Chapter 3

Basic High Performance Liquid Chromatography

3.1 Introduction and History

In Chapter 2, Michael Tswett (1872-1920) was credited as the father of chromatography due to his 1903 separation of the green-leaf pigments into bands of colors, a demonstration of liquid chromatography. While similar work was being conducted in the petroleum industry, Tswett is credited with coining the term “chromatography”. Despite Tswett’s results, chromatography did not develop quickly. The next major developments were the use of thin-layer chromatography (TLC) in 1937-38 and the use of paper chromatography in the mid-1940s, but thin layer chromatography quickly won popularity. Thin layer chromatography was originally developed by Nikolai Izmailov (1907-1961) and his graduate student Maria Shraiber (1904-1992) for pharmaceutical preparations. Early TLC was conducted with microscope slides that were coated with suspensions of calcium, magnesium, and aluminum oxides. As used today, a small spot of solution was placed on one end of the slide, the slide was dipped into a solvent, and the analytes migrated at different rates through the oxide coatings where they were later detected (today by a UV lamp or chemical stain).

TLC advanced slowly during the next few years but a major advancement was made in 1956 by Egon Stahl (1924-1986) when he attempted to standardize the preparation of the sorbents used to make the plates. While these advances and others such as forced flow TLC, significantly matured TLC into an accepted (and reproducible) practice; it was still only a qualitative technique, at best. However, Izmailov and Shraiber’s spot chromatography, commonly known today as TLC, is the workhorse of undergraduate organic synthesis labs where synthesis reactions are conducted and the resulting products are selected for using common glass open columns filled with silica gel (refer to Figure 3.1).

Eluent from these columns is collected in fractions that are then run by TLC to identify which column fraction contains the desired product.

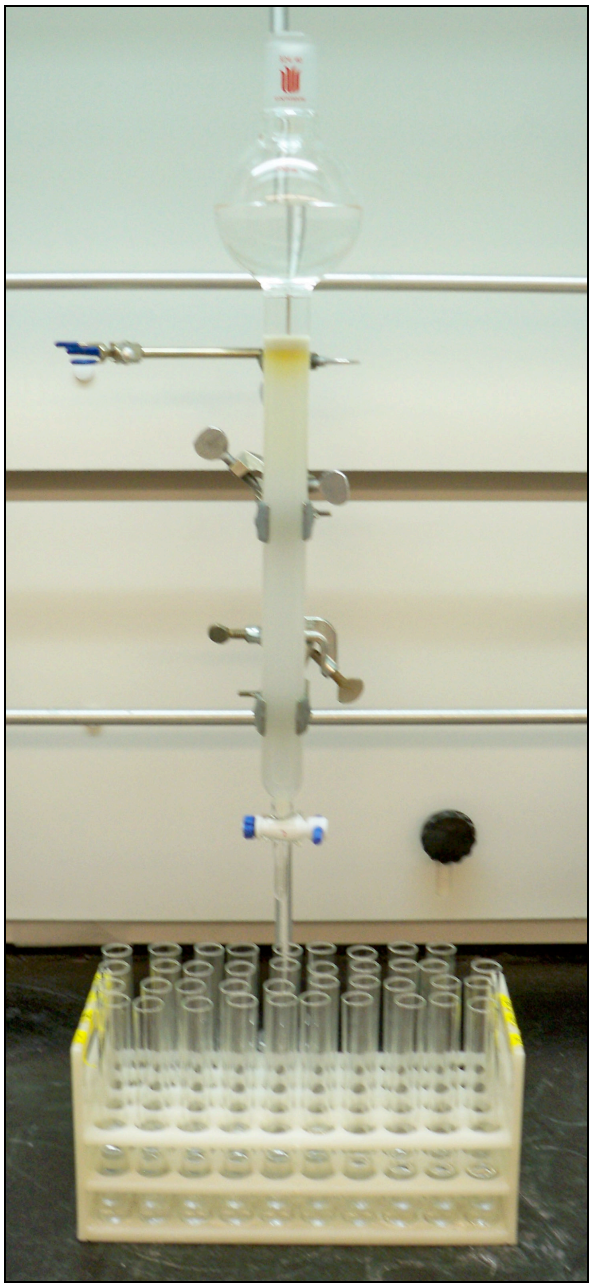


Figure 3.1 An Atmospheric Pressure Open Column Chromatographic Column. Fractions are collected in the test tubes and later ran by TLC to determine the purity of the fraction and the presence of synthesis products.

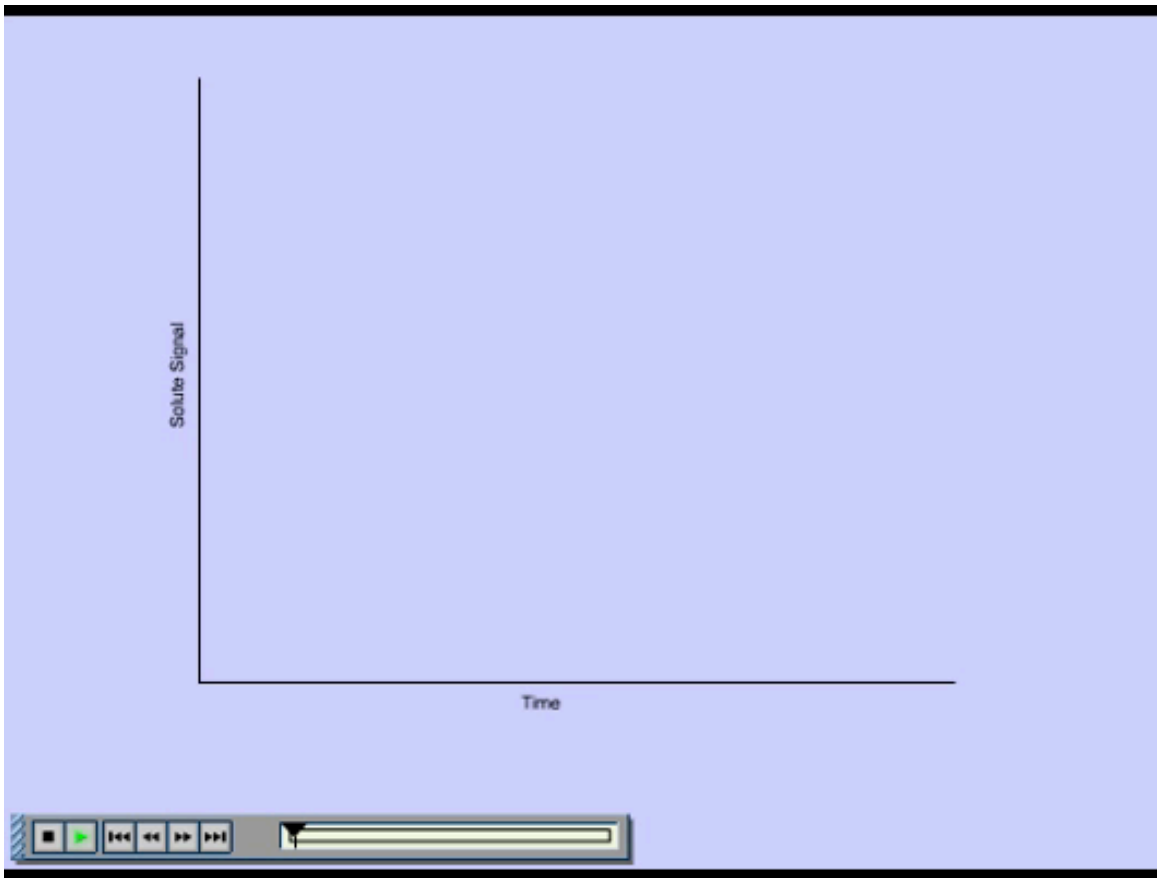
Since the development of TLC, liquid chromatography needed a technique comparable to gas chromatography where a complex mixture of analytes could be quantitatively separated and identified. Several attempts were made to pressurize the relatively large glass preparatory column (shown above) with little success due to the fragile nature of the column. High-pressure liquid chromatography (HPLC) was later developed to meet this goal in the 1970s. The pressure was first delivered by a large syringe, but this approach limited the volume of solvent that would be passed through a column and therefore limited the analysis time. Syringes were later replaced by a single reciprocating pump but these delivery systems experienced flow surges, between strokes of the single piston, interfering with stable detector baselines. The placement of two reciprocating pump, operating opposite to each other with respect to flow, greatly minimized the flow fluctuations which were later removed completely with a pulse damper. This form of chromatography is referred to as HPLC, where the HP stands for high performance or high pressure. Some jokingly refer to the HP as meaning high priced since it replaced TLC plates, that only cost pennies, with $20 000 to $30 000 instruments. The inflation of the cost of a LC analysis is even greater when an HPLC-MS is considered, a minimum of $150 000. But regardless, HPLC-MS is considered the technique of choice for isolating a synthetic product and is widely utilized in most synthesis laboratories.

