chromatography

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What is HPLC?

HPLC is an improved liquid chromatography system where stationary phases in a column is densely packed to increase plate efficiency while the mobile is forced through the system mechanically.

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The Definition of Chromatography

Chromatography is an analytical technique used for separating components of a mixture to aid in their identification or quantitation.

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* Based on the partitioning of solutes between two phases.

Stationary phase is packed or coated into a column and does not move.

Mobile phase is pumped through the column at a regulated speed and carries the sample

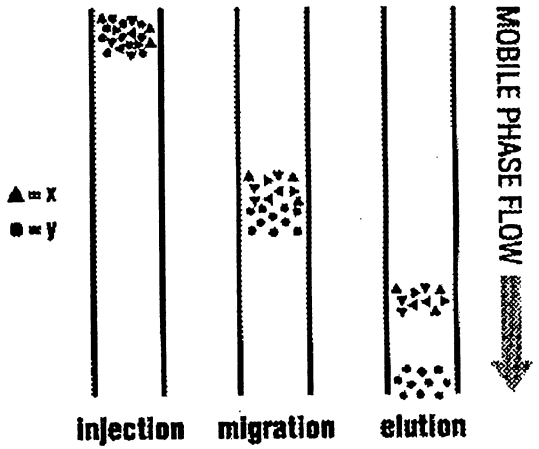

Figure 1-1: Separation of sample solutes

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Solute Partitioning

* If the solute is highly soluble in the solvent but not soluble in the stationary phase, the solute will migrate through the column with little interaction with the stationary phase.

*If the solute is more soluble in the stationary phase, it will migrate more slowly through the column depending on the extent of the interaction.

*If each solute travels the length of the column at different speed, then the solutes will be separated when they elute.

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Different Chromatographic Techniques.

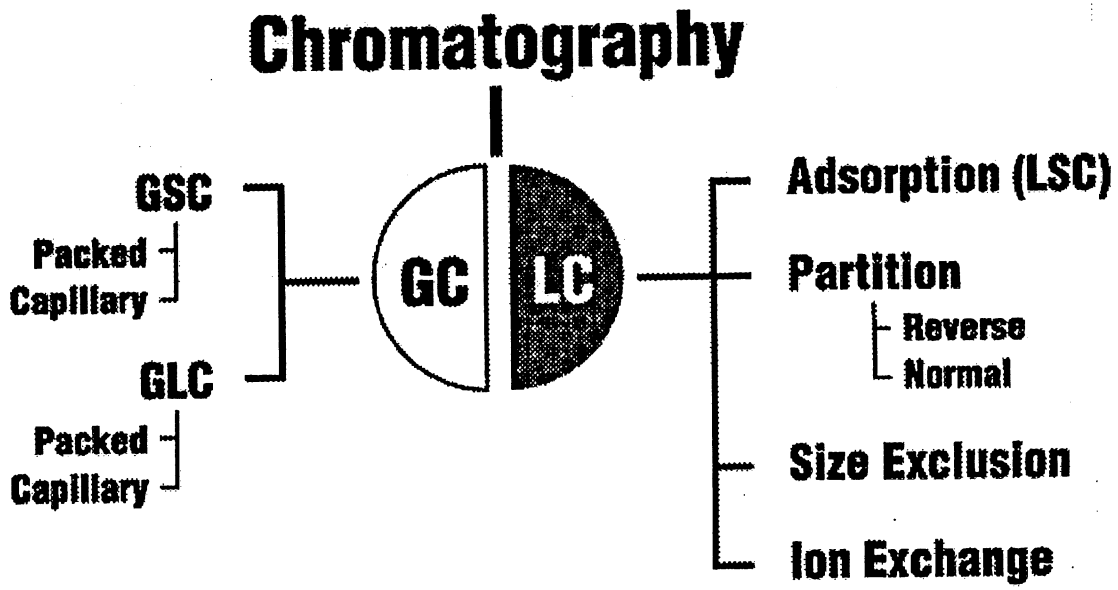

Figure 1-3: Divisions of chromatography

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Modes of HPLC

HPLC Mode	Mobile Phase	Stationary Phase	Separation Mechanism
LSC	non-polar solvent	polar powder (solid)	adsorption
Partition: Reverse Phase	polar solvent	non-polar bonded liquid	hydrophobic interaction
Partition: Normal Phase	non-polar solvent	polar bonded liquid	polar interaction
Size Exclusion	non-interactive solvent	polymeric gel	filtering by size
Ion Exchange	buffer/ion pairing reagent	ion exchanger	charge interaction

Table 1-1: Modes of HPLC separation

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The HPLC Apparatus

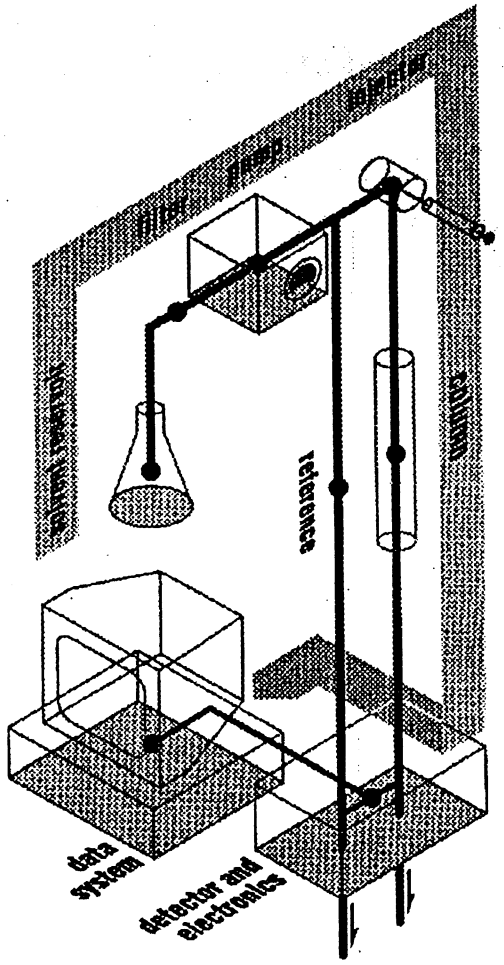

Figure 1-5: A simplified diagram of the HPLC apparatus

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LC vs. GC

HPLC	Capillary GC
non-volatile samples	volatile and thermally stable samples
macromolecules	relatively simple and inexpensive
thermally unstable (labile) samples	rapid analysis with excellent resolution
inorganic and ionic samples	easily interfaced to mass spectrometry

Table 1-4: Comparison of HPLC and capillary GC

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Column Theory

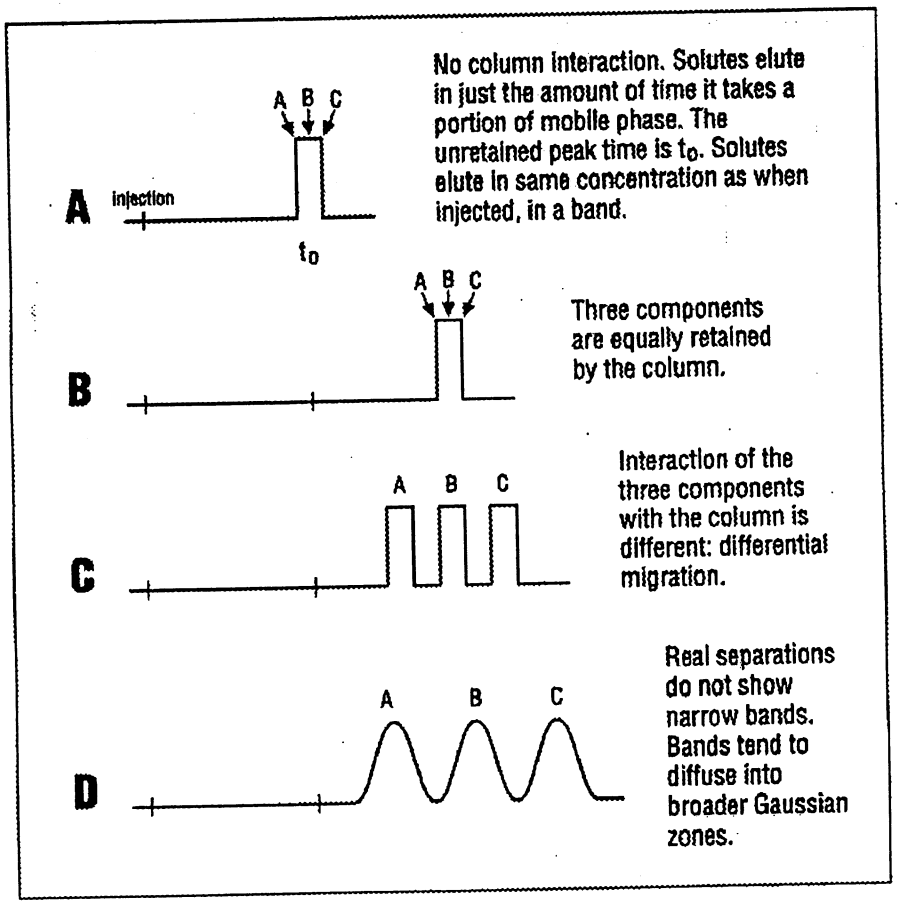

Figure 1-6: Retention and separation

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Capacity Factor

* Measures the solute molar distribution between the stationary and mobile phases

* Determines the amount of sample which can be introduced into the column

$$K' = \frac{t_R - t_o}{t_o} = \frac{t'_R}{t_o}$$

where

t_R = retention time of the solute
t_o = time for an unretained solute
t'_R = adjusted retention time of the solute

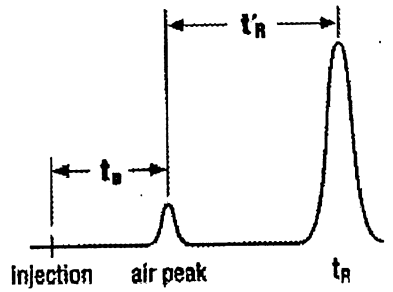

Figure 1-7: Measuring the capacity factor

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Phase Ratio

- Ratio of the volume of mobile phase to the volume of stationary phase inside the column.

$$\beta = \frac{V_m}{V_s}$$

where

V_m = volume of mobile phase inside the column

V_s = volume of stationary phase inside the column

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Resolution

$$R = \frac{1.18\,\Delta t_R}{W_1 + W_2}$$

where

Δt_R = difference in retention times between the two peaks

W_1 and W_2 = widths of the two peaks at half their height

if

R = 1.0, the separation of the two peaks is 98%

R = 1.5, the separation of the two peaks is 99.7%

and

baseline resolution is found only for R > 1.5

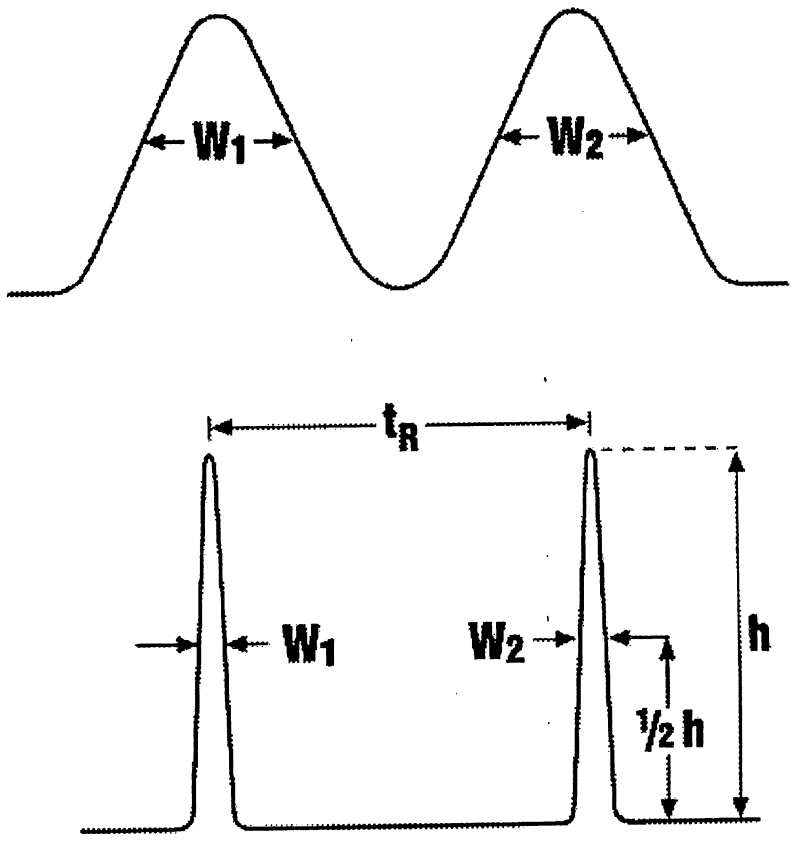

Figure 1-8: Resolution on two different columns

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Resolution Vs Concentration

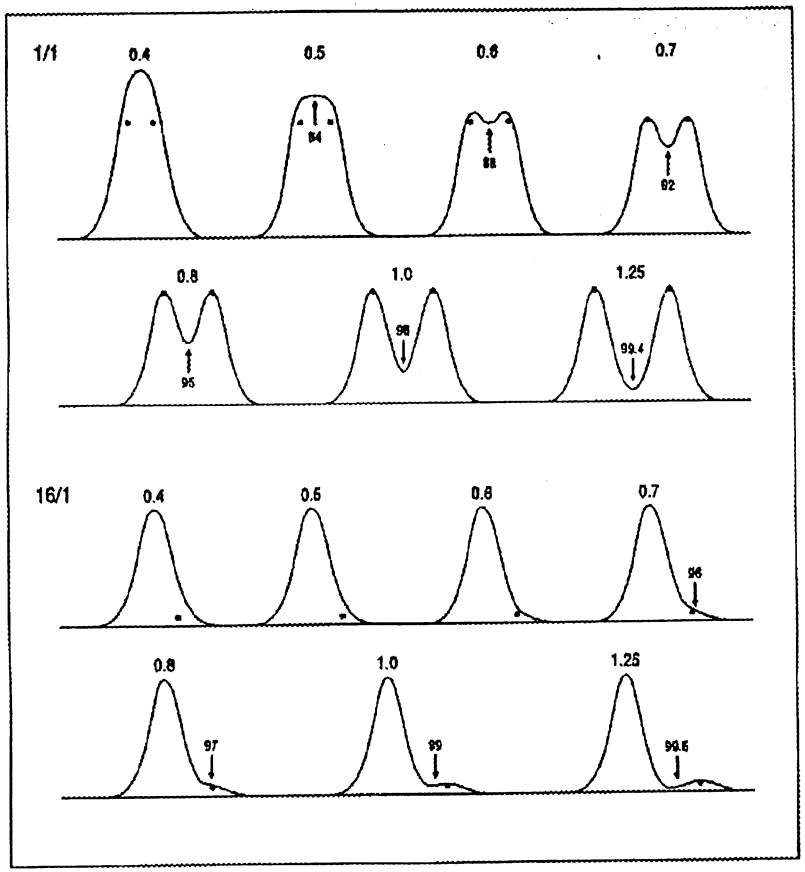

Figure 1-9: Resolution of two components at the same and at different concentrations

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Resolution Vs Peak Shape

Ideal Chromatographic peak shape is a Guassian shape

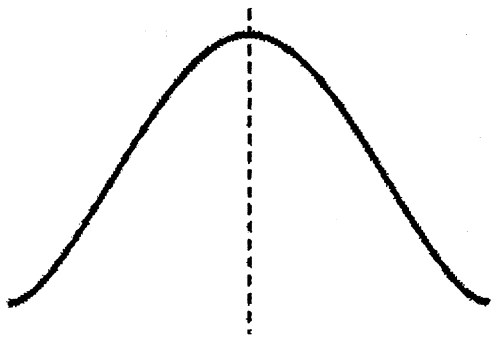

Figure 1-10: Gaussian curve

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Resolution Vs Peak Shape

Gaussian curve represents the resulting statistical distribution of the molecules in the space that they occupy

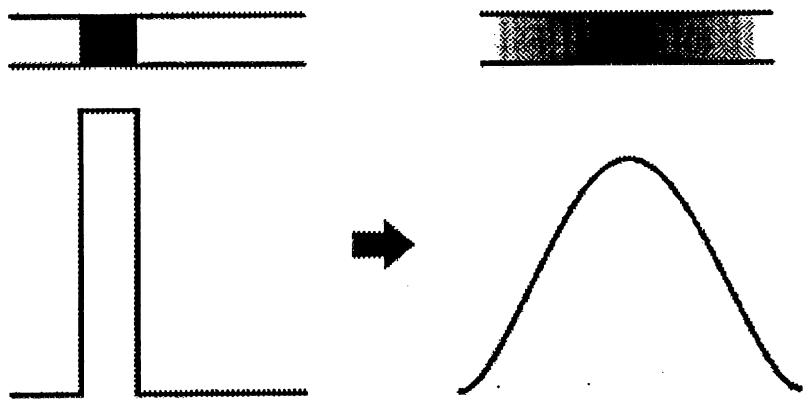

Figure 1-11: Statistical distribution of sample molecules

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Resolution Vs Selectivity, Capacity and Efficiency

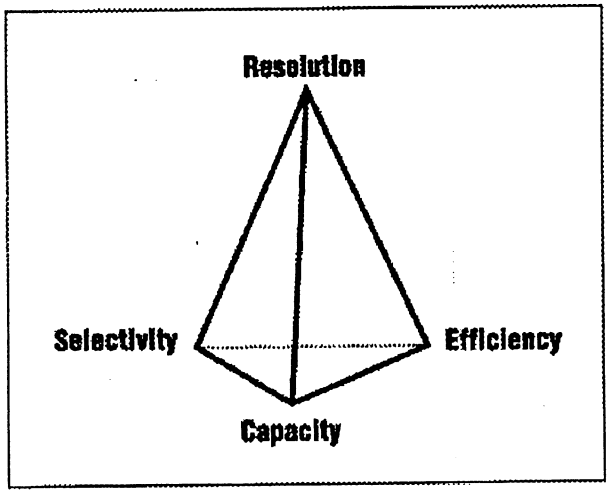

Figure 1-12: The resolution pyramid

$$R = \frac{\sqrt{N}}{4} \times \frac{(\alpha - 1)}{\alpha} \times \frac{\kappa'}{(\kappa' + 1)}$$

where

N = number of theoretical plates (efficiency)
α = relative retention time (selectivity)
κ = partition (capacity) ratio

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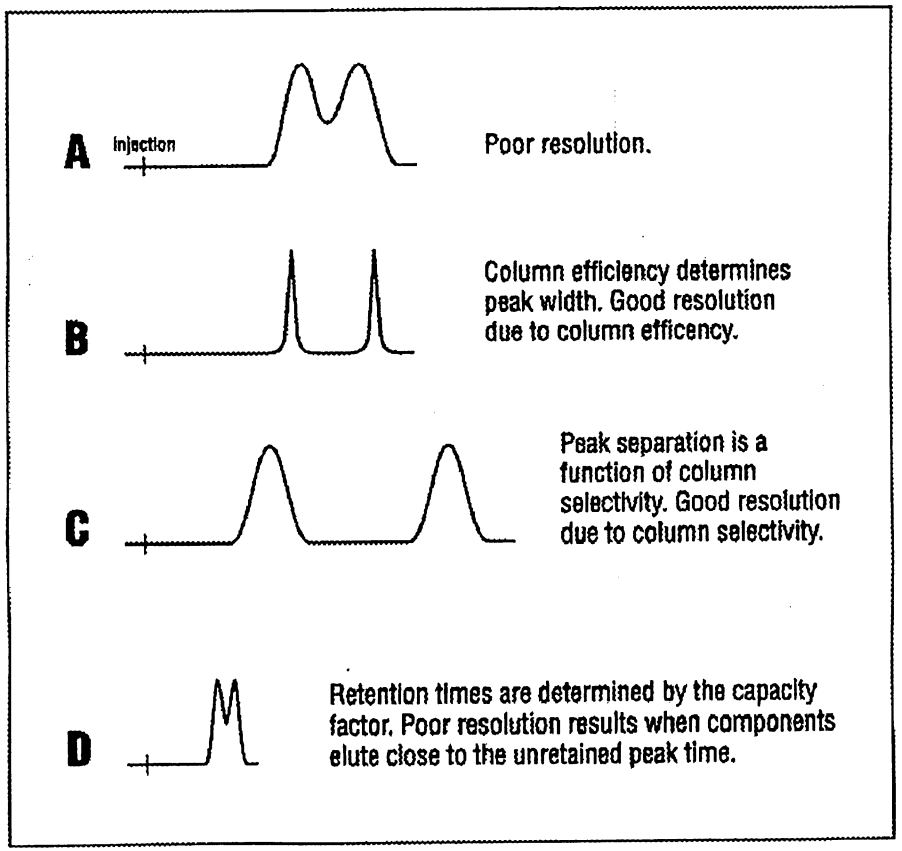
Resolution Vs Selectivity, Capacity and Efficiency