Chromatography, as noted in Chapter 1, is divided into gas, liquid and supercritical fluid techniques. Liquid chromatography can be divided up into a relatively large collection of techniques. Those mentioned above include atmospheric or low-pressure open column chromatography and thin layer chromatography. Pressurized liquid chromatography can be divided into ion exchange, exclusion, partition, and liquid-solid chromatography as summarized in Animation 3.1. View this animation by double clicking on the figure below.

Animation 3.1. A Discussion of the Various Types of Chromatography.

As noted in Animation 3.1, a variety of separation techniques are available with *high pressure* liquid chromatography. Most relevant to this chapter is partition chromatography, although a few others will be discussed in later sections. The most important point here is to distinguish between normal and reverse phase chromatography. HPLC was first developed using normal phase conditions (NP-HPLC), that followed the logic of atmospheric open-column chromatography, where the stationary phase acted as the polar phase and the mobile phase was non-polar, specifically an organic solvent. Normal phase HPLC focused on the separation of analytes that were readily soluble in non-

polar solvents but had slight affinities for the polar stationary phase. However, NP-HPLC required the use of relatively expensive and large volumes of organic solvents that also led to high waste disposal costs. NP-HPLC was effectively replaced by reverse phase HPLC (RP-HPLC) that operates with a non-polar stationary phase and an aqueous, moderately polar mobile phase. Gradient programming, an additional development, changes the composition of the mobile phase during a chromatographic run which greatly enhanced the utility of RP-HPLC. In RP-HPLC, the gradient is initially more polar (i.e. water) and as the chromatographic run progresses, more and more less-polar solvent is added to the mixture (i.e. methanol or acetonitrile) to end the gradient program with pure organic solvent. As noted in Animation 3.2, the retention order of analytes in RP-HPLC is opposite NP-HPLC. For example, the first analyte that eludes on a RP system would elude last is separated by NP-HPLC.



Animation 3.2 Demonstration of Normal and Reverse Phase HPLC.

Additionally, the pH of the polar solvent in RP-HPLC can play an important role in optimizing analyte separations. Ionic or ionizable analytes that would not normally be separated on a RP (non-polar) analytical column can also be analyzed by ion-pair chromatography. In this type of chromatography, the ionic analyte is bound to another ion (usually a large organic counter-ion such as quaternary ammonium or alkyl sulfonate) to form a neutral pair that has selective affinity for the non-polar stationary phase. Even many chiral compounds can be separated on special chiral stationary phases or by the additional of chiral resolving agents that selectively bind to one of the enantiomers. Additional modifications to an HPLC system such as ultra-high pressure LC, ion exchange, and supercritical fluid chromatography will be discussed in Sections 3.5 and 3.6.

One final point should be made with respect to HPLC. Chemists use HPLC for two completely different purposes. Organic chemists, especially in the pharmaceutical industry, use large-scale systems, referred to as preparatory HPLC or flash chromatography, to recover relatively large-scale milligram quantities of their products. The main differences of a preparatory HPLC, as opposed to an analytical HPLC, are the pump flow rates and the size of the columns. In contrast, analytical chemists use HPLC to separate and identify nanogram or smaller quantities of analytes. Which ever practice is needed, the overall chromatography is the same.

3.2 Types of analytes, samples and sample introduction

Gas chromatography is somewhat limited in that the analyte has to be volatile below 300 C and thermally stable with molecular weights less than 1000 Daltons. HPLC greatly expands the range of possible analytes and with reverse phase HPLC and can include many of the same analytes as GC (but with less resolution as compared to capillary column GC). Analysis of compounds commonly used in HPLC increases the analyte range of molecular weights to just less than one million Daltons. Many chemicals, such as petroleum hydrocarbons, solvents, illicit drugs and environmental chemicals, can be analyzed by both GC and HPLC. But with the ability of HPLC to analyze non-volatile chemicals many bio-molecules can be analyzed, including sugars, amino acids, proteins, and a large variety of other non-volatile compounds.

Samples analyzed by HPLC are always in liquid form, in either aqueous or organic solvents. Little to no sample preparation is needed, except that it is good practice to filter all samples through a 0.2 μm low-volume cartridge filter prior or during injection. Filtration of samples and solvents avoids the buildup and eventual clogging of the in-line filter or guard column. Samples containing high concentrations of analytes may need to be diluted in order to avoid overwhelming the capacity of the stationary phase and remaining in the linear range of the detector (governed by Beer's law in UV-Vis applications). Solvent exchange of the samples may be necessary depending on the solvent gradient conditions

needed for separation. Samples containing compounds with significantly different chemical structures may interfere with the detector of the analyte(s) and may require removal with a micro-column clean up (such as silica gel or alumina) or may require the use of a specialty clean up cartridge (such as those used in GC for concentration of the analytes from aqueous samples described in Section 2.2). Samples containing relatively low concentrations of analytes may need to be concentrated using one of these same micro-resin columns.

3.3 The Liquid Chromatograph

3.3.1 Overview:

In recent decades the modern HPLC system has become increasing complex. Automatic injection systems have all but replaced manual injections, manual six-port values are now pressure actuated, the instrument is usually computer controlled, and data are collected and processed via a specified computer method. It should come as no surprise that the cost of a basic HPLC system (without mass spectrometry detection) has risen from $10 000 a few decades ago to nearly $40 000 today. In this section, each component of an HPLC will be presented. Figure 3.2 illustrates an overview of a modern HPLC-MS system.

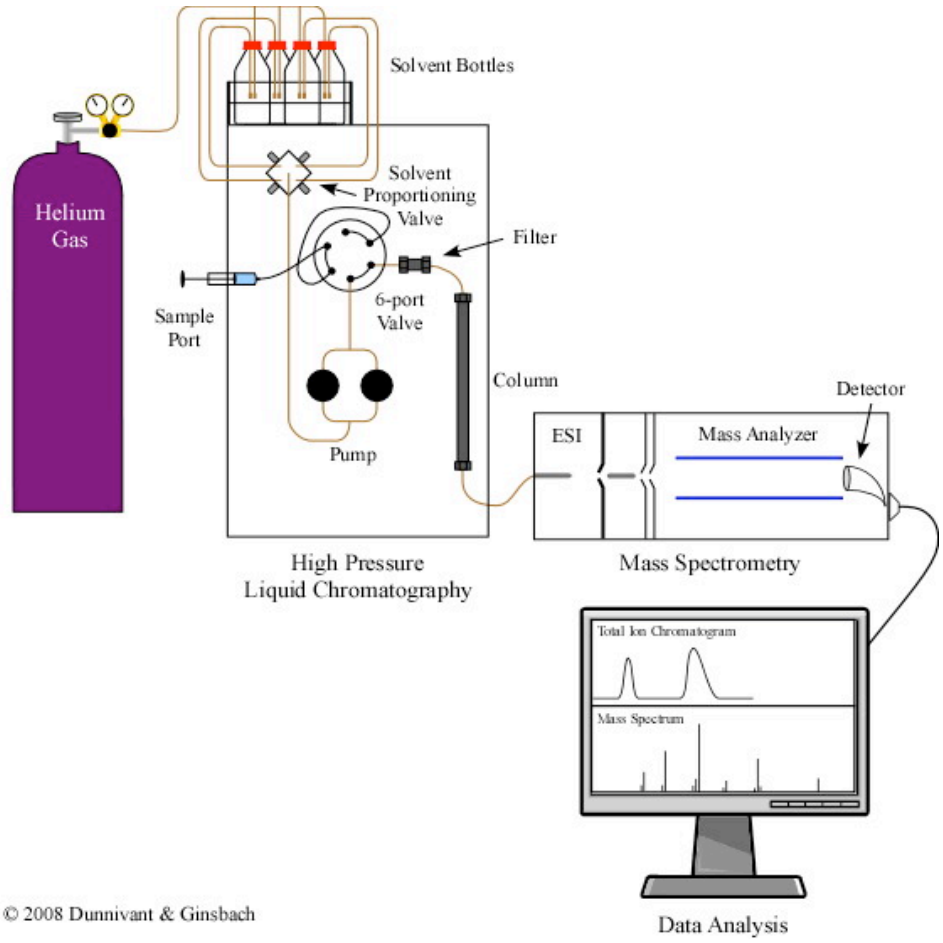


Figure 3.2 An Overview of a Modern HPLC-MS System.

3.3.2 Actuator Gas: Pressurized gas is required to degas solvents and to drive many of the mechanical functions of modern HPLCs. Solvents used as the mobile phase contain trace concentrations (ppm) of atmospheric gases (N_2 and O_2) which create bubbles in the pump (during the low pressure intake of solvents) causing pumping problems. Dissolved gases can also evolve in the low pressure detectors in ultrahigh pressure LC, ion exchange, and supercritical fluid chromatography giving the appearance of a chromatographic peak depending on the specific detector being used. Pressurized gas is also needed to turn the six-port injection valve during automatic injection mode.

3.3.3 Solvents and Solvent Preparation: Solvent purity requirements depend on the type of samples being analyzed. All solvents used as the mobile

phase must be filtered through 0.2 μm filters prior to entry to an HPLC system in order to avoid the scratching of the pump pistons by particles and to avoid clogging the in-line filter or guard column. Organic chemists are usually not concerned with the purity of solvents since their analytes are present in high concentrations and their samples usually contains many side-products and solvents. Hence, relatively low purity, and therefore inexpensive, solvents can be used. In contrast, the analytical chemist must purchase HPLC grade solvents that are considerable more expensive. For example a 20-L metal can of ACS grade methanol costs less than $70, while a 4-L glass bottle of HPLC UV-Vis grade bottle costs $150. Many solvent gradients call for acetonitrile that in recent years (2009-2009) has risen considerable in costs due to a decrease in production, from $100 in 2005 to $250 in 2009 (if supplies allow you to even order it). The increase in cost has tempted many chromatographers to switch their acetonitrile solvent gradients to one based on methanol while maintaining the same polarity index (a measure of the polarity of the solvent mixture that is used to optimize analyte separation). However, as noted by William Campbell in a recent Supelco Analytical Note (volume 27.1, page 13), while analyte retention orders may (or may not) remain the same, the relative retention times and resolution can significantly change, in some cases where analyte separations are no longer achievable. Solvents used for elution are contained in glass reservoirs (usually one liter in capacity) and connected to HPLC with Teflon tubing. The solvent bottle is purged with He gas to remove dissolved gases, mentioned above, and kept under a He atmosphere during HPLC operation.

Tubing and Connections: Different components of an HPLC system operate under different pressures ranging from atmospheric to 4 500 psi (3.10×10^7 Pa) in standard HPLC and to 20 000 psi (1.38×10^8 Pa) in ultra high pressure liquid chromatography. Systems are currently under development that will operate at 100 000 psi. While GC fittings are usually standardized to Swagelok fittings, many HPLC manufactures have established their own type of high pressure fittings. All operate on the same ferrule-nut configuration as in GC but the shape of the ferrule and receiving system is different. A few of the more

common fitting are shown in Figure 3.3. Tubing sizes are much smaller in HPLC as compared to GC. Common tubing materials are stainless steel and PEEK (polyaryletheretherketone), a very strong organic polymer.

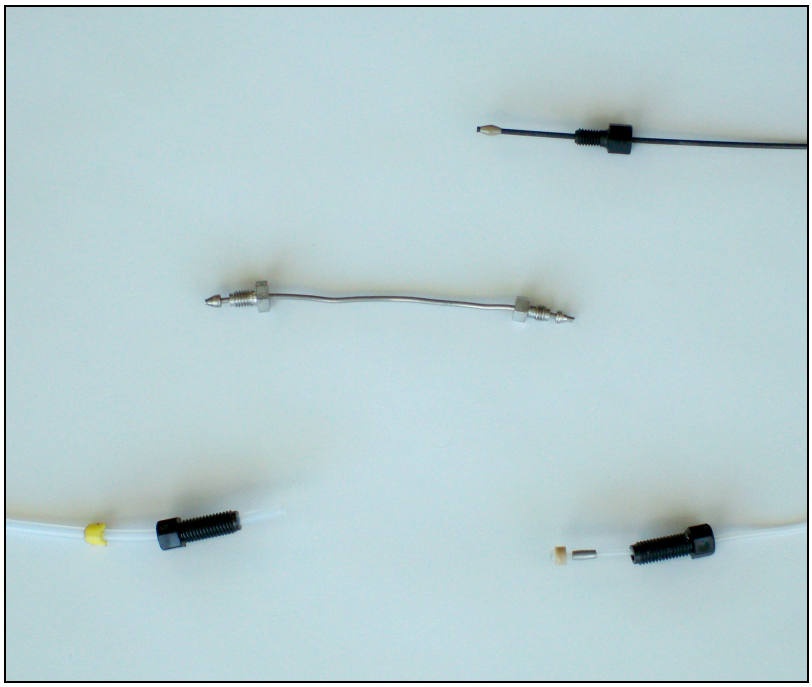


Figure 3.3 A Selection of HPLC Tubing and Connectors.

3.3.4 Proportioning Valve: Up to four separate elution solvents enter the HPLC first through a proportioning valve that adjusts the flow of each solvent to a predetermined amount. A proportioning valve is shown in Figure 3.4. Prior to the development of the proportioning valve, a separate pumping system was needed for each solvent. Since the pumping system (Figure 3.4) is typically the most expensive component of an HPLC system, the proportioning valves help reduce the cost of an HPLC system.

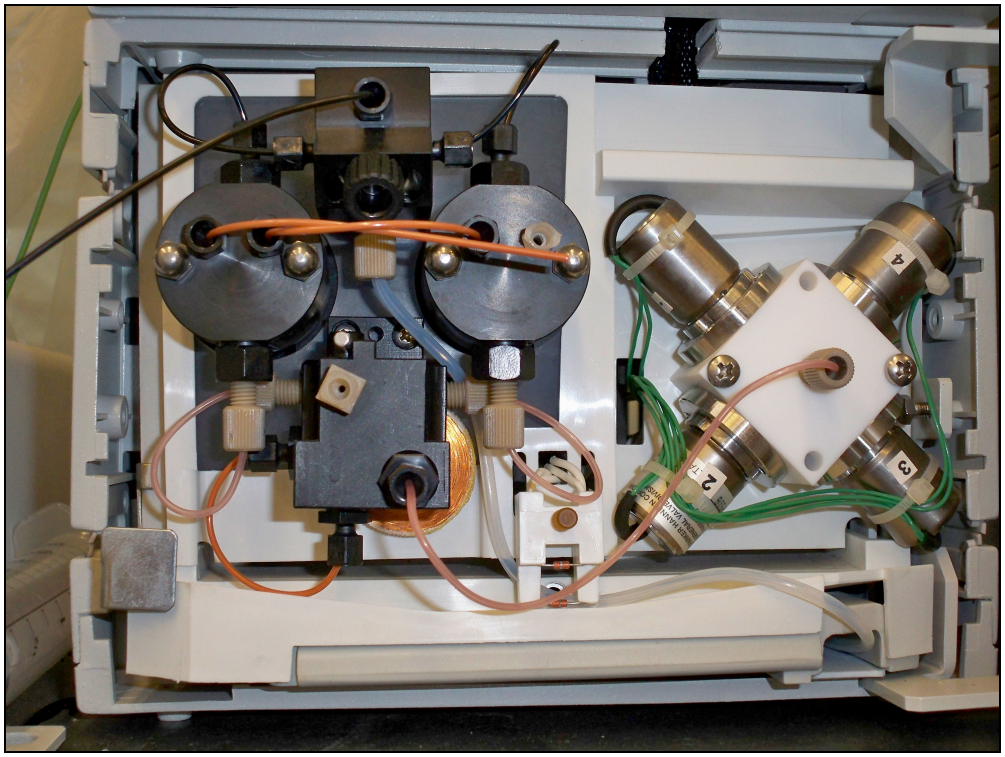
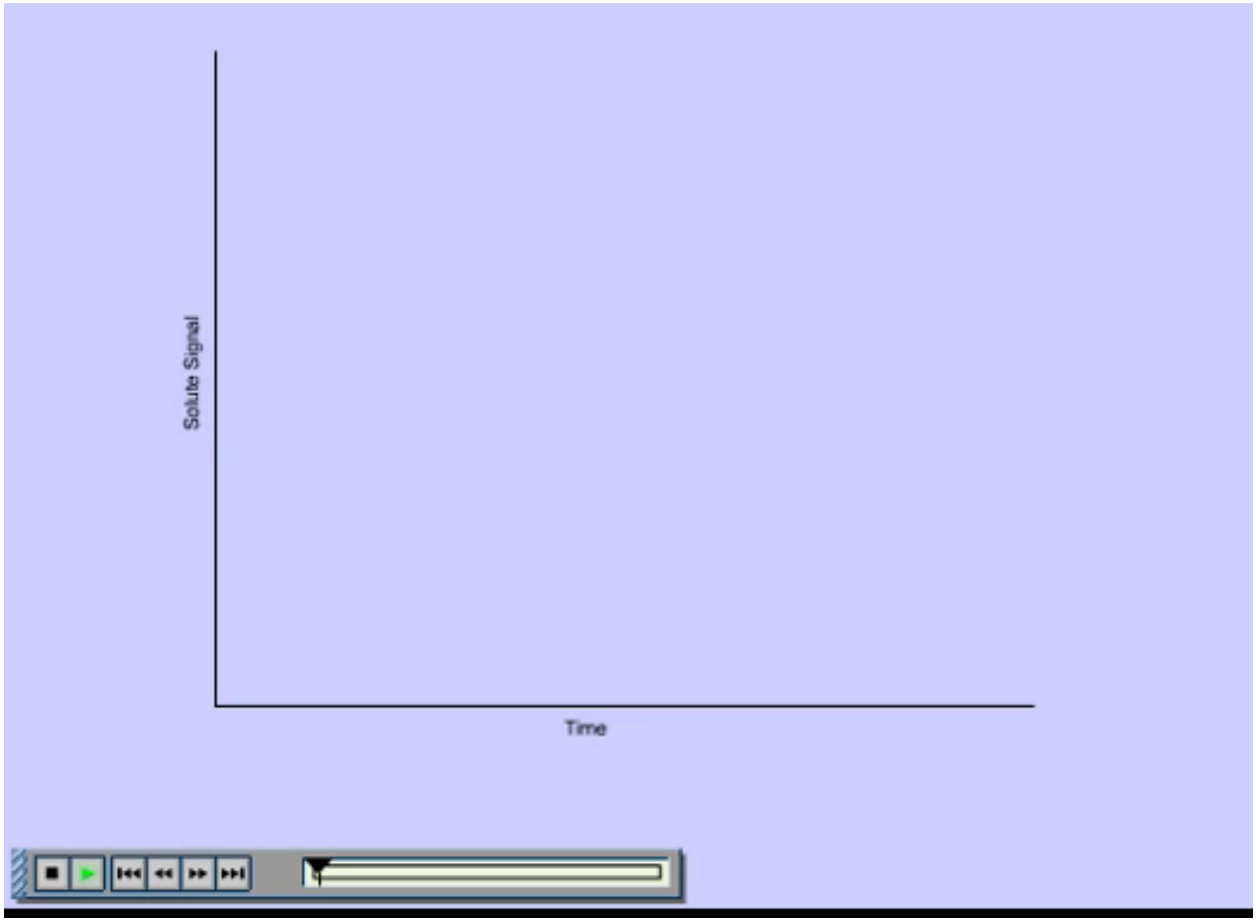