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Selectivity

*Measure of a column's ability to distinguish between two components

*May be adjusted by choice of mobile and stationary phases

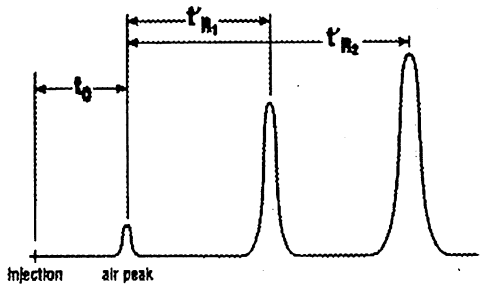

Figure 1-14: Adjusted retention times of two peaks

$$\alpha = \frac{K_2}{K_1} = \frac{t'_{R_2}}{t'_{R_1}}$$

where

K_1 = distribution coefficient of first peak
K_2 = distribution coefficient of second peak
t'_{R_2} = adjusted retention time of the second peak
t'_{R_1} = adjusted retention time of the first peak

if

$a > 1$, separation is achievable

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Column Efficiency

$$N_{eff} = 5.54\left[\frac{t'_R}{W}\right]^2$$

where

t'_R = adjusted retention time
W = width of the peak at half height

* More efficient columns produce sharper peaks for the same retention time (peak broadening)

*Quantitatively expressed as the number of theoretical plates

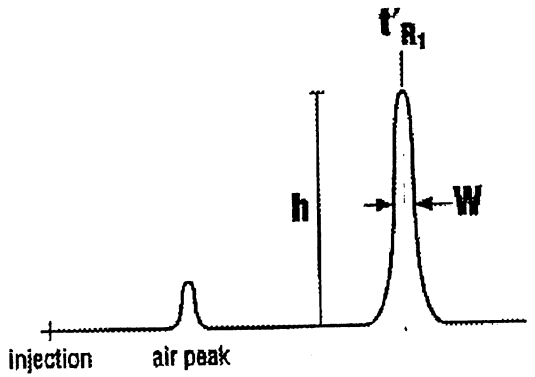

Figure 1-16: Measuring N_{eff}

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Band Broadening

***Column efficiency is primarily a function of the column length and the size and uniformity of the particle**

There are three main sources that contribute to lower column efficiencies

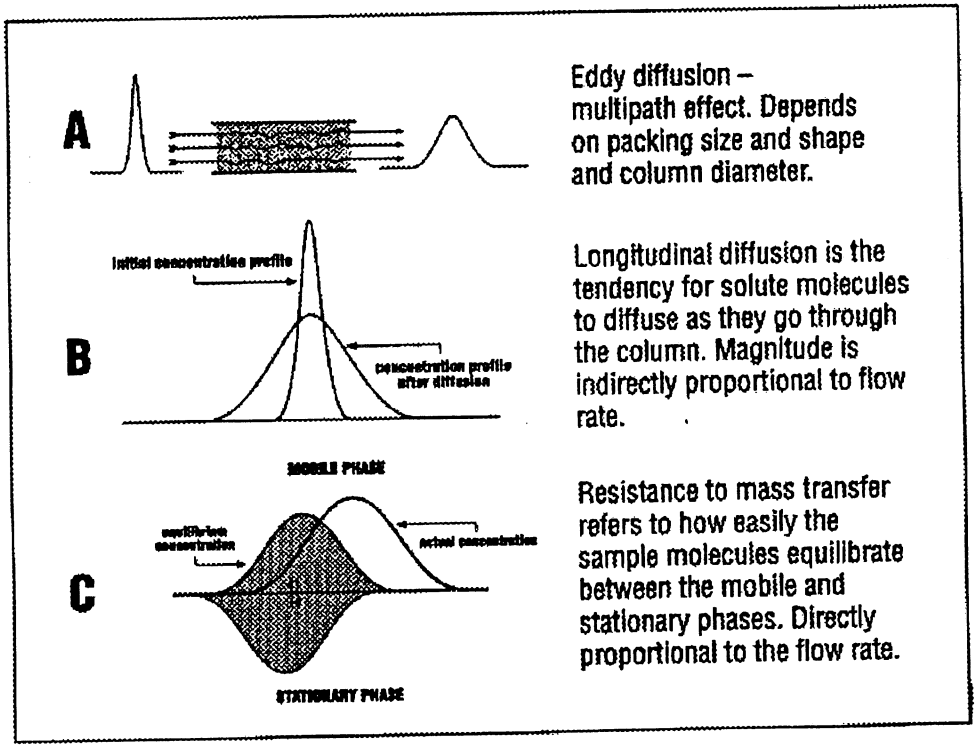

Figure 1-17: Band broadening contributions

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HETP

The Van Deemter Equation is a theoretical means that expresses column efficiency. It is the sum of the three contributing terms for band broadening

$$HETP = A + \frac{B}{\mu} + C\mu$$

where

A = eddy diffusion
B = longitudinal diffusion
C = resistance to mass transfer
μ = mobile phase linear velocity

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HETP Plots

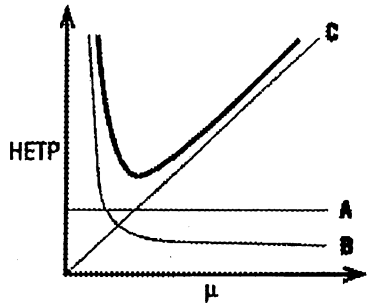

Figure 1-18: HETP vs. mobile phase linear velocity

- The particle size of the packing is the major factor in determining efficiency.

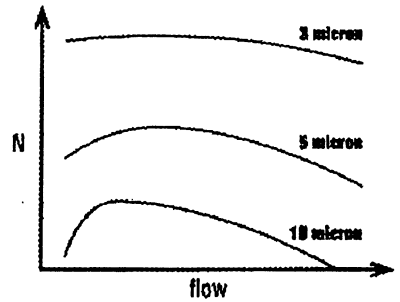

Figure 1-19: Effect of packing size on efficiency

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Measuring HETP

HETP describes the length of the column equivalent to one theoretical plate. Smaller HETP indicates more efficient column

$$HETP = \frac{L}{N_{eff}}$$

where

L = length of the column (mm)

N_{eff} = effective number of theoretical plates

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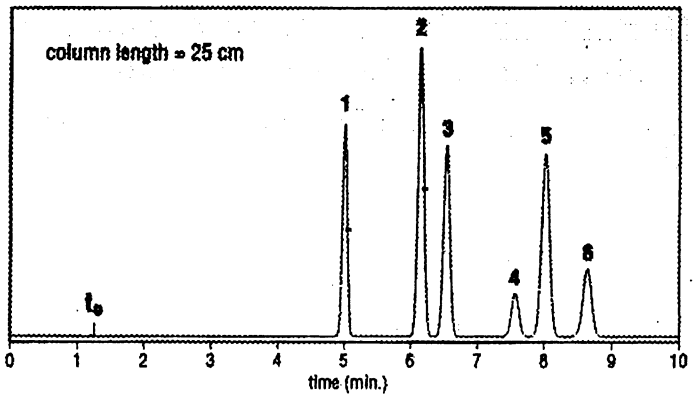
Theory in Practice



$$\kappa' = \frac{t'_{R_1}}{t_o} = \frac{5.0 - 1.3}{1.3} = 2.8$$

$$\alpha = \frac{t'_{R_2}}{t'_{R_1}} = \frac{6.2 - 1.3}{5.0 - 1.3} = 1.3$$

$$R = \frac{1.18\,\Delta t_R}{W_1 + W_2} = \frac{1.18\,(1.2)}{0.13 + 0.15} = 5.0$$

$$N_{eff} = 5.54\left(\frac{t'_{R_1}}{W_1}\right)^2 = 5.54\left(\frac{3.7}{0.13}\right)^2 = 4{,}500$$

$$HETP = \frac{L}{N_{eff}} = \frac{25\,cm}{4{,}500} = .05\,mm$$

VARIAN

Optimizing an LC Separation

To optimize the capacity factor:

*Adjust the solvent strength

*Change the stationary phase

To optimize selectivity:

*Change mobile phase composition

*Use mobile phase additives

*Change the stationary phase

*Change pH and the temperature

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Optimizing an LC Separation

To optimize column efficiency

*Decrease particle size

*Reduce the flow rate towards minimum on HETP plot

*Use two or more columns in series

*Use less viscous solvents

*Increase column temperature

*Increase particle uniformity

3

VARIAN

Introduction to HPLC

SYSTEM

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Safety Concerns

*** Pumps used with HPLC systems are capable of generating pressures higher than 6000psi**

-Safety glasses or a face shield should be worn at all times when operating the instruments

*** Solvents should be kept in a safe place**

-Follow all regulations

-Review MSDS's

-Obey the threshold limit valve(TLV) in your work area

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Safety Concerns (Cont'd)

Learn about the hazards of your samples

-Take precautions whenever you are in doubt

-Protect your eyes, skin, and respiratory system

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Safety Concerns (Cont'd)

Do not look directly into flow cells or at UV light sources

-Turn light source and main power off during trouble shooting and maintenance

-Wear safety glasses with UV protection when detector lamp is exposed during operation

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Safety Concerns (Cont'd)

Hazardous Symbols

Hazard Symbols

Electrical Shock: Hazardous voltages are present inside the covers. Disconnect from main power before removing screw-attached panels.

Chemical: Hazardous chemicals may be present in the instrument. Avoid contact, especially when replenishing reservoirs.

Heat: Very hot surfaces may be exposed. Use caution to avoid severe skin damage.

Eye Hazard: Eye damage could occur either from flying particles or UV radiation. Wear safety glasses when servicing instrument.

Fire: The potential for fire may be present. Follow manual instruction to ensure safe operation.

Explosion: The potential for explosion may exist because of type of gas or liquid used. Follow manual instructions carefully.

Radiation: Ionizing radiation source is contained inside. Follow operator's manual instructions for proper use and disposal.

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Environmental Concerns

***Follow laboratory or local government guideline regarding chemical disposal**

-Label waste vessels accurately

-Never dispose of chemicals in the sink or garbage

*** Consider solvent recycling to lessen waste management and save cost**

*** Do not overlook the disposal of contaminated laboratory glassware, sample vials or autosampler vials**

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The Solvent System

* Mobile phase consists of one or more aqueous salts (buffer) or organic solvents

* Solvent is pumped through the HPLC to carry the sample through the column to the detector and to interact with the sample during separation

* There are many solvent considerations

-Polarity and solvent strength

-Purity, filtering and degassing

-Miscibility with other solvents

-Buffering to maintain pH

-Isocratic or gradient analysis

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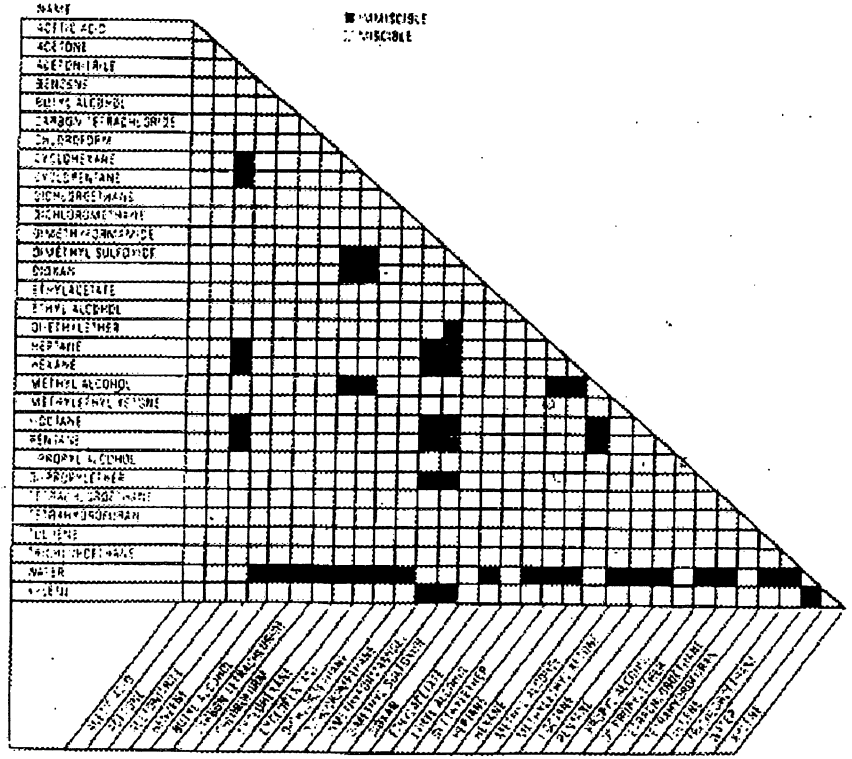
Chemistry of Solvents

Miscibility

* Solvents may form air bubbles from poor mixing

* Premix solvents in a test tube or beaker, if layers do not form between liquids, the solvents are miscible

Table II : Solvent miscibility table.



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Chemistry of Solvents

Viscosity

* Higher viscosities yield higher column head pressure and lower efficiency

* Viscosity does not change linearly with amount of solvent in mix

* In gradient analysis, the point of highest viscosity significantly increases system pressure

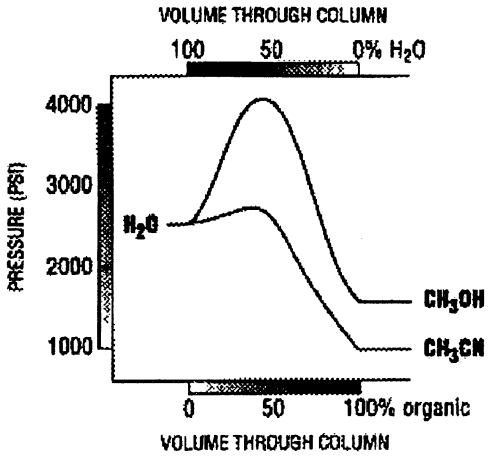

Figure 2-2: Effect of viscosity on pressure

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Chemistry of Solvents

Compressibility

* Affects absolute accuracy of pumps and pulsation level

* With a greater solvent compressibility, the pump must work harder to maintain flow at the desired level

* Most modern pumps have some sort of compressibility compensation built in via software or hardware

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Chemistry of Solvents

Refractive Index (RI)

* A measure of the way different solvents refract light

	good case	fair case	poor case
solute RI	sucrose 1.5376	cyclohexylamine 1.4565	n-decylaproate 1.4423
solvent RI	water 1.3325	chloroform 1.4429	chloroform 1.4429
Δ RI	0.2051	0.0136	0.0006
detection limit	1 ppm	15 ppm	340 ppm

Table 2-1: Effect of the difference in sample and solvent refractive indices on the limit of detection.

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Chemistry of Solvents

Polarity

* Molecule containing an overall dipole moment (μ)

* The polarity index is a relative measure of the degree of interaction of the solvent with various polar test solutes

--Increases with increasing solvent polarity

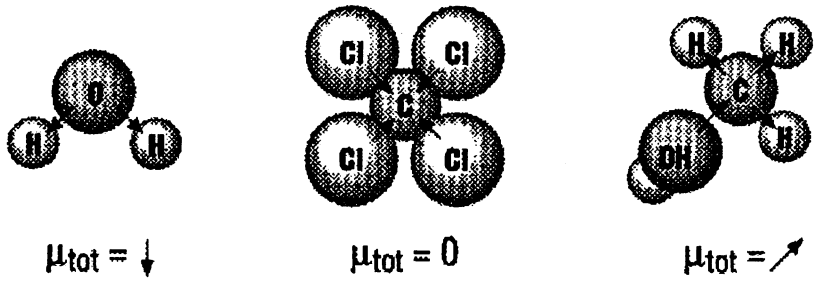

Figure 2-5: Polarity of water, carbon tetrachloride, and methanol

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Chemistry of Solvents

Solvent Strength

* A stronger mobile phase will result in less retention by the stationary phase (shorter analysis time)

* Solvent strength is the sum of three types of intermolecular interactions: dispersion, orientation, and hydrogen bonding

* When there is a good interactive match between solvent and solute, the solvent strength is especially high

* Non polar or organic solvents are stronger for reverse phase

  --A low percentage of organic modifier is a weak solvent and a high percentage of organic modifier is a strong solvent

* Polar solvents are stronger for normal phase

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Chemistry of Solvents

Solvent Strength

Solvent Strength	Reverse Phase	Normal Phase
↓	water	hexane
	DMSO	1-chlorobutane
	methanol	methylene chloride
	acetonitrile	acetonitrile
	tetrahydrofuran	

Table 2-2: Solvent strength

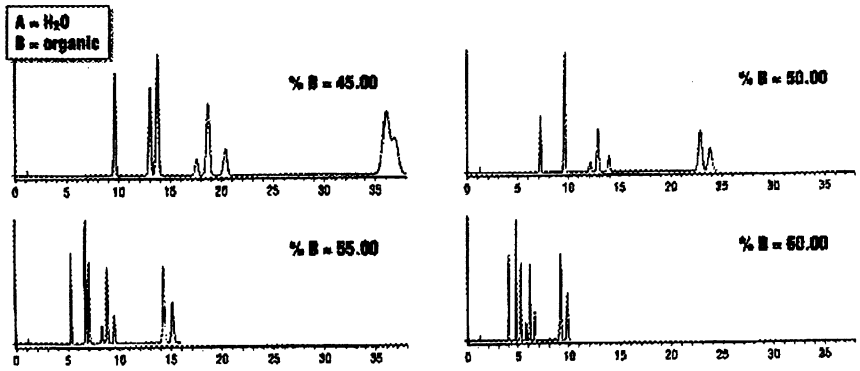

Figure 2-7: Change in retention time due to varying solvent strength

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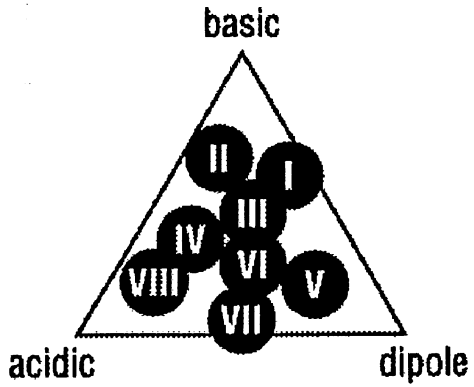
Chemistry of Solvents

Selectivity

* Degree to which a solvent is chromatographically stronger for a particular solute

* Selectivity depends on the functionality of the solvent molecules



Solvent Selectivity Groups

I	alkyl amines, ethers, phosphates
II	alcohols
III	THF, DMSO, pyridines
IV	carboxylic acids, glycol, formamide
V	methylene chloride, ethylene chloride
VI	ketones, esters, nitriles, dioxane
VII	aromatic hydrocarbons, nitro compounds
VIII	chloroform, phenols, water