Figure 3.4 A Dual Piston Reciprocating Pump (left) and a Four-Way Proportioning Valve (right).

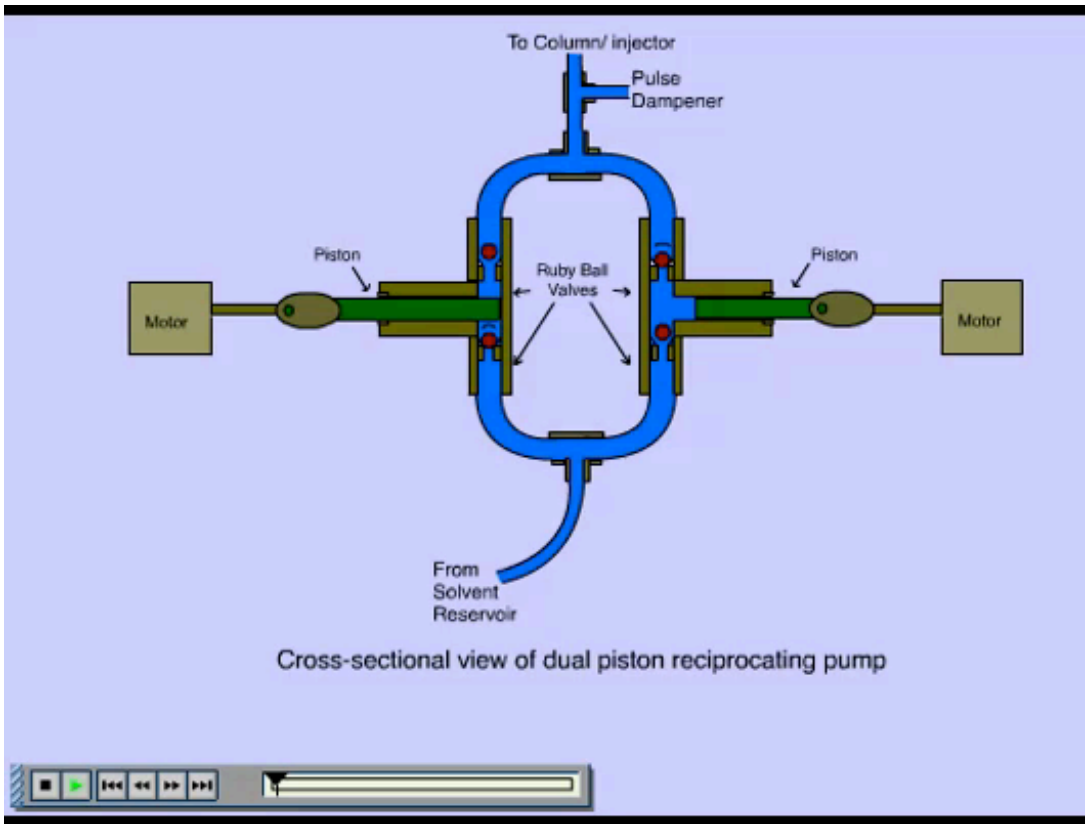
The relatively inexpensive proportioning valve allows the mixing of up to four solvents which enter a single pumping system. The mobile phase can be delivered as a constant composition of solvents (isocratic) or as a changing composition (gradient programming). In gradient programming, the solvent composition changes from a more polar solvent mixture to a more non-polar organic composition. Isocratic operations are used for relatively simple separations, while gradient programming is used for complex separations. In the past, the relative flow rates of multiple pumps were used to control the mobile phase composition. Today the less expensive proportioning valves adjust the composition. The need for gradient programming is illustrated in Animation 3.2 and is similar to temperature programming in GC and is again referred to as the general elution problem. Click on the figure below to start the animation.



Animation 3.2 The Solution to the General Elution Problem in HPLC.

3.3.5 Pump: As noted earlier, HPLC pumping systems have evolved more than any other component of LC. Gravity-fed open column systems were later pressurized with gas, which was replaced by syringe pumps in early HPLC, then by single piston pumps, and today with specially engineered dual reciprocating piston pumps. Today's pumps provide constant pressure and mobile phase flow by alternating the pumping actions between two pumping systems. When either pump is in full stroke, constant flow is easily provided but as each pump reaches the end of a stroke flow rates may be altered. This is overcome in some systems by an oval-shaped caming device that speeds the pumping rate at the end of each piston stroke. At the end of the stroke, one pump speeds up the flow of mobile phase as the other pump head decreases the pumping rate. This combined action provides constant flow rates in modern HPLC systems. While the simultaneous operation of two pumps and their associated pistons, cams, and check valves are difficult to describe in words, an

animation makes the process easy to understand. Such an animation is shown in Animation 3.3. The animation can be viewed by clicking on the figure below.

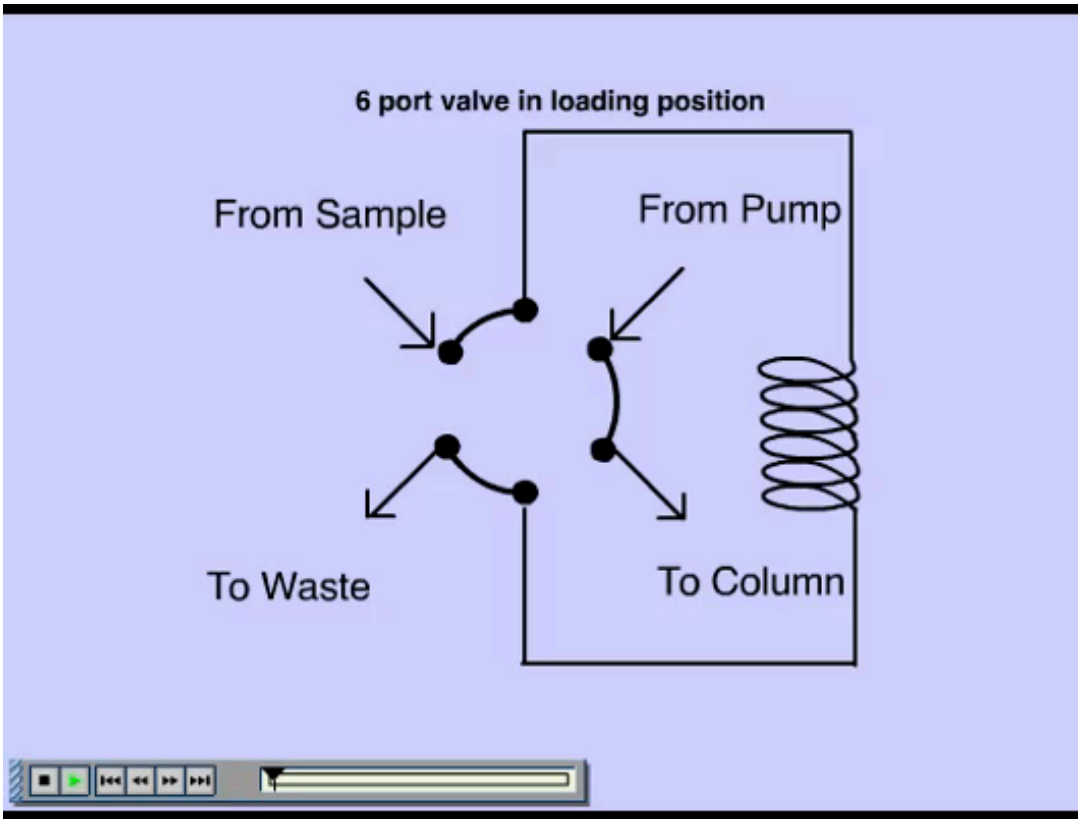


Animation 3.3 Illustration of a dual reciprocating piston HPLC pump.

Pulse Damper: Early HPLC pumps produced pressure, and therefore flow pulses, this was especially true with single piston pumps which required a pulse damper to be installed down-line from the pump. Today's systems, with advanced pump design, produce little pressure fluctuations and either do not require a pulse damper or electronically compensate for the very small pressure fluctuations.

3.3.6 Six-Port Injector: High pressure systems require a special type of sample injection since injections with standard syringes are not possible. Four-,

six-, and eight-port valves are used for high pressure systems. These systems are equipped with a fixed volume loop of tubing that serves as the sample loop that is loaded with a standard blunt-tipped syringe. These multiple port systems allow un-interrupted flow to the column during the loading of a sample on a sample loop and during injection of a sample under high pressure. As with a dual reciprocating HPLC pump, it is easier to show how a six-port valves works through an animation. Animation 3.4 shows the alignment of the valves during the loading of a sample onto a sample loop, switching of the values, and injection of the contents of the sample loop onto an HPLC column. View the animation by clicking on the figure below.



Animation 3.4 Operation of a six-port sampling/injection valve for an HPLC.

The sampling loop of a six-port valve can be loaded by a syringe or by pumping liquid sample into the valve by an automatic sampler. Sample loop

sizes range from 5 - 100 μL for analytical-scale HPLC to milliliter volumes in preparatory-scale HPLC. The advantage of a syringe (manual) injection is that less sample volume is required (since filling of the tubing in an automatic sampler is not required). The obvious advantage of an automatic sampler is that numerous samples can be automatically analyzed by a computer-controlled system.

In-Line Filter: Although samples and solvents are filtered prior to injection or use with an HPLC, an in-line filter is usually present immediately after the injection valve. This filter removes any remaining particles in the mobile phase that may clog the guard or analytical column. In-line filters are one of the most commonly maintained items for HPLC systems.

3.3.7 Columns:

Guard Column: The next component of an HPLC is the guard column. Guard columns are miniature versions of the analytical (separation) column and they contain the same stationary phase. The purpose of the guard column is to adsorb any permanently adsorbing chemicals that could destroy the more expensive analytical column. Guard columns typically cost one-fourth or less of the cost of an analytical column.

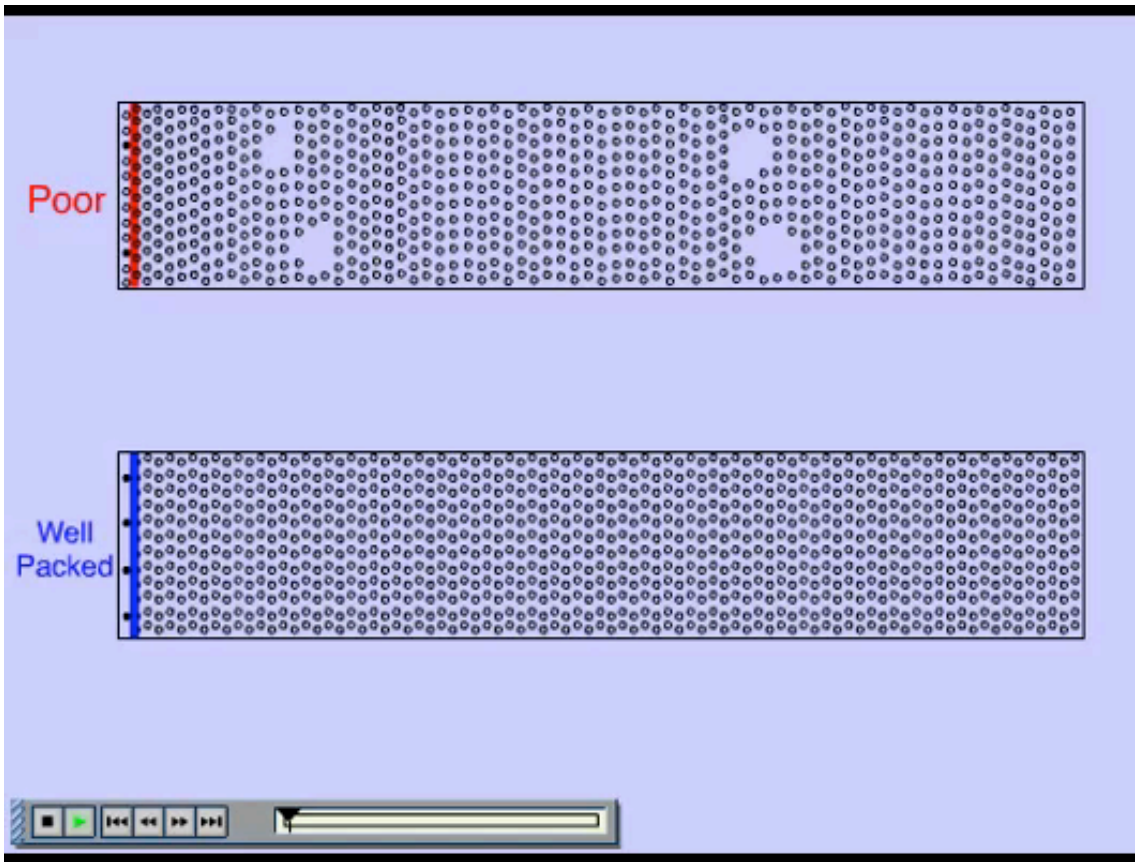
Analytical Column: The heart of an HPLC system is the analytical column. This is where the mixture of chemicals injected in the system is separated into individual analytes that appear as peaks in the chromatogram. Columns are available in a variety of diameters and lengths ranging from large preparatory columns (20 - 50 mm in diameter by 50 - 250 mm in length), to analytical columns (typically 4.5 mm in diameter by 12 - 25 mm in length), to narrow bore analytical columns for improved performance and MS applications (1-2 mm in diameter by 10 cm in length), to capillary columns for MS detectors (from 0.075 - 0.1 mm in diameter). Larger columns require a larger mobile phase volume to push the analytes through the system. A collection of HPLC columns is shown in Figure 3.5.

Figure 3.5 Various Sizes of HPLC Analytical and Quard Columns. Source: Analytical Sales and Services, Inc. Reprinted with permission from Analytical Sales and Services.

With the exception of relatively new capillary columns, all HPLC columns are packed with small resin beads (typically 2-5 μm in diameter or less) that contain a coating of stationary phase. As in GC, stationary phases are selected based on the expected intermolecular forces available to the analyte for bonding. The adage “like dissolves like” is also appropriate for liquid chromatography applications. Separations in HPLC are considerable more complicated then in GC. In GC, the chromatographer is only concerned with the stationary phase and the temperature program. In HPLC, one must also be concerned with the polarity of the mobile phase. Adequate analyte separations are only achieved with an appropriate match of gradient polarity and stationary phase interactions. In more advanced applications, the pH and ionic composition of the mobile phase

must also be controlled. The most common stationary phases used in reverse-phase HPLC include alkylamine, octodecyl (C18), octyl (C8), butyl (C4), cyanopropyl (CN), and methyl functional groups. Additional and custom-made stationary phases can be ordered from many suppliers.

Early chromatography columns that were open to the atmosphere were easy to pack. The chromatographer filled a glass column (1 to 5 cm in diameter) with solvent, slowly poured in silica gel with stirring to remove air pockets, and applied the solution to be separated to the top of the column. These types of columns yielded adequate separation for organic chemists attempting to isolate their synthesis products but have extremely limited use for analytical chemists who require more theoretical plates in the column and on-line detection of column effluents. Today, analytical chromatographers almost exclusively purchase their separation columns from manufactures because of the need for perfectly packed columns with no void spaces. The emphasis here is on perfect column packing since the presence of only a few void areas in a column will significantly affect the theoretical plate height in the column and decrease resolution. A comparison of a poorly packed column and an adequately packed column is shown in Animation 3.5.



Animation 3.5 The Effect of Adequate Column Packing on Peak Broadening in HPLC Columns.

The particle size of the stationary phase also significantly affects the operation of an HPLC by determining the shape of the van Deemter curve. Figure 3.6 shows the van Deemter curves for a variety of particle-sized stationary phases. Recall from our discussions in Chapter 1, better separations occur at the minimum point (linear velocity) in the van Deemter curve and a more stable system operation will occur with a large range of linear velocities. In HPLC, smaller sized stationary phase particles will yield a van Deemter curve that is

essentially flat (refer to Figure 3.6). Thus, a range of flow rates will produce optimum analyte separation.