Figure 2-8: Solvent groups

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Chemistry of Solvents

Solvent Properties

	molecular weight	boiling point (°C)	vapor pressure (Torr @ 20°C)	refractive index	dipole moment debye (D)	solvent group	polarity index (P)	viscosity (cP @ 20°C)	TLV (ppm)	UV cutoff (nm)
acetonitrile	41.05	81.6	88.8	1.344	3.44	6	5.8	0.38	40	200
methanol	32.04	64.7	97	1.328	2.87	2	5.1	0.55	200	225
tetrahydro-furan	72.11	66	142	1.407	1.75	3	4.0	0.55	200	250
water	18.02	100	17.54	1.333	1.87	8	10.2	1.00	not listed	none
hexane	86.18	68.7	124	1.375	.08	0	0.1	0.313	50	225
n-butyl chloride	92.57	78.4	80.1	1.402	1.9	5	1.0	0.45	not listed	240
methyl t-butyl ether	88.14	55.2	240	1.369	1.32	1	2.5	0.27	not listed	250
methylene chloride	84.93	39.8	350	1.424	1.14	5	3.1	0.44	500	240
chloro-form	119.4	61.2	158.4	1.446	1.15	8	4.1	0.57	2	265
ethyl acetate	88.11	77.1	73	1.372	1.88	6	4.4	0.45	400	275

Table 2-3: Physical properties of common HPLC solvents

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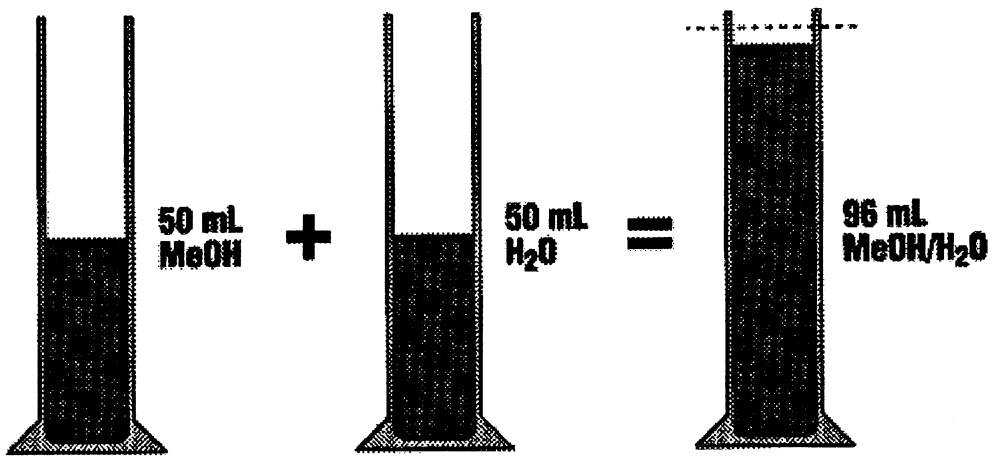
Preparing the Mobile Phase

* When mixing solvents to form mobile phases, the volume of each component should be measured separately before the solvents are mixed

--Volume of the mixture does not usually equal the sum of the separate volume



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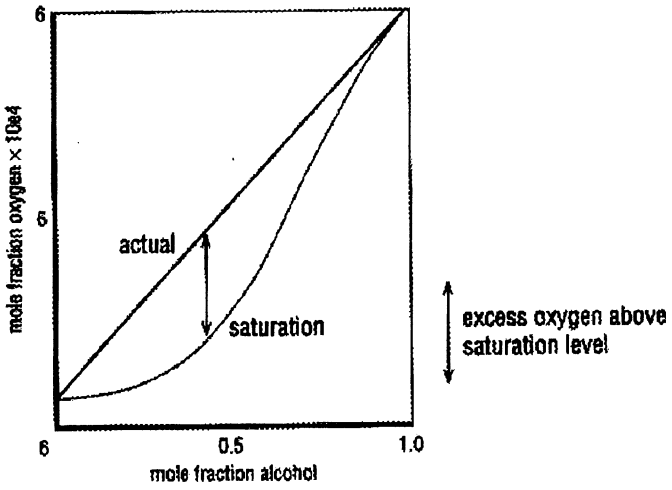
Preparing the Mobile Phase

* **Bubble problems in HPLC result from mixing solvents**

  --Air is generally less soluble in solvent mixtures than in pure solvents

  --Excess gas leaves solution as bubbles



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Degassing

* Degassing may improve reproducibility and detector stability
* Helium Sparging
    --Helium scavenges air from the mobile phase
    --Extended vigorous sparging causes evaporation
* Vacuum degassing

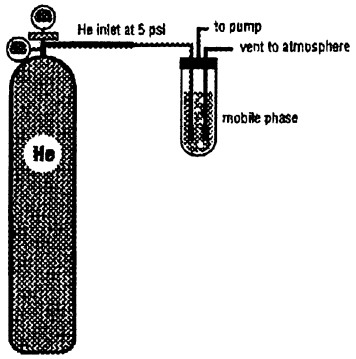
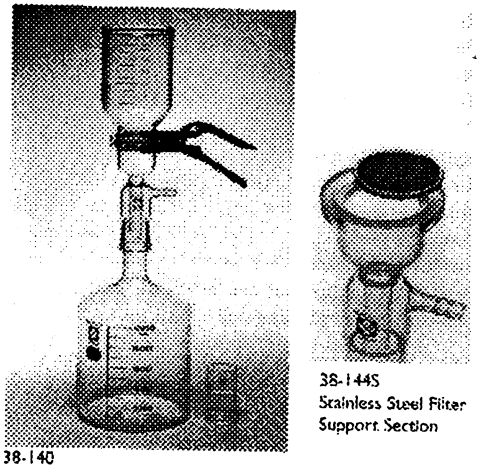


Figure 2-10: Sparging apparatus



38-140
Includes all components shown above

38-144S
Stainless Steel Filter
Support Section

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Filtering

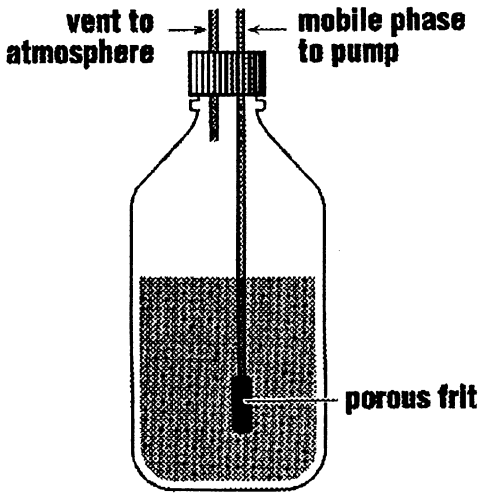
* Filter the mobile phase if particles are visible

  --Particles can damage or block the analytical column and

  --Cause problems in detector cell

* Cap the reservoir to avoid dust

* A porous frit serves as a gross filter



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Additives

* **Buffers are necessary to maintain the pH to limit biological activity or to control acid/base interaction**

  --At least 50-100mM should be added

  --pH>7 may dissolve column packing

  --Precipitation may cause severe damage to hardware

* **Antioxidants may be necessary to protect sample and hardware**

pH range	buffer	UV cutoff (nm)
2–3.5	phosphate	210
3.6–6	acetate	220
6–8	phosphate	210

Table 2-4: Buffers for HPLC mobile phases

VARIAN

Choosing the Correct Mobile Phase

*** The major consideration in choosing a solvent system is choosing one that adequately separates your sample. In addition to a quality separation, the mobile phase should:**

--Not alter the column and its characteristics

--Be compatible with the detector

--Dissolve the sample

--Have a low viscosity

--Permit easy sample recovery, if desired

--Be of high purity and non toxic

--Be commercially available at a reasonable price

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Reverse Phase Solvents

* Most reverse phase LC uses water as the main mobile phase component (A)

--Water is the most common polar liquid

--All but the most polar or ionic solutes experience a strong hydrophobic force that drives them onto the stationary phase and causes retention

--HPLC grade water should always be used

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Reverse Phase Solvents

* Adjusting organic modifier concentration (B) is the primary means of changing retention

--The three most common are acetonitrile, methanol and tetrahydrofuran

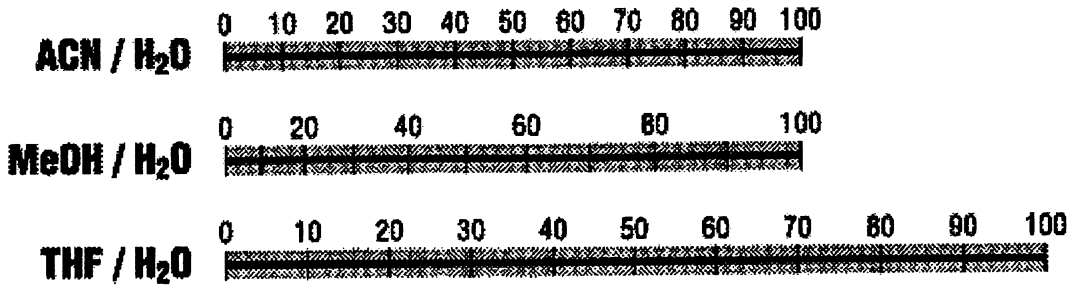

Figure 2-12: Matching solvent strengths

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Normal Phase Solvents

* Normal phase chromatography uses mixtures of a non polar solvent and one or more polar solvent modifiers

  -- Isocratic composition is usually a hydrocarbon, 10-50% chlorinated hydrocarbon, and 1-5% alcohol

* Polar interactions are the primary cause for the separation

  -- The mobile and stationary phases are in competition for the solute

  -- The mobile phase can absorb to the stationary phase

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Ion Chromatography Solvents

* Uses an aqueous buffer at a pH providing for charged samples

  -- For acids, the pH should be greater than 5

  -- For bases, the pH should be less than 5

* As the concentration of the buffer is increased, solvent strength increases and retention decreases

* Organic solvent can be added to change the retention and selectivity

  --Methanol is usually chosen because it provides better solubility

VARIAN

Ion Chromatography Solvents

* Ion pair reagent is usually a short chain ion

--For the separation of protonated bases, pentane sulfonate, hexane sulfonate, or heptane sulfonate is used

-- For separation of ionized acids, tetraethylammonium or tetrabutylammonium is used

VARIAN

Solvent Programming

* Conditions to remember when choosing the optimum strength are resolution, analysis time and the capacity factor

--Resolution should always be at least 1.5 for quantitation

--Shorter analysis times can be achieved by using a stronger solvent for overly resolved peaks

--A good rule of thumb is to have all of the solutes elute with k' between 1-20

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Solvent Programming

* Computer modeling software is available to aid in finding the best mobile phase strength

* Use the methods development triangle for manual method development

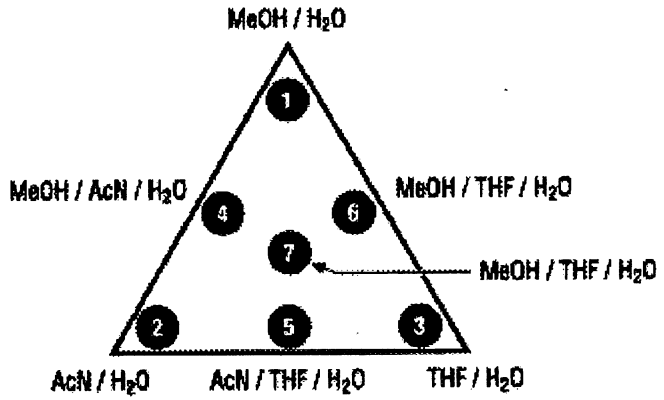

Figure 2-13: Methods development triangle for reverse phase

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Isocratic vs Gradient Separation

* **Isocratic mobile phase cannot achieve all separations**

  --Some samples are too complex

  --Poor resolution of early peaks

  --Band broadening and tailing of later peaks

* **In gradient elution, the mobile phase strength increases by changing solvent composition over time**

  --Chosen for wide range of samples or biopolymers

  --Cleans the column during each run

  --Used to estimate optimum isocratic conditions

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Isocratic vs Gradient Separation

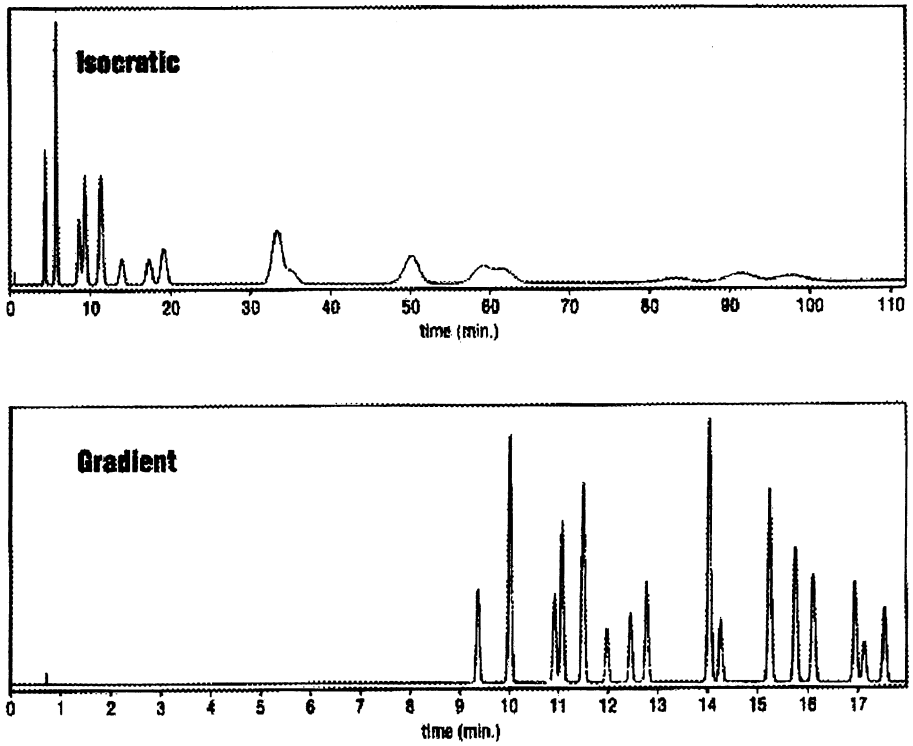

Figure 2-14: Isocratic vs. gradient elution

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Gradient Elution

* The pump must be capable of combining a weak and strong solvent under the control of a gradient program

  -- A gradient can be weak or strong and shallow or steep

* Solutes remain retained at the front of the column until the mobile phase becomes strong enough to start each solute moving

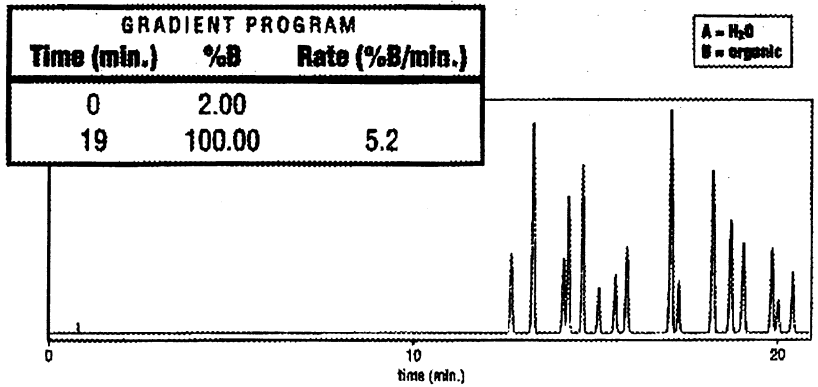
  -- The organic mobile phase should start at a % that is strong enough to start the first solute moving

  -- Once the last band elutes, the gradient can be terminated

VARIAN

Gradient Elution

GRADIENT PROGRAM		
Time (min.)	%B	Rate (%B/min.)
0	2.00	
19	100.00	5.2



GRADIENT PROGRAM		
Time (min.)	%B	Rate (%B/min.)
0	52.00	
1.7	52.00	0.0
5.8	94.00	10.0

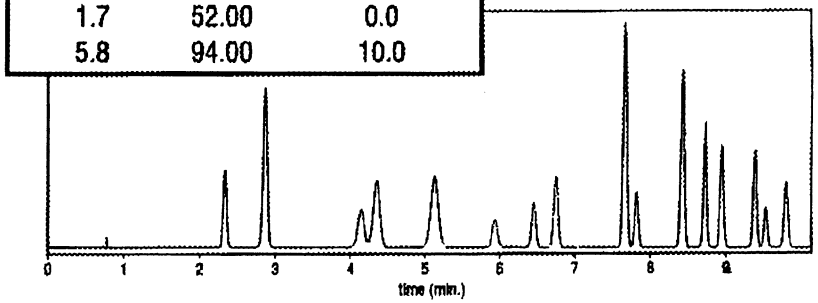


Figure 2-15: Gradient with wasted analysis time and efficient analysis time for reverse phase

VARIAN

Gradient Considerations

* Multi-segment gradients can improve resolution marginally
* Isocratic hold times can be used to separate a poorly resolved cluster
* Changes made to the gradient program do not take place instantly

-- Column dead volume and equipment dwell volume affect when gradient reaches the column

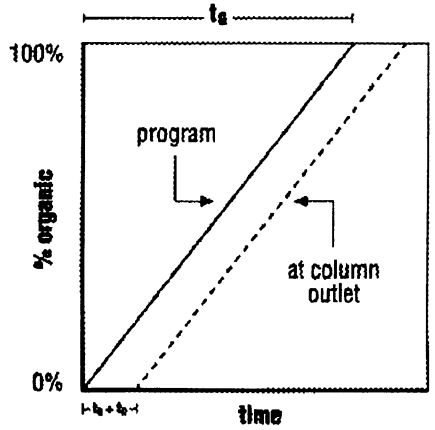


Figure 2-16: Effect of dwell volume for reverse phase

VARIAN

The Pump

* A good pump for HPLC has the following qualities:

--Able to withstand high pressures

--Large programmable flow range

--Flow should be non-pulsing

--Easy to dismantle and repair

--Wetted parts should be inert

--Gradient elution possible

--Rapid solvent change over

--Flow rate reproducibility

VARIAN

Syringe Pump

*** Mobile phase is displaced from a chamber using a stepper motor to turn a screw which drives a piston**

--Pumping is non-pulsed, constant flow

--Flow rate is controlled by varying the rotation of the motor

ADVANTAGES	DISADVANTAGES
Pulseless flow	Limited volume
	Difficult mobile phase changeover

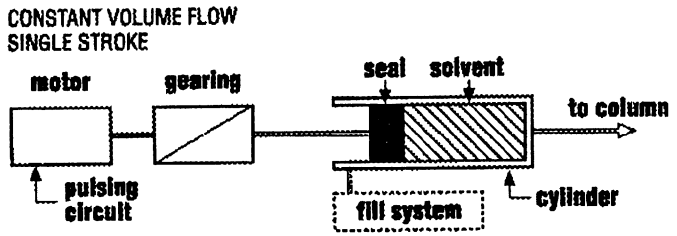


Figure 2-17: Schematic of a syringe pump

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Reciprocating Pump

* A piston is driven in and out of the solvent chamber by an eccentric cam or gear

--Flow rate is varied by changing the length of the delivery stroke.

--Inlet and outlet check valves controls the direction of flow through the pump head

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Reciprocating Pump

*** During the intake stroke, the piston retracts from the pump head, creating a low pressure area**

--Outlet check valve settles onto its seat to prevent downstream mobile phase from entering the pump head

--Inlet check valve actuates, allowing mobile phase to fill the pump head

*** During the delivery stroke, the piston moves into the chamber, increasing the pressure**

--Inlet check valve seats

--Outlet check valve opens, allowing mobile phase to flow into the column

VARIAN

Reciprocating Pump

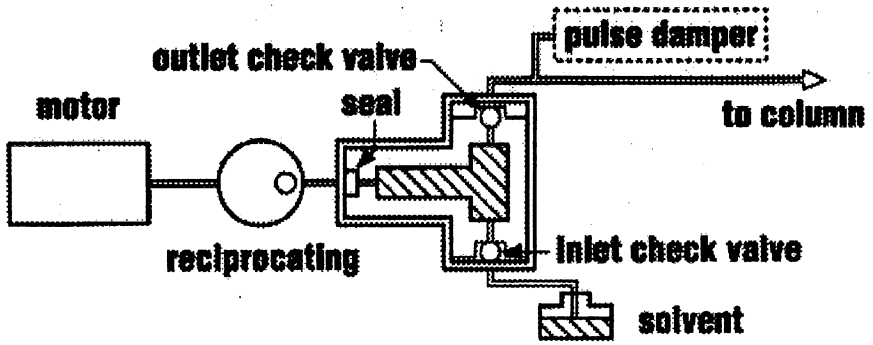
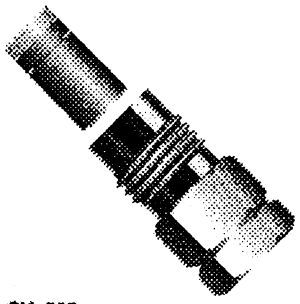


Figure 2-18: Schematic of a reciprocating pump

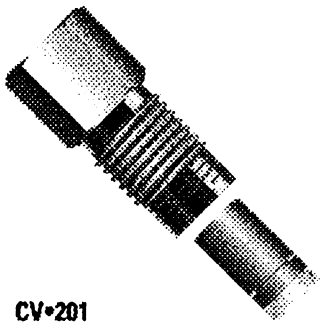
ADVANTAGES	DISADVANTAGES
Unlimited capacity	Pulsed flow
Small internal volume	

VARIAN

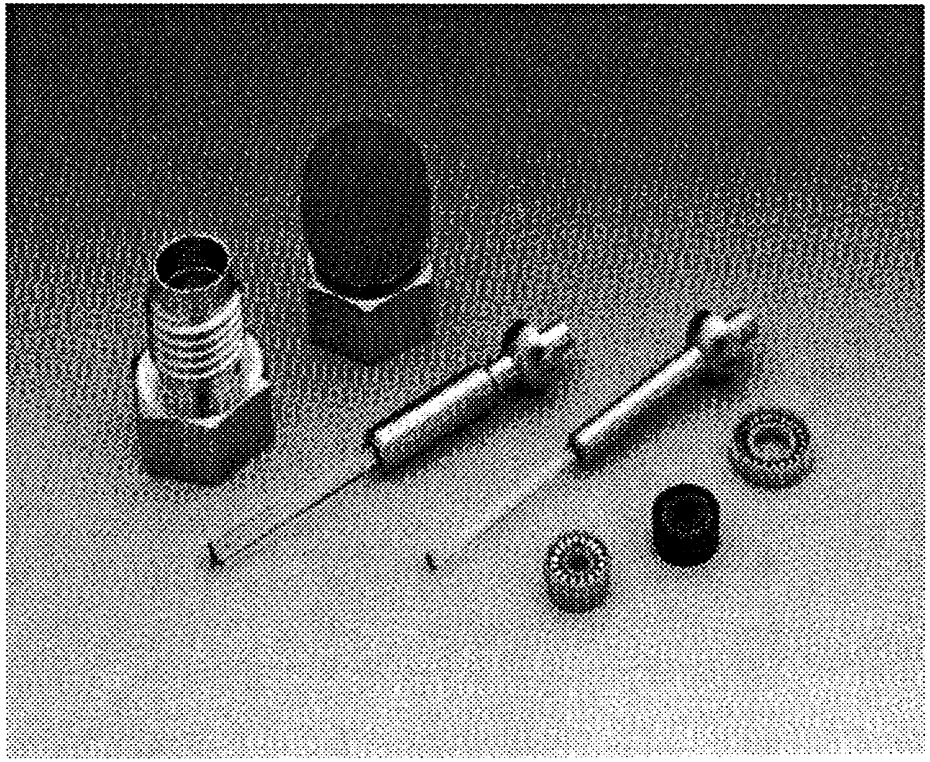
Pump Head Accessories



CV•212
Inlet Check Valve Assembly
(1/4•28 flat-bottom internal threads)



CV•201
Outlet Check Valve Assembly
(1/4•28 coned internal threads for 1/16" OD tubing)



VARIAN

Mixers

* Solvent composition must be well mixed before reaching the column head

* Varian Prostar pumps uses either high or low pressure mixing

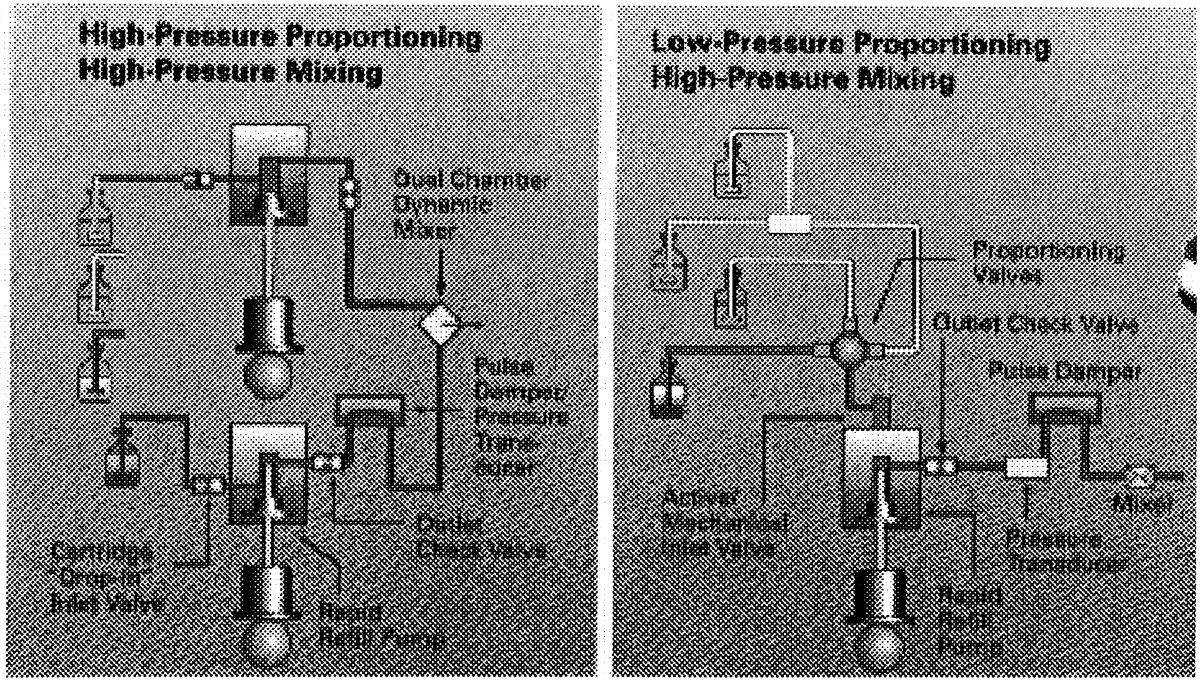
* Dynamic mixers use small moving parts to mix

* Static mixers rely on laminar flow

Figure 2-19: High-pressure, static mixer

VARIAN

Static Vs High Pressure Mixing



VARIAN

Sample Injection Valves and Autosamplers

* A six-port valve houses the injection port and sample loop that delivers the liquid sample into the system

* Turning of valve can be manual or automated

    --Automation improves precision

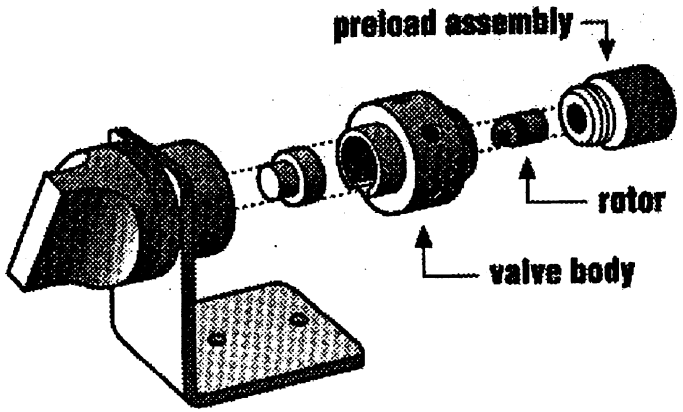


Figure 2-20: Valco injection valve

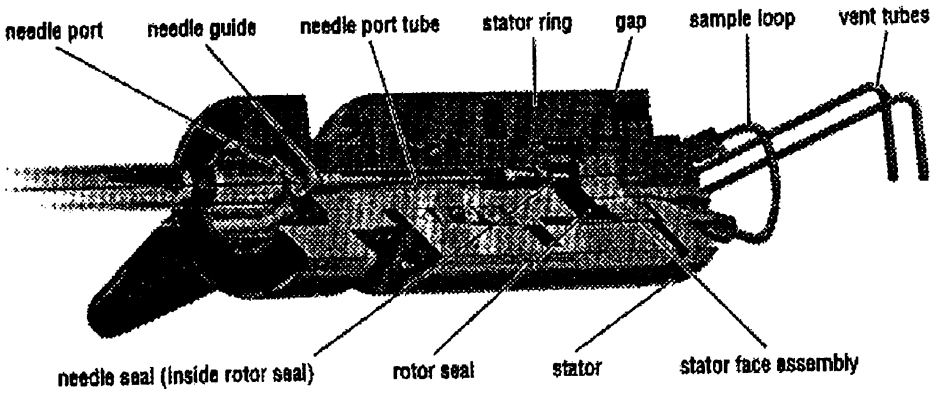


Figure 2-21: Rheodyne injection valve

VARIAN

Filled (Full) Loop Injection

*** Volume of the sample loop determines how much sample is loaded**

--If the injector has a 20ml sample loop, 20ml of sample will be delivered

*** Syringe loading should be at least four times the loop volume for reproducible results**

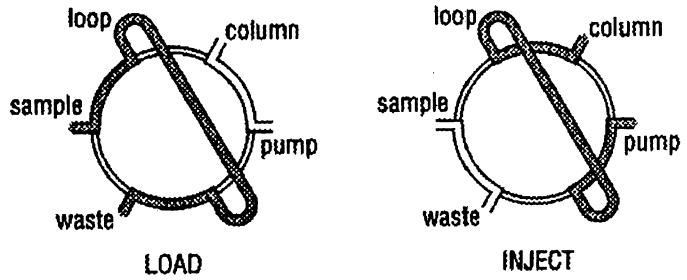


Figure 2-22: Filled loop injection

VARIAN

Effects of Laminar Flow

* Mobile phase in the loop is displaced when you dispense sample from the syringe

  -- Boundary between the sample and mobile phase has a parabolic profile due to laminar flow

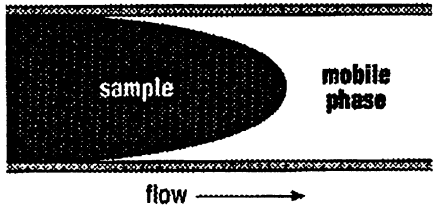


Figure 2-23: Laminar flow of sample

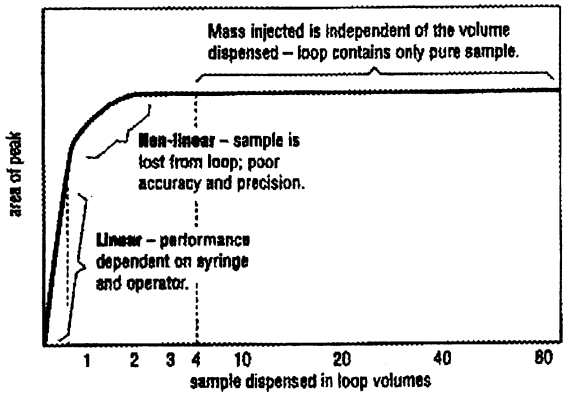


Figure 2-24: Volume of sample dispensed vs. sample mass injected into the column

VARIAN

Partial Loop Injection

* Take advantage of linearity up to one half sample loop

* Volume of sample in syringe determines volume of sample entering system

* Injection precision is increase by use of an internal standard

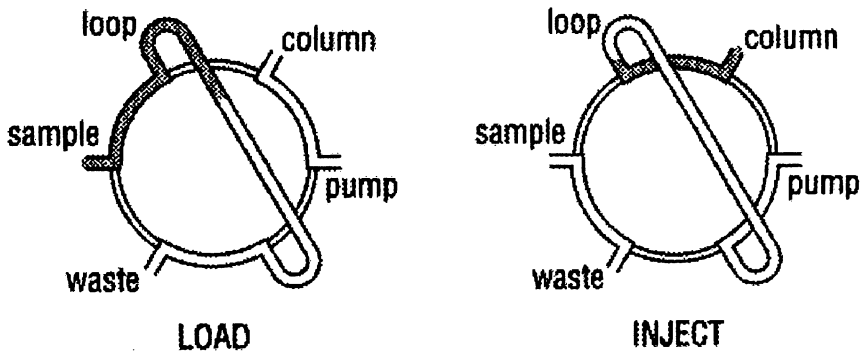
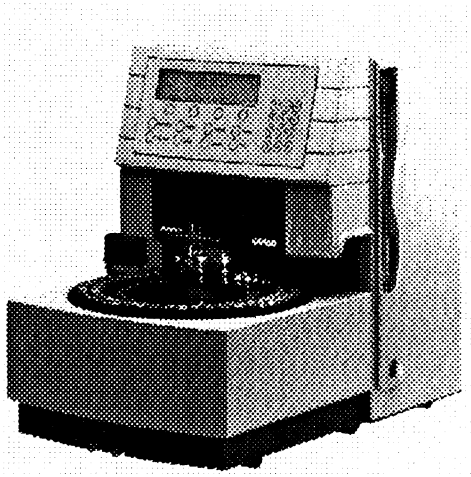
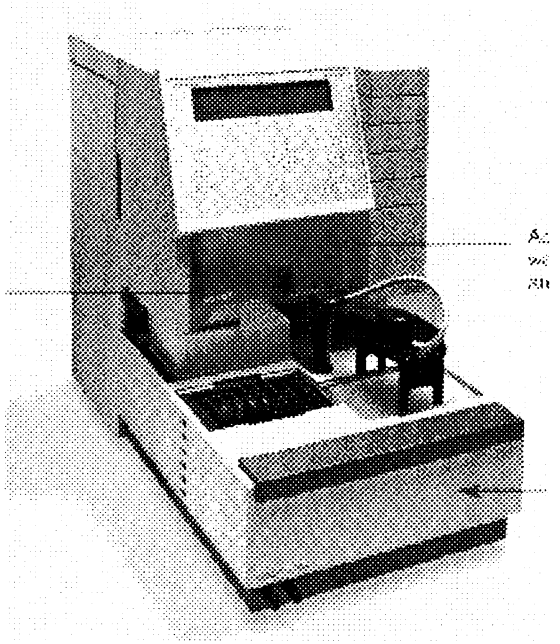


Figure 2-25: Partial loop injection

VARIAN

AUTOSAMPLERS





VARIAN

ACCESSORIES FOR THE INJECTORS

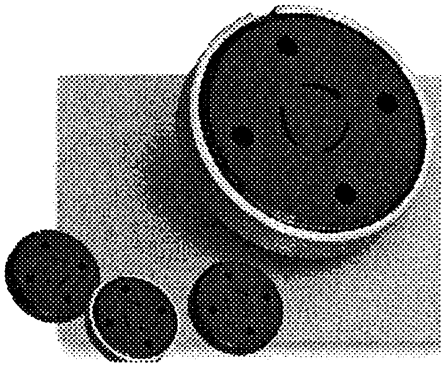
Manual Injectors:

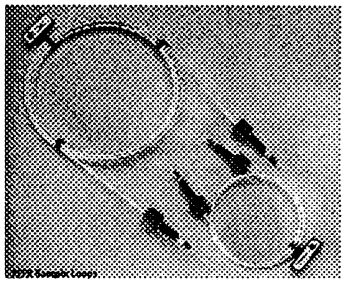
Rotor Seals

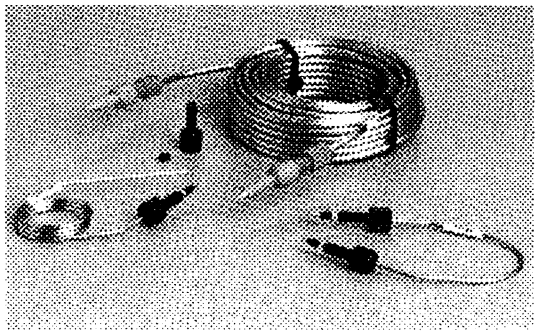
Stator

Sample loops

Injection syringe/needle







VARIAN

ACCESSORIES FOR THE INJECTORS

Autosamplers:

Stator

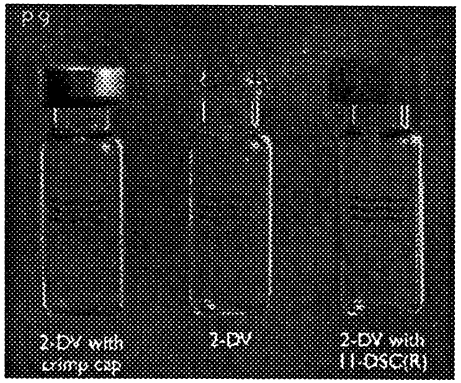
Buffer tubings

Sample loops

Injection needles

Vials

Vial segments



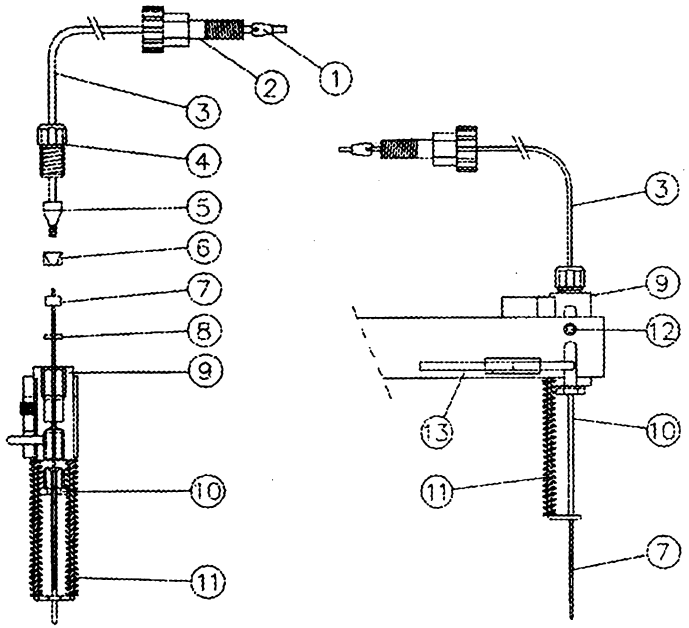


Figure 37: Sample Needle

VARIAN

HPLC Columns

* The column is the heart of the chromatograph

Materials and Construction

* Stainless steel tubing with stationary phase packing

* Stationary phase differs depending on mode of HPLC

  -- Polymeric gels can be used for ion and exclusion chromatography

  -- Spherical, microparticulate, porous silica with chemically bonded substituents can be used for reverse phase, normal phase or ion chromatography

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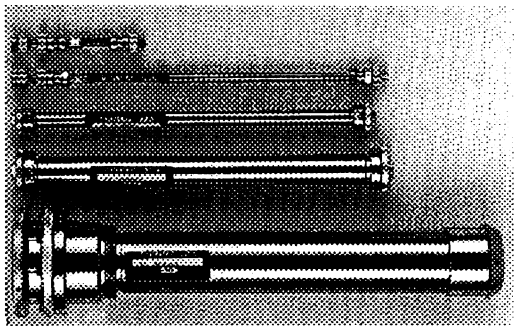
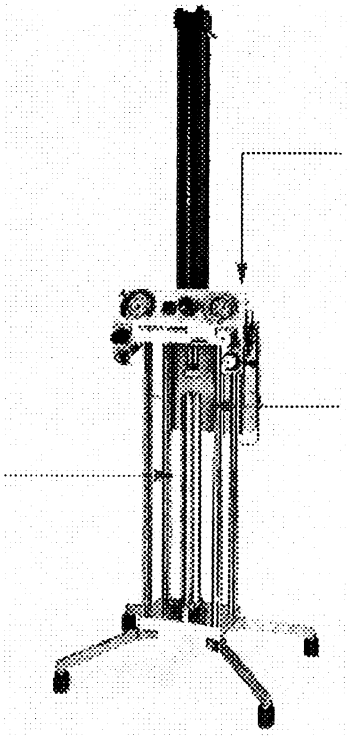
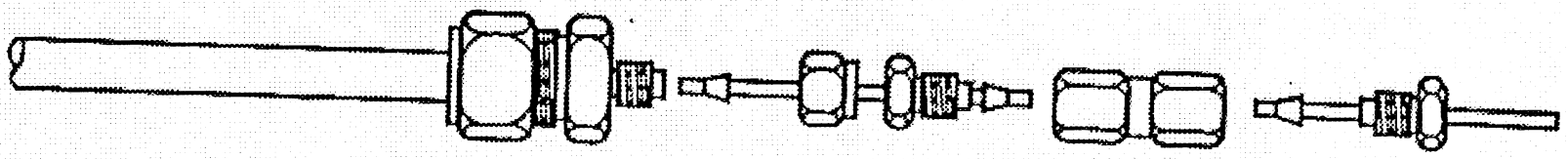
HPLC Columns