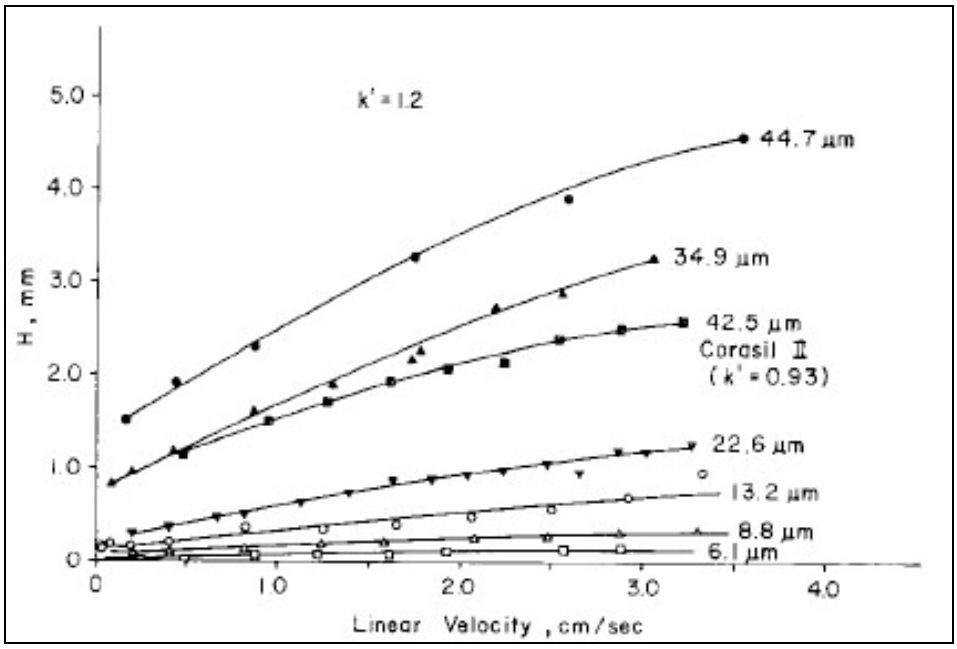


Figure 3.6 The Effect of Stationary Phase Particle Size on the Optimum Flow Rate in HPLC. From R.E. Majors, Effect of Particle Size on Column Efficiency in Liquid-Solid Chromatography, 1973, 11, 88. Reproduced from the Journal of Chromatographic Science by permission of Preston Publications, A Division of Preston Industries, Inc.

3.3.8 Detectors: There are a variety of detectors available for use with HPLC. These range from near universal detectors that will respond to any organic chemical structure to specialty detectors that only respond to a few analytes. Both have their advantages. Universal detectors provide economic use for a large diversity of chemicals but specific analyte detection can be hampered if two or more analytes elute from the analytical column at the same retention time. Specialty detectors provide near-interference free detection of

only a few analytes. Common detectors, along with their use and approximately detection limits are given in Table 3.1.

Table 3.1. Common Commercially Available HPLC detectors, Applications, and Detection Limits.

Detector	Application(s)	Detection Limits
UV-Visible Absorbance	For compounds that absorb in the UV or visible range	pg quantities
Fluorescence	For compounds capable of fluorescence (especially polyaromatic hydrocarbons)	fg quantities
Refractive Index (RI)	For alcohol, sugar, saccharide, fatty acid, and polymer analysis with refractive indices different from the solvent	ng quantities
Electrochemical	For analyzing a wide range of compounds	high pg quantities
Conductivity for IC	Mainly for inorganic ions	~ng quantities
Evaporative Light Scattering (ELS)	For a wide variety of compounds that lack UV/Vis chromophores including triglycerides, sugars and natural products	μg quantities

Fourier Transfer Infrared	For compounds with vibrational functional groups	μg quantities
Mass Spectrometry	A universal detector	μg to pg quantities depending on the type of mass spectrometer

The most common detector is the UV-Vis detector that comes standard on basic HPLC systems. This is a near-universal detector since most organic compounds have a chromophore capable of adsorbing UV or visible wavelengths. The focus of this textbook, mass spectrometer detectors, will be discussed in the next chapter.

3.4 Advanced and Specialty LC Systems

3.4.1 Ultra-High Pressure Liquid Chromatography, U-HPLC: HPLC technology made a significant advance with the development of ultra high pressures systems. These systems, currently operate at 15 000 to 20 000 psi, offer significant time and cost saving per analysis. The increased pressure allows samples to pass through the column faster and results in significant increases in column efficiency (and decreases in theoretical plate height) by minimizing longitudinal diffusion processes. These systems offer higher column efficiencies (resolution per column length) and allow the systems to operate at a much wider range of linear velocities, flow rates, and backpressures. Nanobore-sized columns offer up to 95 percent decreases in solvent use and interface well with mass spectrometers. Higher pressure systems are under development that will possibly operate at up to 100 000 psi and yield even greater column efficiencies.

3.4.2 Ion Chromatography, IC: Ion chromatography is a form of HPLC where the system is modified to analyze for mainly inorganic ions such as

nutrient ions (i.e. nitrate, sulfate, phosphate, etc.) as well as many organic anions and metal cations. The systems require a special ion exchange analytical column that is specific to the analytes of interest, a relatively simple conductivity detector, and a unique ion suppressor column for removing ions other than the analytes that would generate electrical conductivity in the detector. Columns and tubing are usually composed of PEEK to avoid reactions on metal surfaces. Analytical columns are mainly divided into columns for cations and anions and are similar in diameter to standard HPLC columns but slightly longer in length. Cationic exchange columns have active strong acid sites such as sulfonic acid ($-SO_3^-H^+$) or weak acid sites such as carboxylic acid groups ($-COO^-H^+$) that preferentially exchange with analyte cations. Common anionic exchange sites include the strongly basic tertiary amines ($-N(CH_3)_3^+OH^-$) and the weakly basic primary amine group ($-NH_3^+OH^-$). Both exchange groups are usually placed on porous microparticles of silica. As the injected sample passes through the analytical column, shown below for the sulfonic acid exchange surface, the following reaction occurs

$$-R\text{-}SO_3^-H^+_{solid} + \text{Metal Cation}^+_{solution} \; < \text{---} > \; (-R\text{-}SO_3^-)_n\text{Metal Cation}^+_{solid} + nH^+_{solution}$$

Similarly for anion analysis, the reaction would be

$$-NH_3^+OH^-_{solid} + \text{Anion}^-_{solution} \; < \text{---} > \; (-NH_3^+)_n\text{Anion}^-_{solid} + nOH^-_{solution}$$

At appropriate flow rates, an equilibrium migration front is set up for each analyte passing through the column. Since each metal cation will have a different affinity for the stationary ion exchange resin, each analyte will elute from the column at a different time. As in all types of chromatograph (in the absence of mass spectrometry detection), identification is largely based on retention time.

The detector in IC is a simple conductivity detector where a potential is placed across two electrodes. In the absence of ions in the solution passing through the detector, minimal current is transmitted and no signal is generated by the electronics. As each packet of analytes, cations or anions, pass through the detector a current is generated that is proportional to the concentration of analytes. In order to detect low concentrations of analytes, the background signal (current) of the mobile phase must be maintained as low as possible. Since all samples have counterions (an equal concentration of cations or anions) half of the ionic components must be removed prior to entering the detector. For example, detecting the presence of cations require the removal of anions before the column effluent reaches the detector. The removal of these ions is accomplished with an ion suppressor device shown in Figure 3.7. This device is positioned within the ion chromatography system as shown in Figure 3.8.

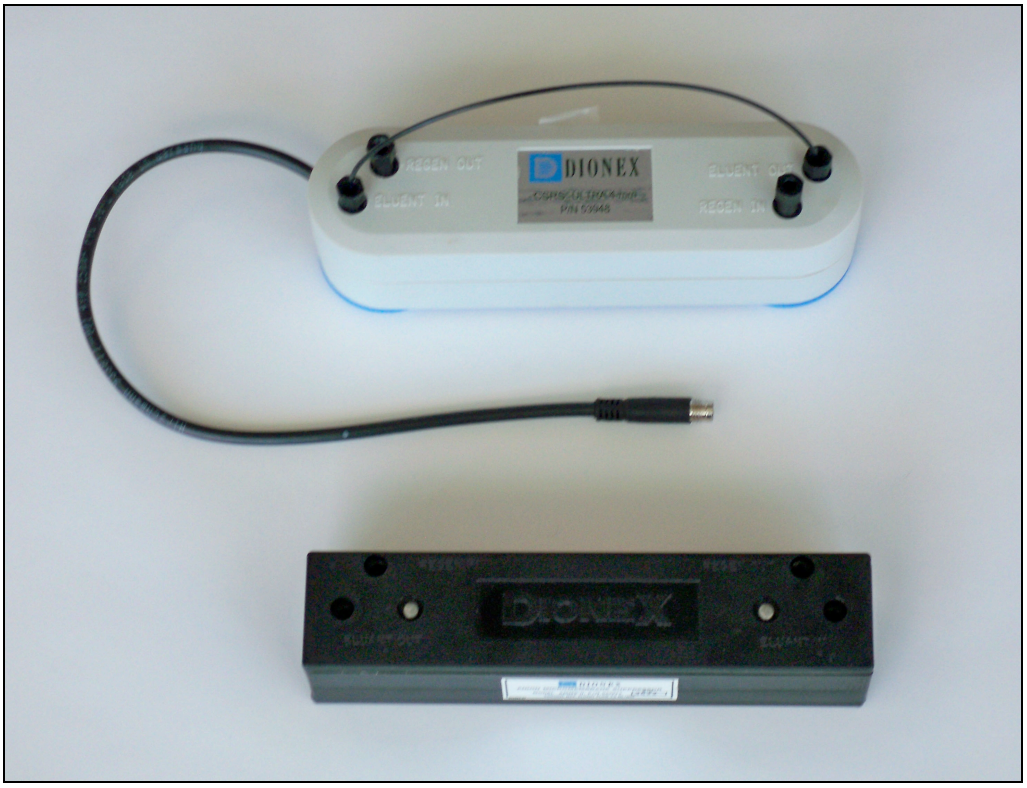


Figure 3.7 Photograph of Two Ion Suppressor Devices.