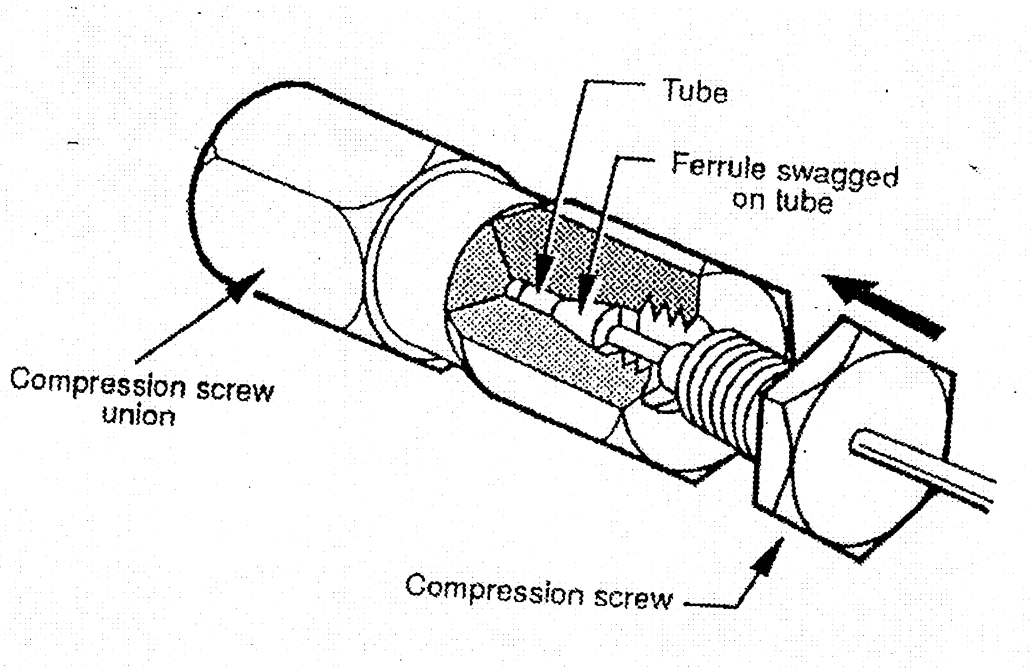
VARIAN

CONNECTING A COLUMN



VARIAN

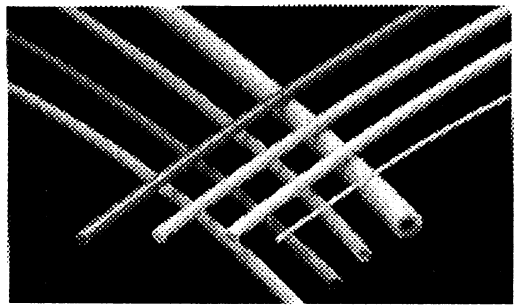
CONNECTING A COLUMN



VARIAN

TUBING USED TO CONNECT SYSTEM

Stainless Steel Vs PEEK (Bio-compatibility)



Analytical: 0.01" I.D. or lower

Semi-preparative: 0.02-0.04" I.D.

Preparative: 0.04" I.D. and above

Tube sizes are identified by color coding

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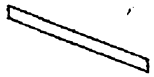
NUTS & FERRULES

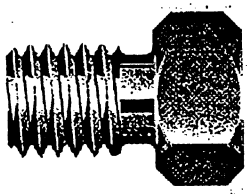
HPLC Accessories



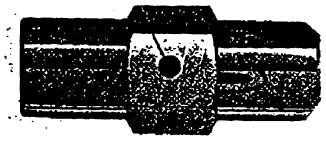
Tubing

Nuts

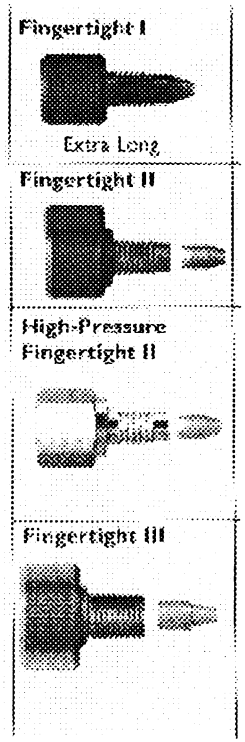
Ferrules



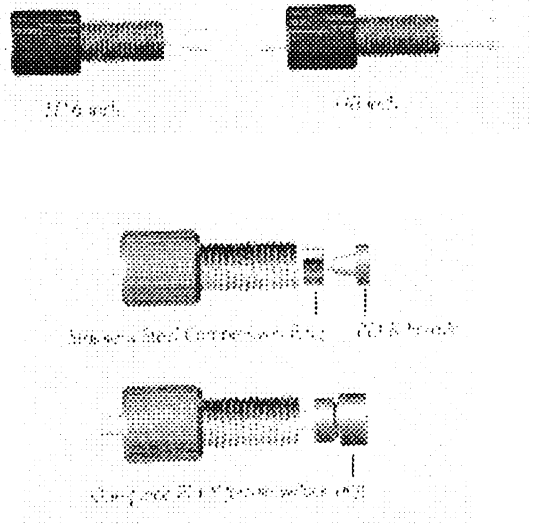
Compression Screws



Union

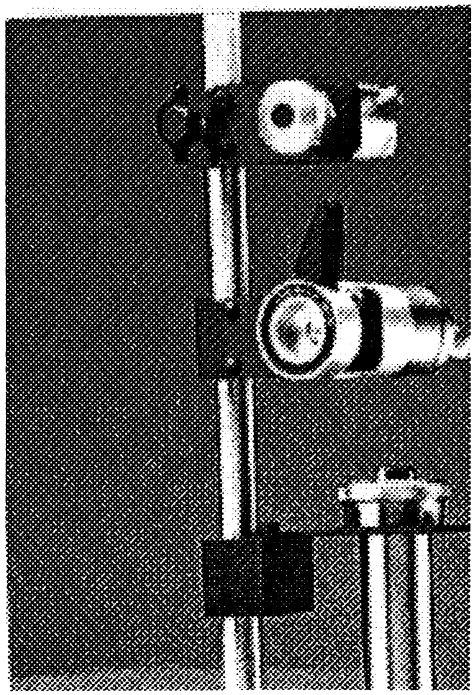


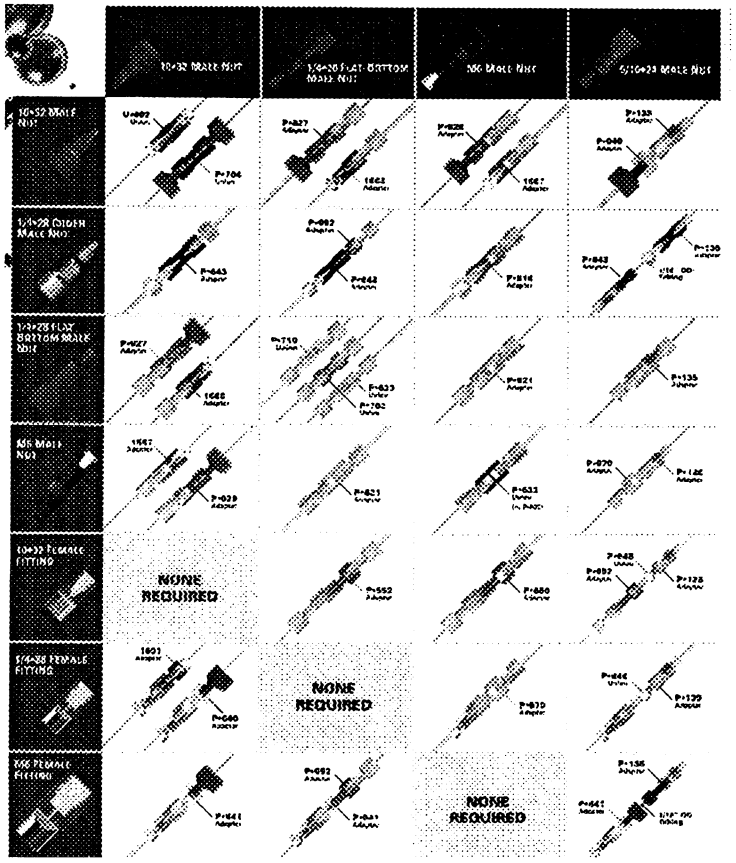
Flangeless Fittings



VARIAN

ADAPTERS & VALVES





VARIAN

Bonded Stationary Phase

*** Takes advantage of the physical strength of silica while minimizing the disadvantages**

-- untreated, porous silica contains silanol groups which are active in hydrogen bonding and acidic reactions

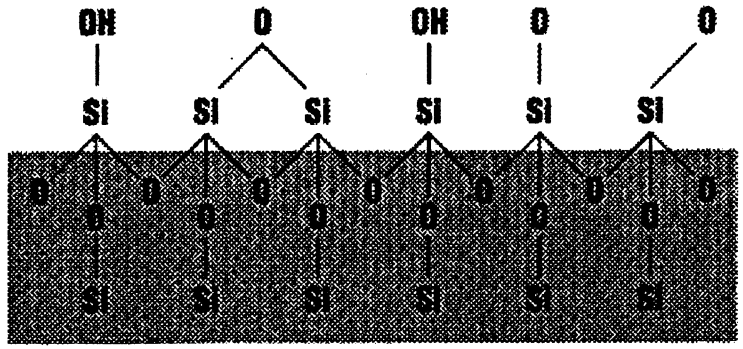


Figure 2-27: The silica surface

VARIAN

Preparing the Bonded Phase

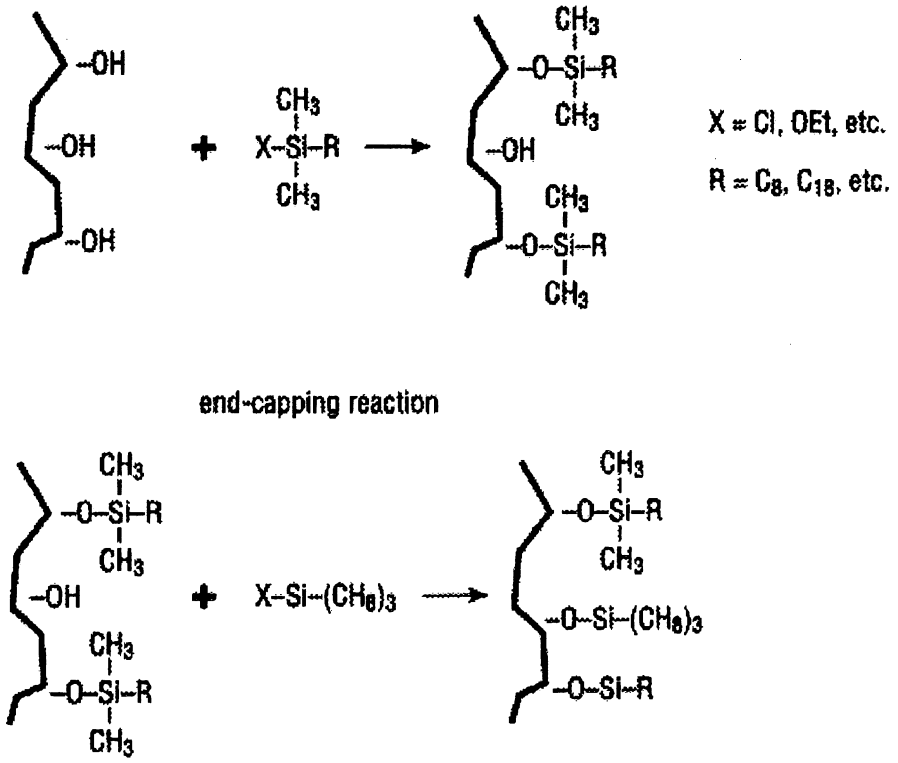


Figure 2-28: Preparation of the bonded phase

VARIAN

Column Selection

* Polarity of stationary phase should be similar to that of sample for reverse phase and dissimilar for normal phase

* column suppliers have good advise for choosing the correct stationary phase for a particular application

Stationary Phase	Description
C-18 or ODS	Rugged; highly retentive; widely available.
C-8	Similar to, but slightly less retentive than C-18.
C-3, C-4	Less retentive; some selectivity differences.
C-1 or TMS	Least retentive; least stable.
phenyl, phenethyl	Moderately retentive; some selectivity differences.
CN	Moderately retentive; used for reverse or normal phase.
NH_2	Weak retention; used for carbohydrates; less stable.
polystyrene	Stable with 1<pH<13 mobile phases; better peak shape; extended column life for some separations.

Table 2-5: Useful stationary phases for reverse phase

VARIAN

Column Selection

* Capacity of the column increases as the diameter increases; however, smaller columns are more efficient and consume less mobile phase

  -- Inner diameters range from 1mm to 25cm

* Resolution increases with the square root of the column length

  -- Lengths of 5, 10, 15 and 25 cm are available

VARIAN

Column Selection

* Smaller diameters of packing produce higher efficiencies

-- 10 micron has adequate resolution for some analytical separations

-- 8 micron can be used analytically, especially if planning to scale
up to prep columns

-- 5 micron is usually recommended

-- 3 micron is highly efficient, but is only available in short lengths
because of increased back pressure

VARIAN

Column Installation

* Avoid dead volume, leaks and damage to hardware

  -- Use correct fittings

  -- Use shortest possible lengths of tubing

  -- Tubing should be squarely cut

  -- Purge air from tubing before making column connections

* Check direction of flow on column label and make column connections

  -- Do not over tighten fittings

* Start the HPLC pump and check for leaks

VARIAN

Column Lifetime

*** Generally, a C-8 or C-18 column will last longer than a shorter chain or more polar column**

*** Low bonded phase load and non-end capped packing will degrade at a faster rate**

*** polymer packing is stable against chemical attack and therefore should last longer**

*** Avoid mechanical shock**

--Turn the injection valve quickly so that pressure does not build

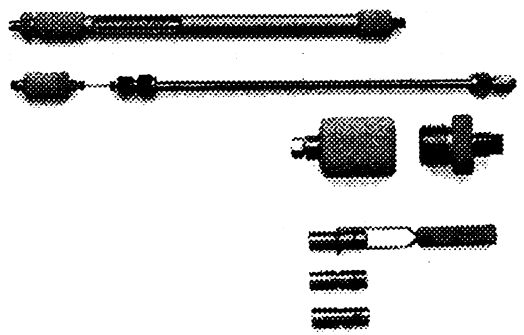
-- Do not drop the column

-- Store column in a vibration free environment

VARIAN

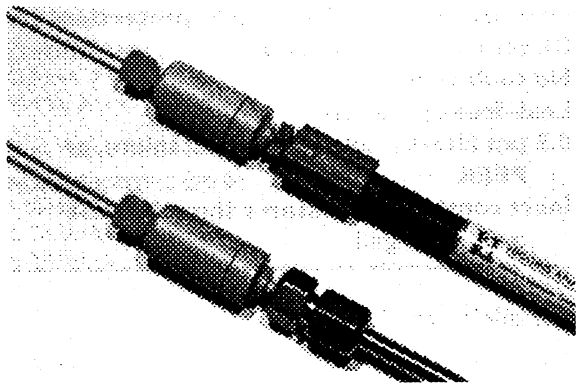
Column Maintenance

* A guard column should be placed between the injector and analytical column



-- Contain same packing material

-- Traps components in the sample or mobile phase that might otherwise accumulate at the head of analytical column



--Replace when contaminated

VARIAN

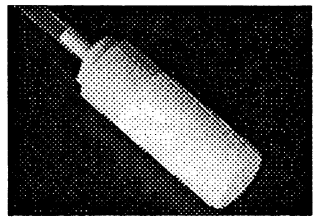
Column Maintenance

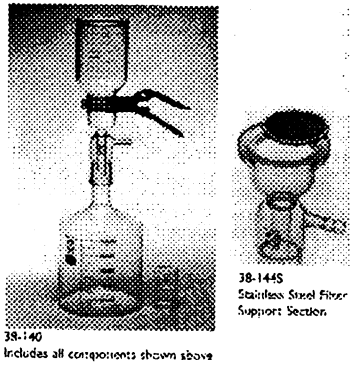
* An in-line filter should be used to remove particles

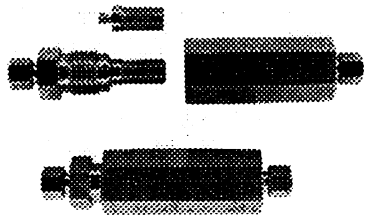
  -- A low volume disc

* A pre-column should be used at pH greater than 8 with ordinary columns

* Solvent should be filtered prior to use







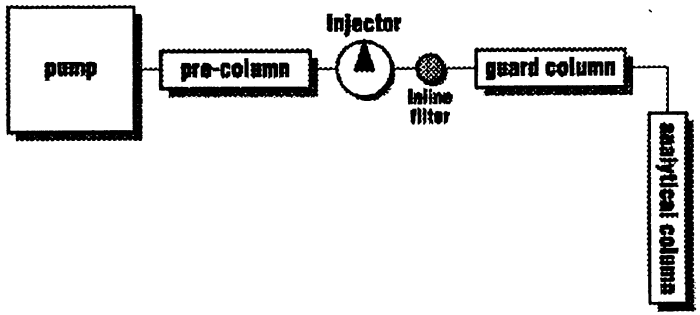


Figure 2-29: Schematic for column protection

VARIAN

Column Maintenance

* Beware of precipitation of incompatible mobile phases

* Never leave the mobile phase that contains buffer or ion pair reagents in the column for more than one day without use

* Dirty samples (environmental of biological) can cause adsorption problems

* Column can be resilanized to restore life after use of high pH or temperature

Stationary Phase	pH Range
octadecyl	2.5–8.5
octyl, cyano, phenyl, diol	3.0–8.0
methyl, amino, butyl	3.5–8.0
silica	1.0–7.0
amino acid	3.5–8.0
urea	3.5–8.0
polystyrene	1.0–13.0

Table 2-6: pH ranges of popular stationary phases

VARIAN

Solvent Regeneration

* If the column begins to exhibit increased back pressure, decreased efficiency, increased tailing, or altered retention characteristics, solvent regeneration may restore performance

* Column is rinsed with solvents of increasing strengths

-- All solvent switching requires miscibility

Phase	Length	Minimum Equil. Vol.	Regeneration Solvent 1	2	3	volume
normal	5 cm	10 ml	hexane	THF	hexane	75 ml
normal	15 cm	30 ml	hexane	THF	hexane	125 ml
normal	25 cm	50 ml	hexane	THF	hexane	200 ml
reverse	5 cm	10 ml	MeOH or CH_3CN	$MeCl_2$	MeOH or CH_3CN	75 ml
reverse	15 cm	30 ml	MeOH or CH_3CN	$MeCl_2$	MeOH or CH_3CN	125 ml
reverse	25 cm	50 ml	MeOH or CH_3CN	$MeCl_2$	MeOH or CH_3CN	200 ml

Table 2-7: Regeneration solvent

VARIAN

Repacking

1. Last resort*
2. Remove top end fitting and ferrule
3. Clean the fitting and ferrule
4. Remove 1-2mm of packing with clean spatula
5. Form a thick paste of the same packing material using methanol or hexane
6. Firmly press and smooth packing to lip of the column tube
7. Position a new frit
8. Replace the end fitting and carefully tighten

*Dynamax column modules can not be repacked

VARIAN

Column storage

* Save column end caps and replace them whenever the column is not being used

* Keep the column wetted with the recommended storage solvent

Stationary Phase	Storage Solvent
octyl	aqueous acetonitrile or methanol (80% organic)
octadecyl	aqueous acetonitrile or methanol (80% organic)
methyl	aqueous acetonitrile or methanol (80% organic)
butyl	aqueous acetonitrile or methanol (80% organic)
cyano	aqueous acetonitrile or methanol (80% organic)
phenyl	aqueous acetonitrile or methanol (80% organic)
amino	hexane
diol	hexane
silica	hexane
amino acid	hexane
urea	hexane

Figure 2-8: Recommended storage solvents for columns

VARIAN

Detectors for HPLC

* detectors monitor the column eluent for a chemical or physical property of the sample such as absorbance or fluorescence

--If the solutes absorb or fluoresce at the input wavelength,

an electrical signal proportional to samp[le concentration is produced

* Refractive index and electrochemical detectors are also popular

VARIAN

Detectors for HPLC

The detector should be capable of the following:

- **--Detecting the solutes of interest in the sample (selectivity)**
- **--High sensitivity (low noise)**
- **--Multiple ranges (linearity)**
- **--Non-destructive to the sample**
- **--Low dead volume**
- **--Reliable and easy to maintain**

VARIAN

Detectors for HPLC

Ultraviolet (UV)

* Light is absorbed by the sample passing through a flow cell
* Amount of light absorbed is related to the amount of sample
* Light is absorbed at a specific wavelength channel
* Different lamp produce different
* UV light is generated by deuterium lamp
* Visible light is generated by tungsten lamp

VARIAN

Detectors for HPLC

Fluorescent

* UV light generated by xenon lamp bombard sample molecules
* At certain wavelength, sample molecules got excited
* Emitted fluorescent light at certain emission wavelength
* Emitted light is corresponding to the amount of sample.