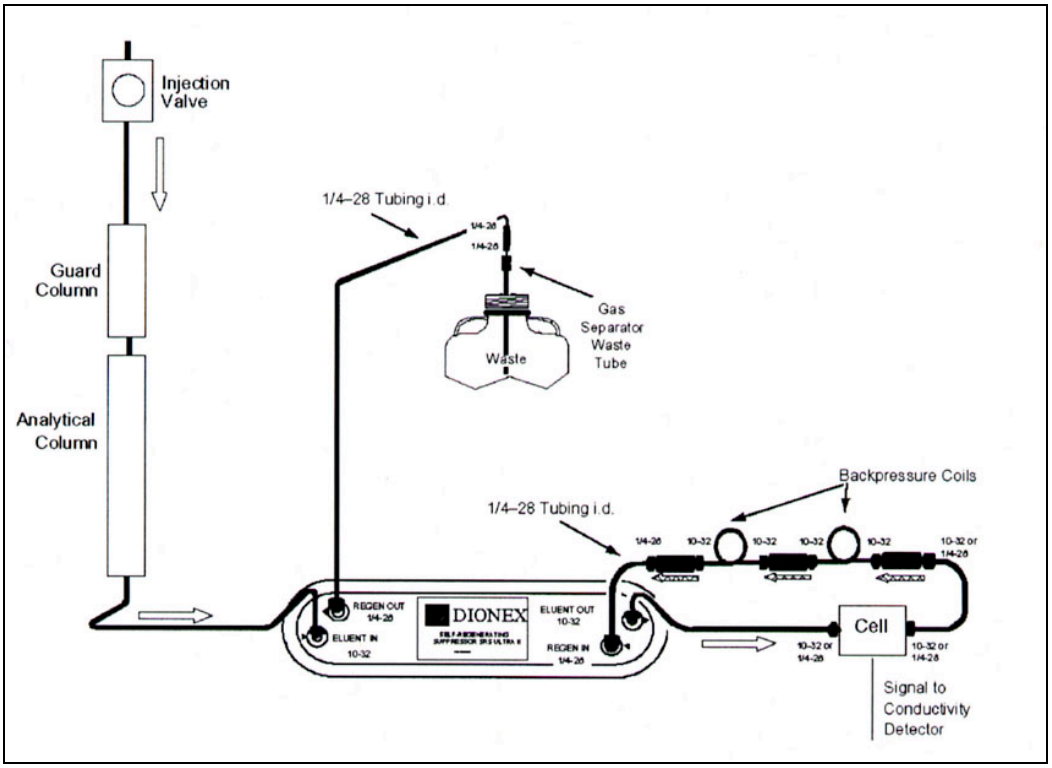


Figure 3.8 Overview of the Components used to Separate Analytes in an Ion Chromatography System. Source: Dionex Corporation Product Manual ASRS 300 CSRS 300, Figure 7, page 20. Reprinted with permission, courtesy of Dionex Corporation, Sunnyvale, California.

As presented in Figure 3.8, the sample first passes through the analytical column where the cations or anions are separated relative to their affinity for the solid-phase exchange resin. The effluent from the analytical column then enters the ion suppressor device, along with acid or base to keep the ion suppressor column active. For anionic analysis, strong acid (H^+) is used in the suppressor device; for cationic analysis, strong base (OH^- or COO^-) is used in the device. In both cases the analytes of interest are unchanged and unretained. As a consequence, the following suppression reactions occur depending on the mode of operation (cation or anion analysis).

In anion analysis, cations in the sample are exchanged for H^+ in the suppressor column and neutralized in the center flow through portion of the suppressor column by the following reaction

$H^+_{solution} + Cl^-_{solution} + resin^+OH^-_{solid} \text{ --- >}$

$resin^+Cl^-_{solid} + H_2O$

Thus, only anions, such as Cl^-, contribute to the conductivity in the detector.

In cation analysis, anions in the sample are exchanged with HCO_3^- (or OH^-) in the suppressor column and neutralized in the center flow through portion of the suppressor column by the following reaction

$cation^+_{solution} + HCO_{3\,solution}^- + resin^-H^+_{solid} \text{ --- >}$

$resin^-cation^+_{solid} + H_2CO_{3solution}$

Thus, only cations, such as Ca^{2+}, contribute to the conductivity in the detector.

Again, note that these reactions convert all ionic species, except for the analytes, to non-conductive chemicals. These reactions in relation to the flow through the ion suppressor are illustrated in Figure 3.9. In both cases, non-ionic species are produced and the interfering ions are removed (suppressed) from the solution. This significantly decreases the background ions in solution and allows for low detection limits (parts per trillion to parts per billion in the injected sample). Ion chromatography systems also operate well with MS detectors.

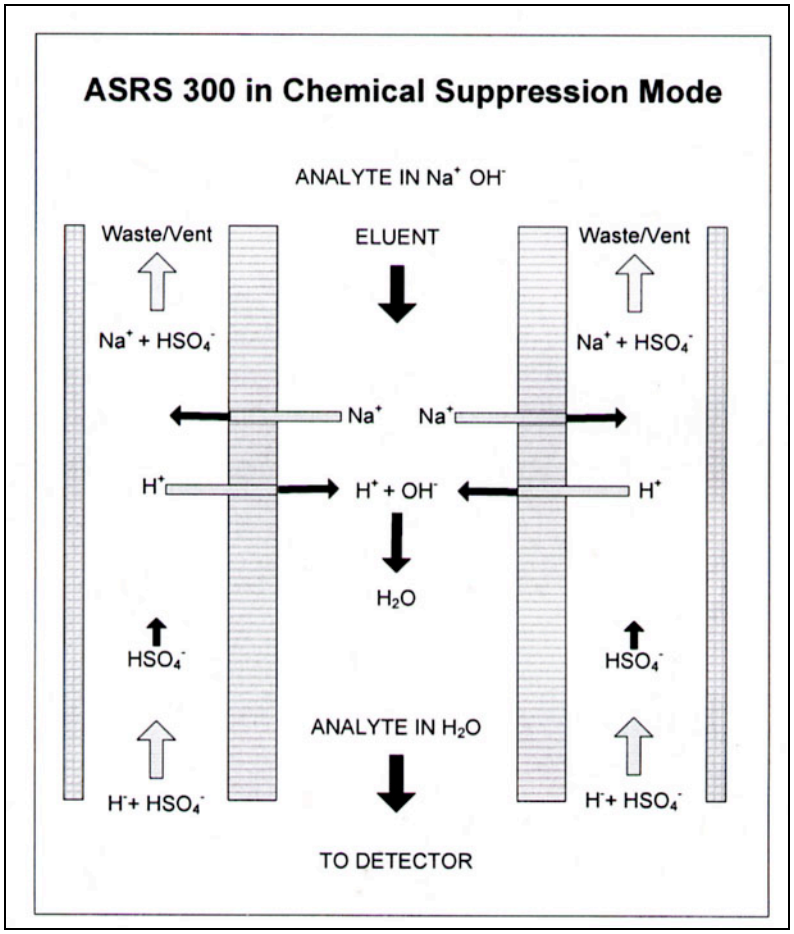


Figure 3.9 Cation suppression in an ion suppression device. Source: Dionex Corporation Product Manual ASRS 300 CSRS 300, Figure 4, page 9. Reprinted with permission, courtesy of Dionex Corporation, Sunnyvale, California

3.4.3 Super Critical Fluid Chromatography, SCF: A liquid turns into a super critical fluid (SCF) when its temperature rises above the critical temperature—the temperature where it can no longer exist as a liquid no matter how much pressure is applied. Super critical fluids exist in a state between a liquid and a gas and have the penetration abilities of a gas and the dissolving power of a liquid. As a result, SCF is a mixture of GC and HPLC and in some cases is superior to GC or HPLC. Super critical CO_2, the same matrix used to selectively extract caffeine from coffee beads and nicotine from tobacco products, can be used as a mobile phase in chromatography. Super critical fluid

chromatography is a special form of HPLC where a near-identical system is used but the mobile phase, as noted above, is super critical $CO_{2,}$. The system is therefore pressured and temperature controlled to maintain the super critical fluid. SCF is a form of normal phase chromatography that is used for the analysis of thermally labile molecules. The same types of packed and capillary columns that are used in HPLC are utilized in SCF. Due to the nature of the super critical fluid, packed columns can actually contain more theoretical plates than capillary column. Also, the shape of the van Deemter curve is different from those observed in GC and HPLC in that a minimum plate height exists over a very broad range of linear velocities. SCF can be used for a variety of separations but it is most commonly used in the separation of chiral compounds in the pharmaceutical industry. In place of a solvent gradient the chromatographer uses pressure programming and the affinity of the stationary phases to separate complex mixtures of analytes. Pressure programming in SCF is analogous to temperature programming in GC and gradient programming in HPLC. Methods of detection include UV/Vis, mass spectrometry, FID (as in GC but unlike in HPLC) and evaporative light scattering.