VARIAN

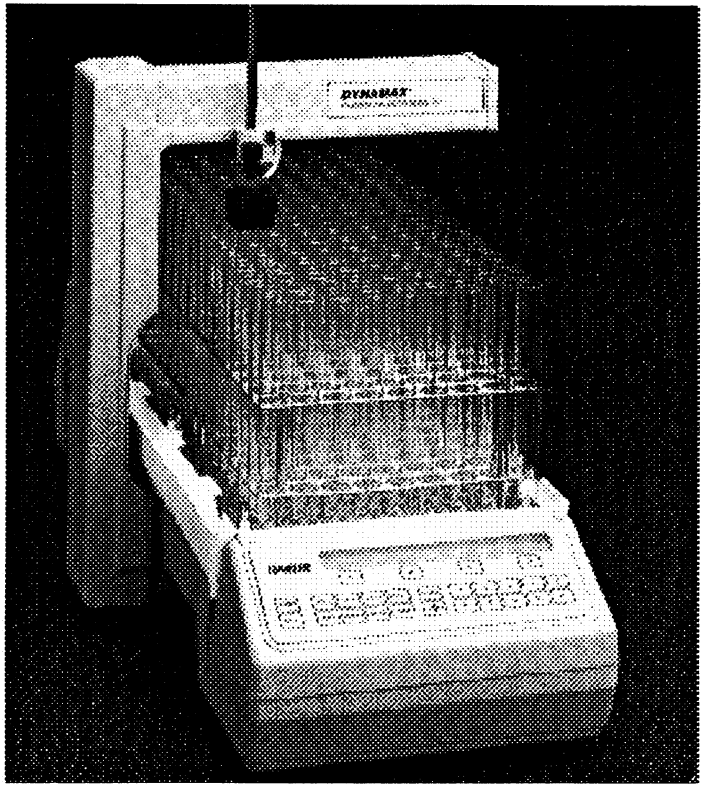
Detectors for HPLC

Photo Diode Array (PDA)

* Light passes through the sample is broken up into color spectrum
* Color spectrum generated by holographic grating or prism
* The image of the samples generated by this color spectrum is stored as a spectral image after it hits the diode
* Spectrum is collected continuously
* Sample spectrum provides us with purity information.

VARIAN

HOW ARE SEPARATED SAMPLES COLLECTED?



VARIAN

Output Devices

* Detector voltage signal is made useful for the user

  --Chart Recorder

  --Integrator

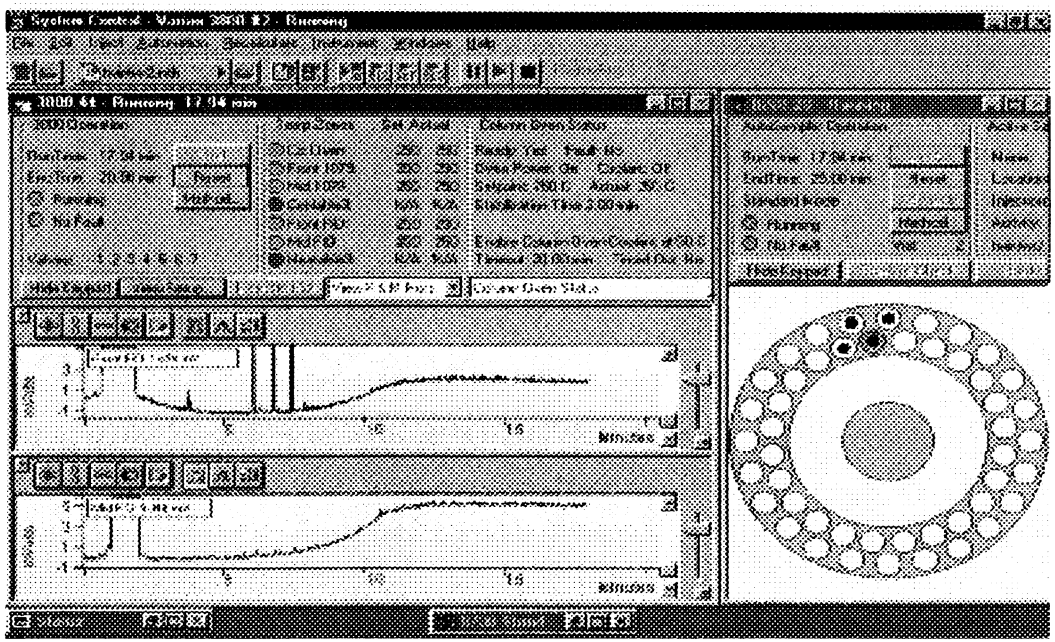
  --Data processing

* LC Star Workstation with system control allows control of the LC system, collects two channels of data, employs many calculation types and integration parameters, prints reports automatically, and saves data indefinitely

VARIAN

COLLECTION OF DATA:

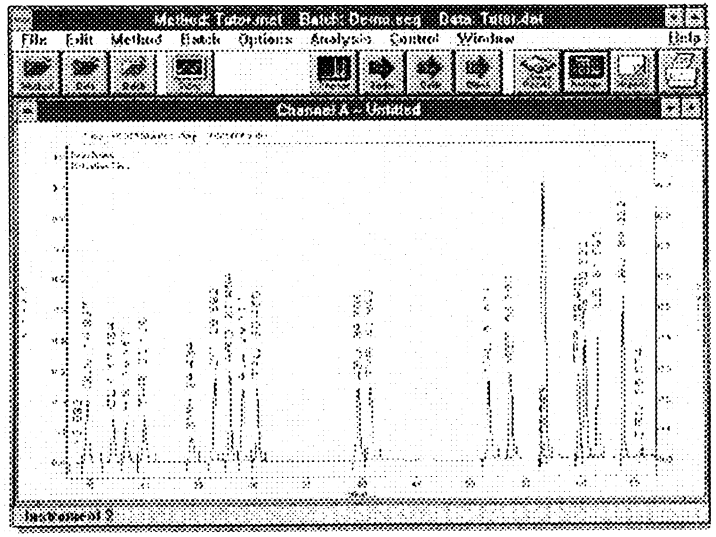
STAR WORKSTATION

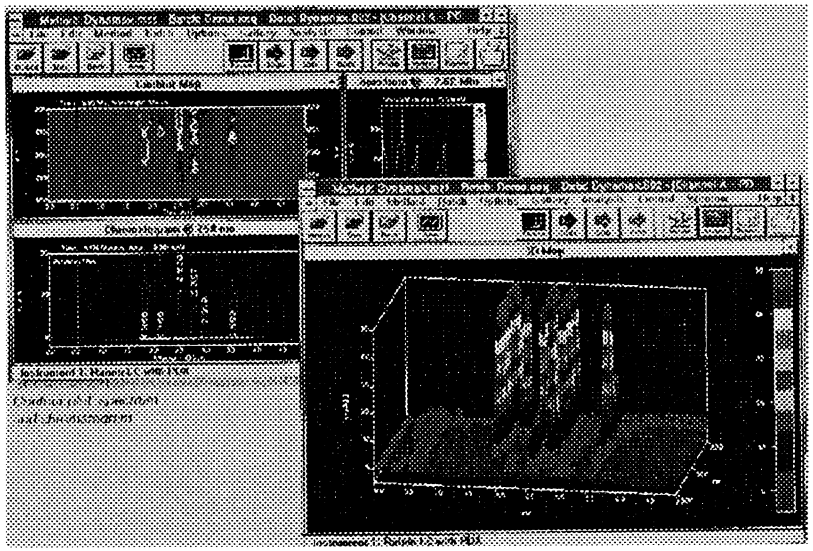


VARIAN

COLLECTION OF DATA:

Dynamax PC



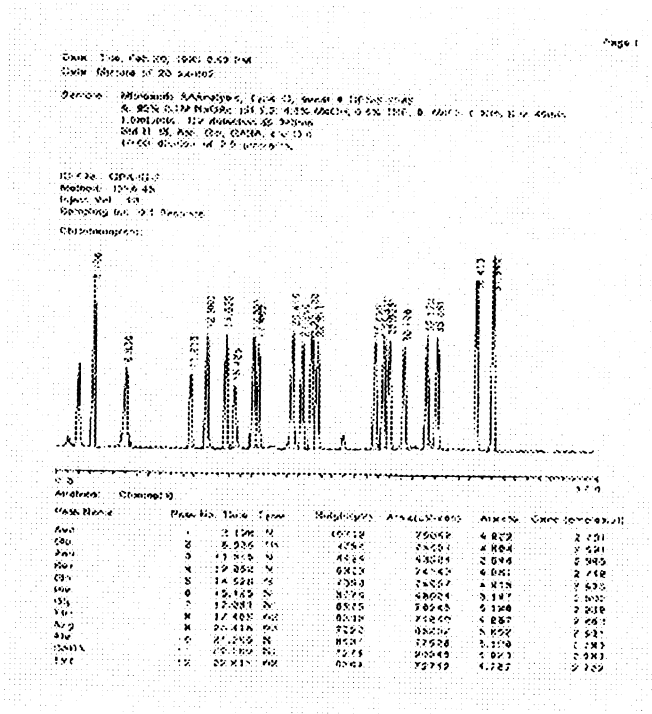


VARIAN

COLLECTION OF DATA:

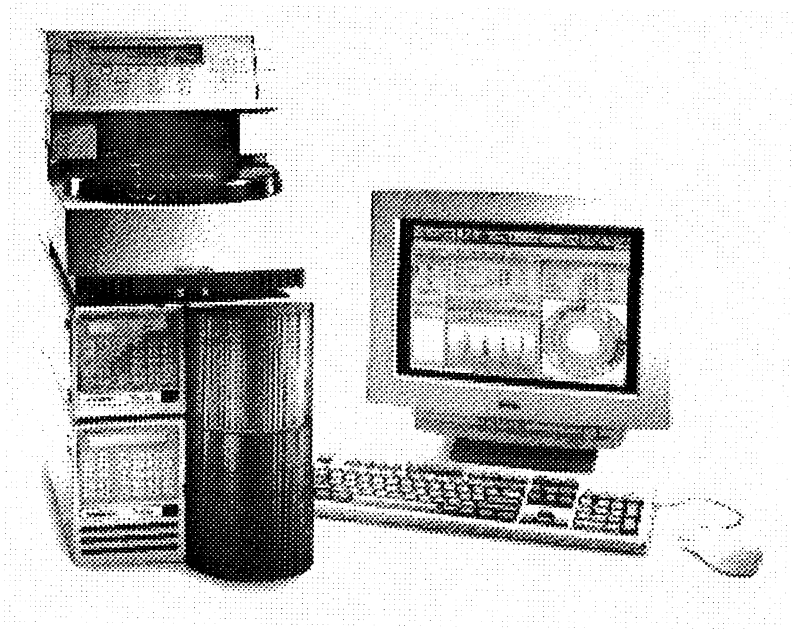
Dynamax Method Manager (Macintosh)

MacIntegrator II



VARIAN

THE HPLC SYSTEM



4

VARIAN

General Approach to Trouble Shooting in HPLC

VARIAN

Do I have a problem with my LC system or not?

Rules of Thumb:

1) Change only one thing at a time when isolating a LC problem

2) A problem isn't really a problem until it occurs at least twice

3) Reinstall original parts if the substituted new part does not solve the problem

4) Always write down what you have done and changed for future reference

VARIAN

Five categories of symptoms for LC problems

A) PRESSURE

B) LEAKAGE

C) QUANTITATION OR DATA QUALITY

D) HARDWARE

E) CHROMATOGRAM

VARIAN

PRESSURE SYMPTOMS

No Pressure or flow :

Solvent delivery not functioning

Check if the pump is plugged in, turned on and check if the pressure limit is set correctly

Check valves malfunction

Check if the ruby in the check valve is stuck; clean or replace

No mobile phase

Check if solvent reservoir is empty, refill and prime pumps

VARIAN

PRESSURE SYMPTOMS

No Pressure or flow :

Air in pump

Prime/purge the pump

Leakage in plumbing

Locate and repair
(tighten connection or replace fitting/ferrule)

VARIAN

PRESSURE SYMPTOMS

Pressure up but no flow

Flow blockage

Change tubing of the appropriate section - Start from the end of system and remove one piece at a time until pressure drops

Check in-line filters for blockage

Check column inlet or outlet frits for clog

Check guard column or column itself for blockage

VARIAN

PRESSURE SYMPTOMS

Erratic pressure

Leakage from pump to column

Locate and repair

Dirty pump head check valve

Clean check valve by sonication in methanol or flush the valve with 1M nitric acid followed by water (replaceif cleaning is not sufficient)

Air in the pump

Prime pump, when flow rate is achieved, loosen both check valves slightly to let air out

VARIAN

LEAKAGES

Leakages are obvious hardware related, locate the problem and repair

Pump

Leakage from pump head usually occurs due to worn out pump seal, remove piston from its housing and replace the seal

Plumbing

Most leakage occurs at tubing connections where ferrules are not seated properly, ferrules are worn, or the threads of the nuts are not catching. Locate problem and tighten or replace fittings

Detector

Leakage could be due to cracks in flow cell or if the flow cell is blocked

VARIAN

QUANTITATION OR DATA QUALITY

Determine efficiency loss and tailing factor problems

Determine efficiency N of the column with $N=16(V_1/W_1)^2$ and compare to the original value

Tailing factor T can be calculated via $T=W_{0.05}/2f$

if T=or< 0.5, the peak is fronting

if T=or> 2.0, the peak is tailing

VARIAN

QUANTITATION OR DATA QUALITY

Probable causes of efficiency loss or tailing problems

Column

Packing fracture (Inlet void), Mixed mode separation; Chemistry altered; Plugged frit or packing material; Contaminated solvent or sample

Injector

Leaking (scratch on rotor seal); Blockage

Detector

Broken, leaking, or blocked cell; Leaking, poorly- madeconnections; Large volume cell/connections

VARIAN

QUANTITATION OR DATA QUALITY

Probable causes of efficiency loss or tailing problems

Tubing/Fittings

Tubing ID too large; Leaking fittings, ferrules not seated properly which creates mixing chamber.

NOTES: Cause of the efficiency loss or tailing can be determined by removing the column from the system and then injecting a sample. If the peak is more than 100ul wide, problem is possibly with the system. If the peak is less than 100ul wide, the column is likely the problem. Clean or replace column.

VARIAN

QUANTITATION OR DATA QUALITY

Retention time Shifting

Pump

Faulty check valve(s); Leaking seals; Trapped air bubbles; Electrical (board) malfunction

Injector

Plugged or leaking; Stuck valve

Mobile Phase

Air bubbles (not mixed or degassed well); Evaporation of solvent (or modifier) caused incorrect solvent composition

VARIAN

QUANTITATION OR DATA QUALITY

Retention time Shifting

Miscellaneous factors

Variable temperature; Column is not well equilibrated or is contaminated; Sample solvent is too strong; Leak in system

NOTE: Correct retention time shift by

Checking flow rate- clean/replace check valves if deficient flow, removing trapped air, degassing solvent and if the shift is consistent in same direction, problems can be corrected by installing column heater, adding more equilibration time for the column, using new mobile phase, cleaning-up sample and dissolving sample in mobile phase

VARIAN

QUANTITATION OR DATA QUALITY

Change in Detection Sensitivity

Detector

Wrong sensitivity setting or wavelength; Detector lamp has low energy; Dirty flow cell needs to be cleaned; Electrical malfunction

Injector

Wrong volume injected; Injector seal, fitting, or loop is plugged or leaking

Sample

Sample over-diluted, sample is unstable, sample adhering to vial or system, incomplete derivatization of sample; sample loss during preparation

VARIAN

QUANTITATION OR DATA QUALITY

Change in Detection Sensitivity

NOTE: Corrective action

Check the system for leakage

Inject fresh standard to ascertain if instrument sensitivity is OK, if sensitivity is still low, then it is a hardware problem

Exchange detector and re-run sample to determine if detector is source of problem

VARIAN

QUANTITATION OR DATA QUALITY

Baseline Problem

Random noise

Excessive random noise is generally caused by contaminated flow cell; poor connections to detector; contaminants in mobile phase or eluted from the column; external electrical interference; failing internal electronics

Cyclical noise

Generated by pulsation in pump flow or mixer problem

VARIAN

QUANTITATION OR DATA QUALITY

Baseline Problem

Spiking

Air bubbles in flow cell or

Electronic noise due to factors such as poorly made electrical connections or from
fluctuations from the power supply

Drifting

This is the result of long term changes. It includes temperature changes in the column, inadequate column equilibrium, column bleeding, contaminated mobile phase and drifting electronics.

VARIAN

QUANTITATION OR DATA QUALITY

Baseline Problem

Corrective Actions

Turn off pump, monitor baseline. If problem remains, detector or mobile phase is at fault. Clean detector flow cell, change mobile phase

Remove column, connect tubing to detector, turn on pump. If problem remains, pump or mobile phase is at fault. Check for air bubbles, clean/replace check valves and change mobile phase

If the system checks out, the column is at fault. Re-equilibrate the column, remove air bubbles trapped in the column by increasing the flow. If column bleeding exists, it is best to replace the column

VARIAN

HARDWARE PROBLEMS

Pump-related problems

Check-valve contamination

Pressure and flow instability---- Flush with solvent or replace with new valve

Air entrapment

Pressure and flow instability---- Open purge valve, pump at high flow

Bleed check valves

Pump degassed methanol

VARIAN

HARDWARE PROBLEMS

Pump-related problems

Seal wear

Inability to pump at high pressure, leakage behind pump head ----
Replace pump head seal

Flow Rate problems

Deficient flow generally related to check valves malfunctioning
clean or replacecheck valves

Seal incompatibility

Excessive baseline noise; mobile phase appears to be contaminated; short column life ---- Check with manufacturer for compatibility; Test compatibility by soaking in solvent; Replace seal; Wash contaminants from system with strong solvent.

VARIAN

HARDWARE PROBLEMS

Pump-related problems

Piston breakage

Visual check of broken piston; no flow ---- Replace piston

Piston scoring

Pumphead leaking, but not corrected by seal replacement; scratches seen under microscope ----

Replace piston

Piston seizing

No flow; pump overheating ---- Remove piston and head, replace piston and inspect head for damage.

VARIAN

HARDWARE PROBLEMS

Flow Cell Problems

Bubbles

Noise spikes in chromatogram; possible off-scale signal ---- Degas solvent and add back pressure regulator after the detector

Immiscible solvents

Noise spikes in chromatogram following solvent changeover ---- Flush system with isopropanol then with mobile phase

Blockage

High back pressure; leaky gaskets --- Clean flow cell, may have to replace cell

VARIAN

HARDWARE PROBLEMS

Flow Cell Problems

Dirty cell

Noisy baseline; increasing bubble problems ---- Clean cell with 6N Nitric acid

Damaged gasket

Leaks at cell body ---- Check for blockage or excessive back pressure, rebuild cell

Damaged or loose fittings

Leaks at fittings ---- Tighten or replace fittings

Sample/reference mismatch

Can't zero baseline ---- If air reference, check for fluid in reference cell, blow dry with nitrogen; If liquid reference, flush thoroughly with mobile phase

VARIAN

HARDWARE PROBLEMS

Wavelength Problems

Poorly chosen wavelength

Irreproducible peak heights; poor method reproducibility between detectors ----
Choose wavelength at adsorption plateau

Calibration problems

Peak heights smaller (higher) than expected ----
Recalibrate with appropriate standard at the specific wavelength
Check sample injector for leaks

Low-wavelength problems

Extra peaks in chromatogram, high baseline level ---- Normal for low wavelength detection; Try acetonitrile instead of methanol or THF (lower solvent UV-cutoff); Helium sparge solvent to remove oxygen; Purge optical path with nitrogen to remove ozone

VARIAN

HARDWARE PROBLEMS

Wavelength Problems

Time constant too large

Broadened peaks especially for early peaks ----
Use smaller time constant (e.g. 10% of width of first peak of interest)

Pump pulsation

Cyclic baseline fluctuations, corresponding to piston cycles ----
Check for and remove bubbles in pump; Add pulse damper

Temperature problems

Excessive baseline drift, especially with RI detection ----
Thermostat column and tubing between column and detector; Remove LC from drift (AC)

VARIAN

HARDWARE PROBLEMS

Wavelength Problems

Mixing problems

Cyclic baseline fluctuations corresponding to mixer cycle; split peak created ----
Verify by changing mobile-phase composition; Add, repair, or replace mixer; Use only hand-mixed mobile phase

Cable connections

Signal too high (low) at recorder/data system, generally off in multiples of 10X expected value ---- Use appropriate detector output for recorder or data system

Ground loop

Short-term noise in baseline ----
Check for proper cable connections for detector output, don't ground at both ends

VARIAN

TYPICAL COLUMN PROBLEMS

Pressure increase; Poor peak shape; Loss in efficiency

Blocked frit ---- reverse column; replace frit, replace solvent filter in Dynamax column

Column void ---- Compress (axial compression column); top off or replace column

Sample blocking column ---- flush column that dissolves sample, (Methanol; protein, use denaturing agent and flush with water)

Change in retention time; Poor peak shape; Loss in efficiency

Loss of bonded place ---- replace column

VARIAN

PREVENTIVE MAINTENANCE FOR COLUMN

Use in-line filters for all columns

Use guard columns for dirty samples

Change flow rates in 1ml/min steps

Keep pH between 2.5 and 7.5 for silica columns

Check samples for particulates and compatibility with mobile phase(filter if necessary)

Pretreat dirty sample (Sample preparation columns)

Flush column daily

Store columns in organic/water mixtures or add azide to prevent bacterial growth

TROUBLESHOOTING

In the routine usage of a HPLC system, problems will occur. Troubleshooting of a HPLC is an essential task that must be mastered. Although this is a rather complex matter, there are essential guidelines to simplify the procedure of troubleshooting.

TROUBLESHOOTING RULES OF THUMB

1) Change only one thing at a time when isolating a LC problem.
2) A problem really isn't a problem until it occurs at least twice.
3) Reinstall old parts if the substituted new part does not solve the problem.
4) Always write down what you have done for future reference.
5) If a problem does exist, most symptoms can be classified under these five arbitrary categories:
    a) PRESSURE
    b) LEAKAGES
    c) QUANTITATION OR DATA QUALITY
    d) HARDWARE
    e) CHROMATOGRAM

PRESSURE SYMPTOMS

SYMPTOM	CAUSES	CORRECTIVE ACTIONS
No pressure or flow	Solvent delivery not functioning	Check if the pump is plugged in, turn on and check if the pressure limit is set right.
	Check valves malfunction	Check if check valve ruby is stuck, clean or replace.
	No mobile phase	Check if solvent reservoir is empty and refill.
	Air in pump	Prime/purge the pump.
	Leakage in plumbing	Locate and repair.
Pressure up but no flow	Flow blockage	Change tubings of the appropriate section, after testing each section one at a time. Check in-line filters for blockage. Check column inlet or outlet frits for clog. Check possible leakage. Check if guard column or column itself for clog.
ratic pressure	Leakage from pump to column	Locate and repair.
	Dirty pump check valve	Clean check valve by sonication in methanol or flush the valve with 1M nitric acid followed by water.

	Air in the pump	Prime pump, when flow rate is achieved, loosen both check valves slightly to let air out.

b) *LEAKAGES*

Leakages are obviously hardware related, locate the problem and repair.

i) PUMP — Leakage could occur due to worn out pump seal, remove piston from its housing and replace the seal.

ii) PLUMBING — Most leakage occurs at tubing connections where ferrules are not housed properly or the threads of the nuts are not seated properly. Locate problem and replace fittings.

iii) DETECTOR — Leakage could be due to crack in flow cell, or if the flow cell is plugged.

c) *QUANTITATION OR DATA QUALITY*

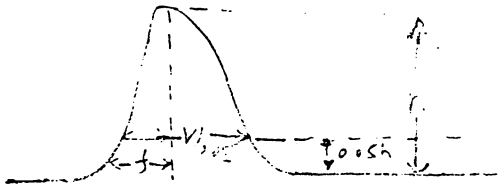
i) Determining efficiency loss and tailing factor problems.

To determine efficiency loss, it is necessary first to calculate the efficiency value of the column.

$N = 16(V_1/W_1)^2$ where V_1 = retention volume of the peak and W_1 = peak width at 1/2 peak height

Tailing factor T can be determined by the following formula,

$T = W_{0.05}/2f$



If T = or < 0.5, there is fronting of the peak.

If T = or > 2.0, there is tailing of the peak.

ii) Probable causes of efficiency loss or tailing problems.

COLUMN	• mixed mode separation
	• chemistry altered
	• plugged frit or packing
	• contaminated solvent or sample
	• packing fracture
INJECTOR	• plugged
	• leaking
	• stuck valve
DETECTOR	• broken, leaking plugged cell
	• leaking, poorly made fittings
	• large volume cell/connections
TUBINGS/	plugged or large i.d.
FITTINGS	leaking fittings, ferrules on a tubing must be fitted. Too short creates mixing chamber.

'ermination of the cause of efficiency loss or tailing can be done by removing the column from the system, ..en inject a sample. If the peak is more than 100ul wide, problem is with the system. And if the peak is less than 100ul wide, the problem is the column. Clean or replace the column.

iii) Shifting of retention time.

This could be caused by

PUMP	• Faulty check valve(s)
	• Leaking plunger seals
	• Trapped air bubbles
	• Electrical malfunction
INJECTOR	• Plugged or leaking
	• Stuck valve
MOBILE PHASE	• Not mixed well or did not degassed well enough, therefore, presence of air bubbles.
	• Evaporation of solvent cause incorrect solvent composition.
MISCELLANEOUS	• Variable temperature
	• Column is not well equilibrated or is contaminated
	• Sample solvent is too strong
	•Leakage in the system

The following approach could be undertaken to correct retention time shifting.

- Check pump pressure and flow and check for leaks
- If time shift is variable, most like there is trapped air or poor mixing. Degas and stir the mobile phase.
- If time shift is consistent in the same direction, the problem is likely due temperature change, column contamination/non-equilibrium, or sample solvent is too strong. In this case, problems can be corrected by installing column heater, allow more time to equilibrate the column, make new mobile phase, clean up sample and dissolve sample in mobile phase.

iv) Change in detection sensitivity

Most probable cause for response or sensitivity problems is caused by the following.

DETECTOR	• Setting is wrong for low sensitivity or wavelength
	• Detector lamp is getting old
	•Electrical malfunction
	• Dirty flow cell or reference cell is not flushed cleanly
INJECTOR	•Wrong volume injected
	• Injector seal/fitting is plugged or leaking
SAMPLE	• Over-diluting of sample, sample is not stable, or adheres to vial/column
	• Sample is not fully derivatized or there is loss during sample preparation.

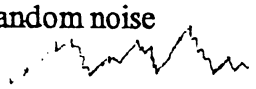
The following ways could be used to correct the problem

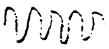
- Check settings for detector, injector and recorder/computer, as well as check the system for leakage.

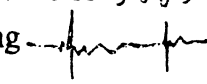
- Inject fresh standard. If the sensitivity is normal, the problem is with the sample. Rework the sample and inject again. If the sensitivity remains incorrect, the problem is with the hardware.
- Swap detector with a known "good" detector and re-run the sample. If trouble still remains, then swap another piece of the hardware one at a time to locate the problem. Correct or replace the problem hardware. If the problem still exist, then it is time to replace the column.

v)Baseline problem.

Baseline noise can be classified as RANDOM, CYCLICAL NOISE, SPIKING or DRIFTING.

Random noise  Although present in more chromatogram, excessive level is usually caused by failing internal electronics, contaminated flow cell, poor connections to detector, contaminants in mobile phase, contaminants eluting from column and external interference such as electrical input.

Cyclical noise  This is most often associated with pulsation in pump flow.

Spiking  This is purely electronics due to factors such as poorly made electrical connections or from fluctuations from the power supply.

Drifting  This is the result of long term changes. It includes temperature change in the column, inadequate column equilibrium, column bleeding, contaminated mobile phase and drifting electronics.

Correcting baseline problems can be achieved as followed:

- Turn off pump, monitor baseline. If problem remains, the detector or mobile phase is at fault. Clean detector flow cell, change mobile phase.
- Remove column, connect tubing to detector, turn on pump. If problems remains, pump or mobile phase is at fault. Check for air bubbles, clean check valves and change mobile phase.
- If all other parts of the system has been checked out, the column is at fault. Re-equilibrate the column, remove air bubbles trapped in the column by increasing flow rate. If column bleeding exists, it is best to replace the column.

d) *HARDWARE PROBLEMS.*

Problems with hardware in the system can be classified into the following categories, PUMP, DETECTOR (lamp, flow cell), and COLUMN. Symptoms and corrective actions are listed in the following table.

Pump-Related Problems and Solutions

Cause of problem	Symptom	Solution
Check-valve contamination	Pressure and flow instability	1. Flush with solvent series 2. Replace with new/rebuilt valve
Air entrapment	Pressure and flow instability	1. Open purge valve, pump at high flow 2. Bleed check valves (See text) 3. Pump degassed methanol
Seal wear and incompatibility	Inability to pump at high pressure, leakage behind pump head	1. Replace pump seal (See text)
Seal incompatibility	Excessive baseline noise, mobile phase appears to be contaminated, short column life	1. Check with manufacturer for compatibility 2. Test compatibility by soaking 3. Replace seal with compatible seal 4. Wash contaminants from system with strong solvent
Piston breakage	Visual check of broken piston, no flow, no movement of piston indicators	1. Replace piston
Piston scoring	Seal leakage not corrected by seal replacement, scratches seen under magnification	1. Replace piston
Piston seizing	No flow, no movement of piston indicators, pump overheating	1. Remove piston and head 2. Replace piston 3. Inspect head for damage, replace if necessary
Other problems	Pressure meter failure, leaks, electronic problems, etc.	(See text)

Cause of problem	Symptom	Solution
Lamp problems		
Lamp failure	No signal	1. Replace lamp
Insufficient warmup	Noisy baseline	1. Allow about 30-min warmup for normal operation, 1 h for maximum sensitivity 2. Allow new lamps at least 1 h warmup before use
Lamp aging	Increased short-term noise, occasional noise spikes	1. Replace lamp
Cell problems		
Bubbles	Noise spikes in chromatogram, possible off-scale signal	1. Degas mobile phase 2. Add restrictor after cell
Immiscible solvents	Noise spikes in chromatogram following solvent changeover	1. Flush system with 2-propanol to remove contaminant, then with mobile phase
Blockage	High backpressure, leaky gaskets	1. Clean cell (Sect. 12.2) 2. Replace cell if cleaning unsuccessful
Dirty cell	Noisy baseline, increasing bubble problems	1. Clean with nitric acid (Section 12.2)
Damaged gasket	Leaks at cell body	1. Check for blockage or excessive backpressure, 2. Rebuild or replace cell
Damaged or loose fittings	Leaks at fittings	1. Tighten fittings 2. Replace fittings
Sample/reference mismatch	Can't zero baseline	1. If air reference, check for fluid in reference cell, blow out with dry nitrogen

Cause of problem	Symptom	Solution
Cell problems (continued)		
		2. If liquid reference, flush thoroughly with mobile phase
Wavelength Problems		
Second-order wavelength problems	Very high baseline in high-wavelength region	1. Use proper UV-blocking filter
Poorly chosen wavelength	Irreproducible peak heights, poor method reproducibility between detectors	1. Choose wavelength at adsorption plateau
Poor wavelenth selection technique	Can't duplicate previous peak heights when wavelength is reset	1. Dial wavelength from same direction each time (Section 12.2)
Calibration problems	Peak heights smaller (higher) than expected	1. Check calibration at 486, 582, or 656 nm, recalibrate if necessary
Low-wavelength problems	Extra peaks in chromatogram, high baseline level	1. Normal for low wavelength detection 2. Try acetonitrile instead of methanol or THF in mobile phase 3. Helium-sparge mobile phase to remove oxygen 4. Purge optical path with nitrogen to remove ozone (Section 12.2)
Time constant too large	Broadened peaks, especially for early peaks	1. Use smaller time constant (e.g., 10% of width of first band of interest)
Pump pulsations	Cyclic baseline fluctuations, corresponding to piston cycles	1. Check for and remove bubbles in pump 2. Add pulse damper to pump 3. Work at less-sensitive detector setting

Table 12.3 Detector Problems and Solutions *(continued)*

Cause of problem	Symptom	Solution
Temperature problems	Excessive baseline drift, especially with RI detection	1. Thermostat column and tubing between column and detector 2. Remove LC from drafts 3. In extreme cases, thermostat reservoir and injector in addition to step 1
Mixing problems	Cyclic baseline fluctuations corresponding to mixer cycle	1. Verify by changing mobile-phase composition 2. Add, repair, or replace mixer 3. Use only hand-mixed mobile phase
Cable connections	Signal too high (low) at recorder/data system, generally off in multiples of 10x expected value	1. Use appropriate detector output for recorder/data system
Ground loop	Short-term noise in baseline	1. Check for proper cable connections for detector output, don't ground at both ends (Section 12.2)
Self-diagnostics	Warning lamps, error messages, buzzers etc.	1. Check detector operator's manual

TYPICAL COLUMN PROBLEMS

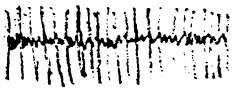
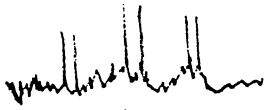
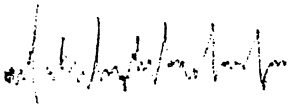
SYMPTOM	PROBLEM	CURE
Pressure increase poor peak shape loss in N	blocked frit	reverse column or replace frit
	column void	top off, compress or replace column
	sample blocking column	flush column with solvent that disolves sample
change in retention poor peak shape loss in N	loss of bonded phase	replace column

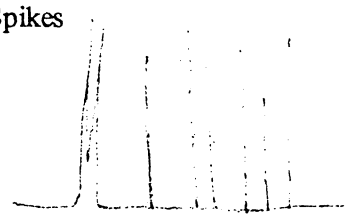
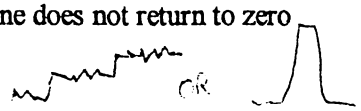
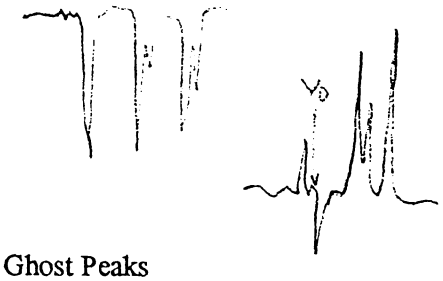
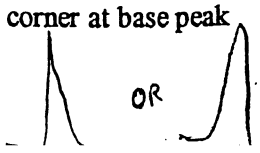
PREVENTIVE MAINTENANCE FOR COLUMN

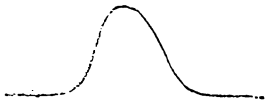
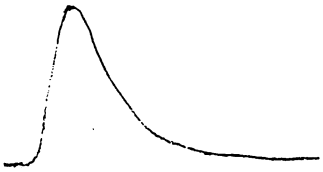
1. USE IN-LINE FILTERS FOR ALL COLUMNS
2. USE GUARD COLUMNS FOR DIRTY SAMPLES
3. CHANGE FLOW RATES IN 1ML/MIN STEPS
4. KEEP pH BETWEEN 2.5 AND 7.5 FOR SILICA COLUMNS
5. CHECK SAMPLES FOR PARTICULATES AND COMPATIBILITY WITH MOBILE PHASE
6. PRETREAT DIRTY SAMPLES
7. FLUSH COLUMN DAILY
8. STORE COLUMNS IN ORGANIC/WATER MIXTURES OR ADD AZIDE TO PREVENT BACTERIAL GROWTH

CHROMATOGRAM

The following is some graphical representation of fluctuations in some HPLC runs. Most of the symptoms and methods for circumventing problems that have arised have been described in the previous sections. The purpose of this section is provide graphical representation of problematic chromatograms.to provide help for correlating the problems.

SYMPTOM	CAUSES	CORRECTIVE ACTIONS
Noisy Baseline	Suddenly increase flow rate to flush out the bubble. Sample or reference cell contamination	Clean flow cell with 1M nitric acid followed by water.
	Bubbles trapped in system	Suddenly increase flow rate to flush out the air bubbles. and degas the solvent.
	RI changes due to pump pulsation	Add a pulse damper or restrictor to pump outlet.
	Temperature effect on flow stream entering the detector.	Install temperature regular for the system, add heat exchanger to the detector flow cell.
	Leak in system	Locate leak and repair
	Faulty detector source (lamp)	Check or replace lamp
	Wavelength too low for solvent, i.e pass UV cutoff of the solvent.	Change solvent or use higher wavelength
	Mixing of solvent incomplete	Improve mixing by dynamic or static mixer.
	Contaminated detector cell	Flush cell with 1M nitric acid, water, new solventor clean and replace window.
	Temperature fluctuation over the system	Allow system to warm up slowly, insulate column, install column heater/temperature heater.

Spikes 	Bubbles in system	Degas solvent and suddenly raise flow rate to flush out the bubble
	Particulate materials pass through cell	Remove and clean the flow cell.
	Electronic connections or irregular power supply	Check for proper grounding, and check all electrical connections.
	Electronic interference	Check for other equipment turning on and off on the same circuit.
Stepping baseline, flat-top peaks. baseline does not return to zero 	Faulty detector source, Setting of damping on instrument is too low.	Properly adjust gain, check and replace source.
	Improper grounding	Check system ground.
Negative peaks 	Incorrect polarity of detector output.	Reverse detector output or stop leakage.
	Impure mobile phase used	Change to purer solvent.
	Conditions change at Vo. Pressure surge from sample introduction	Don't quantitate peaks at Vo. use mobile phase as sample solvent.
Ghost Peaks 	Contaminated sample injection valves or syringe	Clean valve loop and syringe.
	Air dissolved in sample	Degas sample solution.
	Impurities in solvent	Use purified solvents.
	Late eluted from last run	Be sure sample are eluted prior to starting the next run.
Poor peak shape 	Column-sample interaction	Check sample chemistry and change column or mobile phase
	Column dried out	Re-equilibrate or replce column
	Column overload	Reduce sample size.
	Contaminated flow cell	Clean flow cell
Sharp corner at base peak 	Air bubble in detector	Flush air out
	Buildup at detector	Clean flow cell

Very rounded peaks 	Operating beyond dynamic range of the detector	Reduce sample size
	Recorder gain is too low	Reset gain.
Tailing 	System mismatch	Change to another column packing
	Site heterogenity	Decrease sample size, addition of polar ion-pairing agents to mobile phase. Change from isocratic to gradient elution.
	Extra column effect	Replumb LC system, use smaller flow cell for detector, larger volume column.
	Poorly packed column	Test standards, replace column.

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General procedure for quantitation in HPLC

- Develop a method that completely separates the compounds of interest
- Run a standard of known concentration
- Measure some property of the peak (Height or area)
- Run the samples
- Measure the same property of any peak at the same retention time
- Calculate the amount of the unknown

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Method

- Must separate the compounds of interest from everything else that makes the detector respond
  - Can use a selective detector
  - Baseline resolution desirable
  - Must separate compounds from those that might be present

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Standards

- Must be the same compound
- Must have a known purity
- Must be run using the same method and the same type and model of detector

VARIAN

Peak processing - 1

- Detect a peak
  - Easy manually
  - More difficult for a data system
- Identify peak start and end
  - Valley or baseline
- Identify the peak apex (retention time)
- Position the baseline
  - Often differences of opinion occur here

VARIAN

Peak processing - 2

- Area
  - Better for a data system
  - Better for small peaks
  - Flow rate must be constant
  - Reflects the actual concentration better
  - Change in peak shape does not effect area
- Height
  - Better manually
  - Better for the larger peak of a fused pair
  - Flow rate independent
  - Changes with column efficiency

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Area %

- Sum area of all peaks
- Divide each peak area by the total area
- Not really quantitation for HPLC (closer for GC FID)
- Convenient report for methods development

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External standard

- Run standard of known quantity
- Calculate response factor = Amount A/area A
- Measure area of unknown at the same retention time
- Multiply area by response factor
- Everything must be done the same on the standards and the samples

VARIAN

Internal standard - 1

- Find a compound that acts like the compounds of interest and can be separated
- Add this Internal Standard (IS) to both the standards and the samples
- Calculate the relative response factor for the standard
  - (Amount A * Area IS)/(Area A * Amount IS)
- Run the sample
- Amt A = (RRF * Amt IS * Area A)/(Area IS)
  - Usually Amt IS is the same in sample and standard

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Internal standard - 2

- Corrects for changes in sample prep
- Corrects for changes in the chromatographic instrumentation
- Must find a compound that can be separated

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Multipoint calibration

- Use multiple standards to calculate a curve the reflects the detector response
- Useful only if the detector is linear
- Takes a long time to run standards
- Works best when unknown concentrations are in area between standard data points

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Non linear calibration

- Use multiple standards and a non linear curve
- Used is the detector response is non linear
- Used if sample prep is non linear
- Results only valid if they are within the standards
- Causes confusion
- Note: HPLC detectors are linear
- Note: Work in the linear range

6

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METHOD DEVELOPMENT

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Factors which optimize the development of a HPLC method:

- Column Selection
- Solvent Selection
- Sample Preparation
- Choice of Detector
- Elution Evaluation

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Elution Evaluation

Cautions:

- Use pure solvents, water and reagents
- Mobile phase must be transparent to detector
- Use caution not to increase gradient solvent strength too rapidly
- Test to determine sufficient time for re-equilibration between runs
- Remember your LC gradient delay volume and inaccuracy
- Always run a blank before injecting a sample
- If all peaks of interest elute less than 10% change, run isocratic
- Try to avoid run aqueous buffer to 100% organic since it may induce salt precipitation

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Elution Evaluation

Rules for Gradient:

Make a gradient increase slowly to improve resolution

Increase gradient rapidly where there is more than sufficient resolution

If resolution at the front end is sufficient, decrease initial strength

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Elution Evaluation

Improving poor resolution at the end of a run,

- Increase flow at the beginning and decrease at the end
- Decrease the final strong solvent %

Improving poor resolution at the beginning

- Delay the rate of increase at the beginning, decrease at end
- Decrease the starting mobile phase strength

Too steep a gradient could cause bunching of the peaks

Re-equilibration time must be included in the program to equilibrate the column to the starting condition

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Elution Evaluation

Developing a gradient:

Cause for changing a gradient

Poor overall resolution

Poor resolution at front or back end

To improve poor overall resolution,

Decrease initial solvent strength

Increase run time

Increase flow rate

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Elution Evaluation

Step gradients

Series of isocratic steps linked to develop a stepwise change in flow rate.

Continuous gradients

Dual pump, dual solvent

Infinite programmable change in composition and flow rate

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Elution Evaluation

Gradient Elution versus Isocratic Elution

Isocratic elution:

- Simple, no change in composition.
- Solvent is pre-mixed
- Cannot alter composition to help to improve resolution.

Gradient elution:

- Flexibility in altering composition
- Programmability
- Provides finer change in solvent polarity

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Choice of Detector

Electrochemical Detector

Detection of compounds with native electroactivity such as various classes of drugs, amines, ions.

Mass Spectrometer

Post-run detection of mass spectrum of the compound

Most sensitive

Definitive proof of molecular structure with the distinctive spectrum finger print.

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Choice of Detector

UV-detectors:

Single and dual wavelength, Photodiode Array.

For compounds that can absorb UV-light. (Such as having conjugated double bonds, e.g. peptides)

Refractive Index detector

No UV sensitive compounds, such as carbohydrates

Flourescence detectors:

Must have chromaphore that floureses

More sensitive than UV-detector

Compounds are derivatized to tag on a florescent molecule.

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Sample Preparation

Sample Filtration

Precipitates in a sample can plug a column. Normally large precipitates can be removed by centrifugation. Small particulates can be removed by filtration.

Solid Phase Extraction

This is a procedure used to fractionate your sample into different fractions depending on their polarity and chemical properties. Fraction of interest can further be separated by HPLC.

- Removes contaminents
- Concentrate samples

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Solvent Selection

<u>Mobile Phase Impurities</u>:

- **Rapid Increase of back pressure**
- **Change column chemistry**
- **Creates difficulties in quantification**
- **Produce high EC detector background**
- **Cause collection of impure fractions**

Prevention:

- Use HPLC grade solvent
- Do not use expired buffer because bacteria can grow. 0.1% azide can help.
- Plastic bottle can contribute plasticizer.
- Filer mobile phase
- Degas solvent

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Solvent Selection

Effects of salts. Increase in salt concentrations shorten the retention time

Ion Pairing Agent. Additive which changes (increase) polarity of the solvent.

For example: TFA, TEAA.

Buffers. Use to maintain constant pH for the mobile phase.

Buffer	Buffer Range	UV Cutoff (nm)
Acetate	3.8-5.8	240
Citrate	2.1-4.1	230
	3.7-5.7	
	4.4-6.4	
Formate	2.8-4.8	210
Phosphate	1.1-3.1, 6.2-8.2, 11.3-13.3	210
TRIS	7.3-9.3	210

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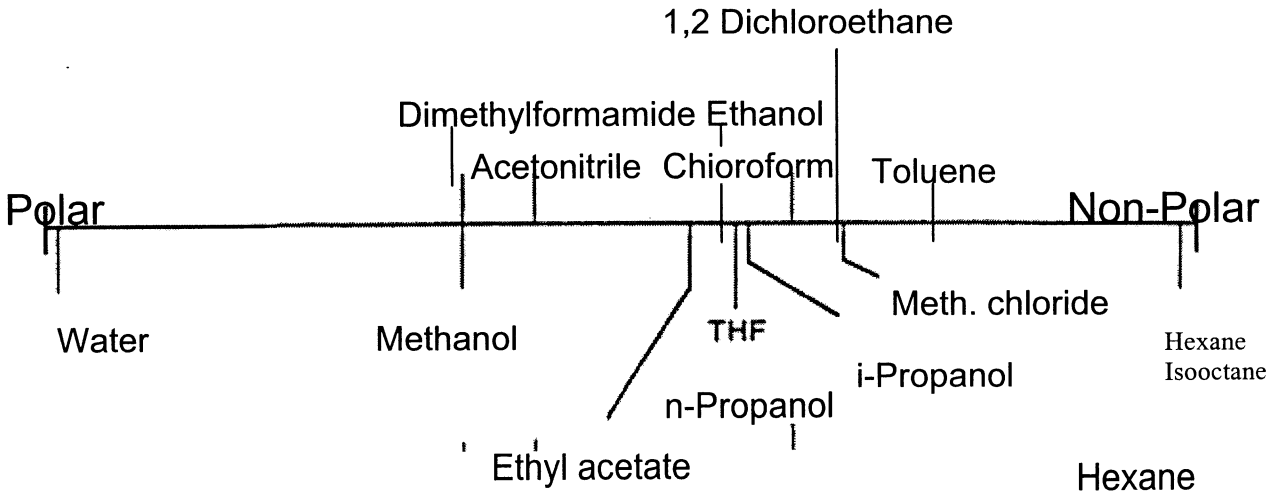
Solvent Selection

Solvent Polarity



Maintaining constant polarity but changing A and B% can enhance selectivity of the solvent mixture.

Total mobile phase polarity index = (%A)(PI of A) + (%B)(PI of B)...

Table 7.4 Solvent strength in reverse-phase LC.

Solvent	*P'*	Decrease in *k'* for each 10%v addition of solvent to water[a]
Water	10.2	---
Dimethyl sulfoxide	7.2	1.5-fold
Ethylene glycol	6.'9	1.5
Acetonitrile	5.8	2.0
Methanol	5.1	2.0
Acetone	5.1	2.2
Dioxane	4.8	2.2
Ethanol	4.3	2.3
Tetrahydrofuran	4.0	2.8
i-Propanol	3.9	3.0

[a]Rough values that are averages calculated as in Chapter 6, using experimental data of *(26a)*.

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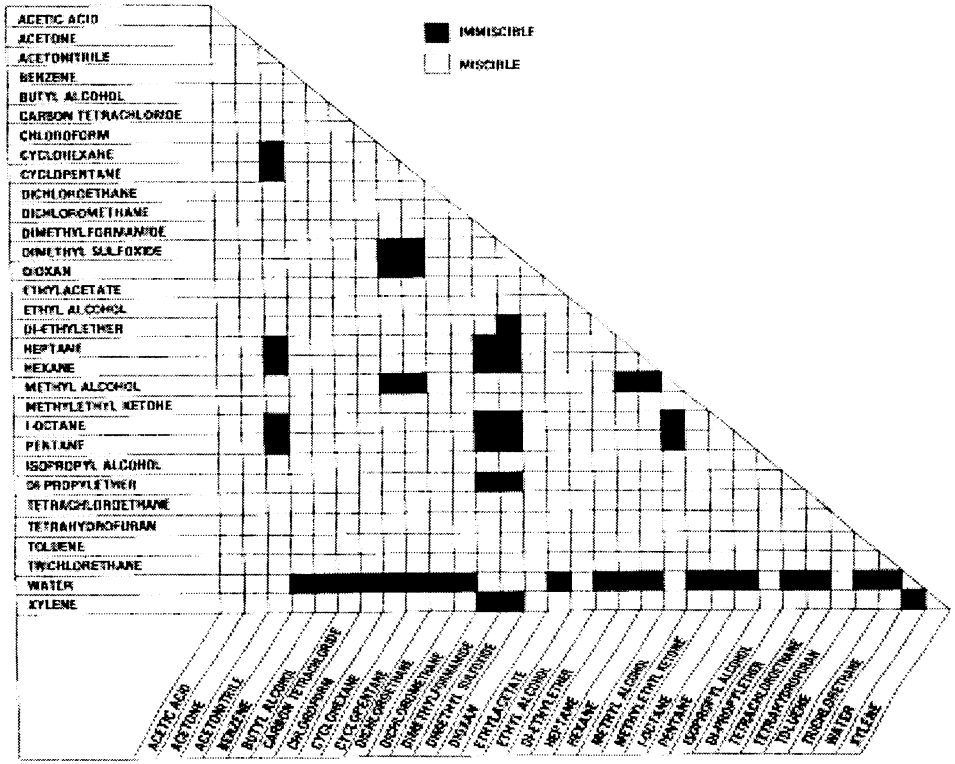
Solvent Selection

Physical Characteristics:

Solvent must be miscible and with low viscosity



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Solvent Selection

Solvent Considerations:

- Physical Properties
- Readily available
- Compatible with the LC detector
- Unreactive
- Low boiling point and low viscosity
- Safe to use and reasonably priced

VARIAN

Solvent Selection

Solvent Considerations:

- Physical Properties
- Readily available
- Compatible with the LC detector
- Unreactive
- Low boiling point and low viscosity
- Safe to use and reasonably priced

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Column Selection

- Know the property of the sample
- Know the available column types
- Know the available separation technique
- Know the available technical literatures
- Know your colleagues

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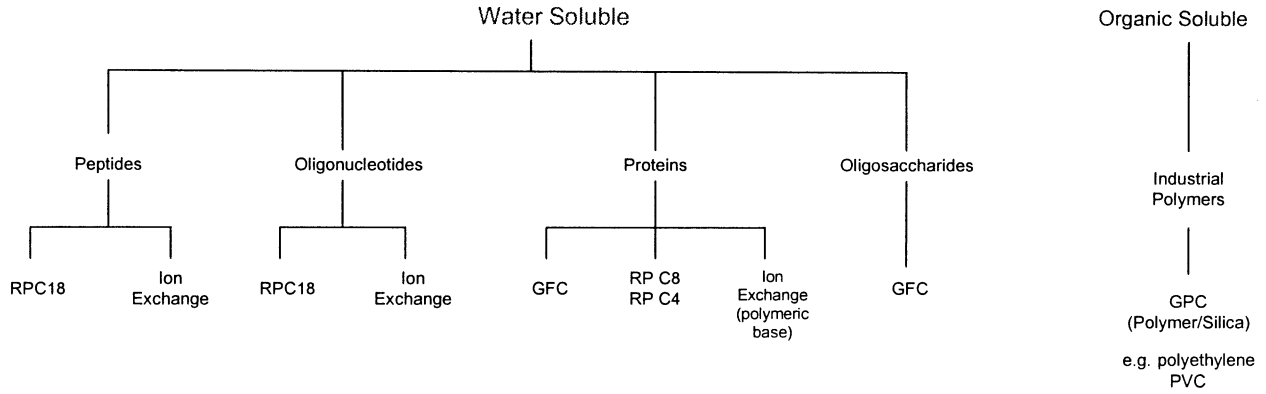
Column Selection

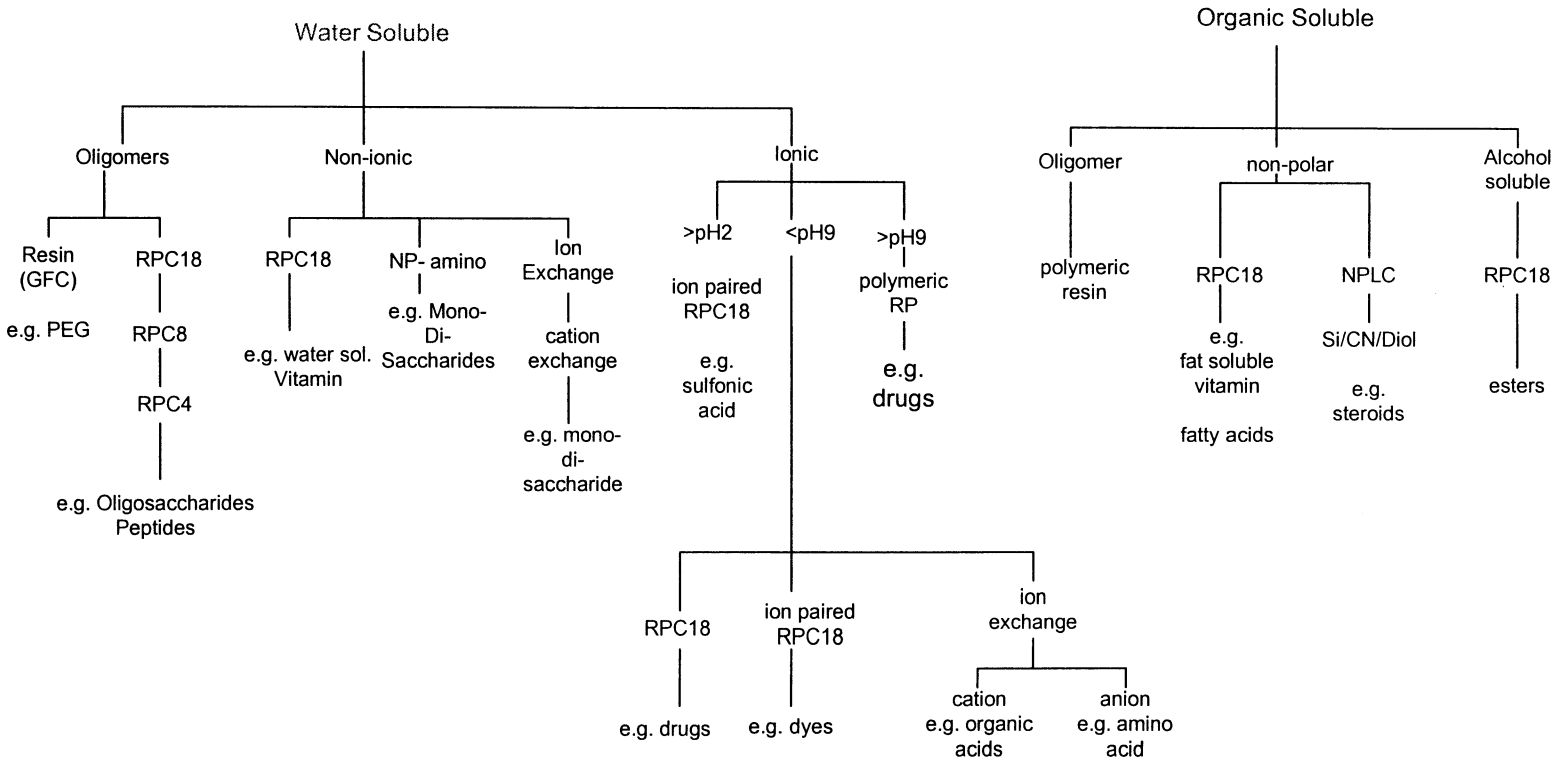
Simplified Column Selection Guide for Samples with M.W. < 2000



VARIAN

Column Selection

Simplified Column Selection Guide for Samples with M.W. <2000



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Column Selection

Some Specialty columns (Analytical size, 4.6x250mm):*

*For characteristics and other column sizes, please refer to the appropriate catalog.

Carbohydrates:	Chrompack Hypersil APS-2, p/n 29769 Chrompack Carbohydrate Ca++, p/n 28351 Chrompack Carbohydrate Pb++, p/n 29010 Varian Micropak carbohydrate, p/n 00-997222-00
Proteins & Peptides:	Chrompack P300RP, P300GFC, P1000GFC, P1000SAX, P4000SAX Rainin Puregel, SAX, SCX.
Lipids (Triglycerides):	Chrompack ChromSpher 5 lipid, p/n28313
Organic Acids	Varian Micropak aromatic organic acid, p/n 00-997-224-00 Chrompack Organic Acids, p/n 28350
Amino Acids:	Rainin AAAnalysis TypeO, p/n R0080OPAK3
DNA	Rainin PureDNA, R0083P03F5
Environmental:	
Pesticides:	Chrompack ChromSpher 5 Pesticides, p/n 29278
Polycyclic Aromatic hydrocarbons	Chrompack ChromSpherPAH, p/n 29276 Hypersil Green PAH, p/n 01-900017-00
Inorganic	
Anions:	Chrompack IonoSpher 5A, p/n 29280
Cations	Chrompack IonoSpher 5C, p/n 29282
Chiral (optical Isomers):	Chrompack Sumichiral OA-2000, OA-3300, OA-4900, OA-5000 Macherey-Nagel, Chiral-1, Chiral-2, Chiral-3