3.5 Applications

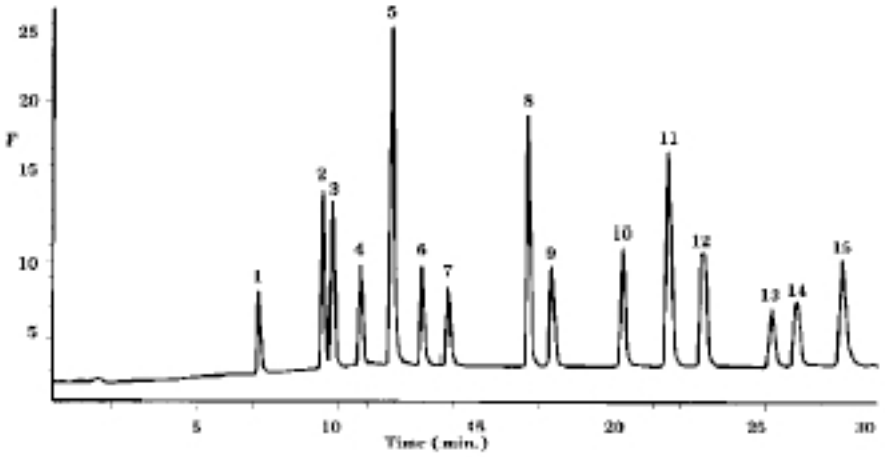
HPLC extends the capabilities of chromatographic separations past the abilities of capillary GC by allowing the analysis of thermally unstable analytes but at the expense of poorer resolving power due to the packed column nature of the system. These limitations are being slowly overcome with the use of capillary columns and ultra high pressure systems. This section highlights a few HPLC applications. As with GC, major column manufacturers and distributors provide very useful websites that contain example chromatograms for common analytes. These sources can be extremely helpful in selecting the appropriate column for your needs. In addition, technical help is available from professional chromatographers at these companies with more complex separations. The chromatograms below were selected from numerous ones that are available from Agilent technologies at http://www.chem.agilent.com/en-

us/Search/Library/Pages/ChromatogramSearch.aspx and Dionex Corporation at http://www.dionex.com/en-us/columns-accessories/ion-chromatography.

The first example we will illustrate is for polyaromatic hydrocarbons that can be analyzed very efficiently by GC. While HPLC also offers adequate separation (as shown in Figure 3.10 below) as compared to GC, HPLC allows improved detection limits with the use of fluorescence detection.

PAHs on 3 mm Columns

1. Napthalene
2. Acenaphtalene
3. Fluorene
4. Phenanthrene
5. Anthracene
6. Fluoranthene
7. Pyrene
8. Benzo(a)anthracene
9. Chrysene
10. Benzo(a)fluoranthene
11. Benzo(k)fluoranthene
12. Benzo(a)pyrene
13. Dibenzo(a,h)anthracene
14. Benzo(g,h,i)perylene
15. Indeno(1,2,3-cd)pyrene



Sample:	PAH Standard
Column:	LiChrospher PAH, 3.0 X 250 mm, 5 μm, (P/N: 79925PA-583)
Mobile phase:	A = Water, B = Acetonitrile
Gradient:	0 min 50% B, 3 min 60% B, 15.4 min 100% B, 23.5 min 50% B
Flow rate:	0.8 mL/min
Temperature:	27°C
Detection:	254 nm

Figure 3.10 Separation of PAHs by HPLC. Source: Copyright 2006 Agilent Technologies, Inc. Reproduced with Permission

Applications that cannot be performed by GC include the isolation of Vitamin A (Figure 3.11), the separation of proteins (Figure 3.12) and the separation of food coloring agents (Figure 3.13).

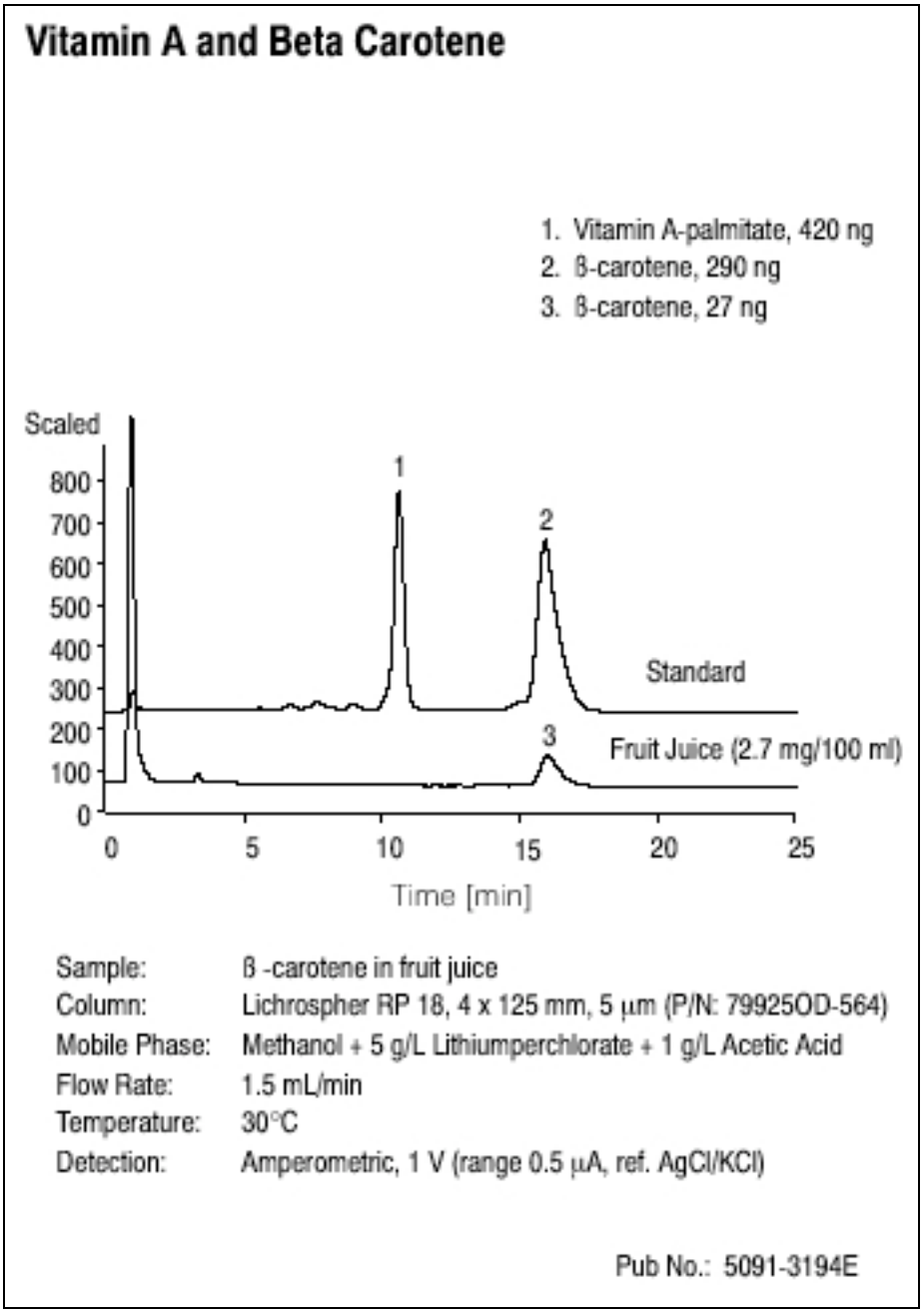


Figure 3.11 Isolation of Vitamin A by HPLC. Source: Copyright 2006 Agilent Technologies, Inc. Reproduced with Permission

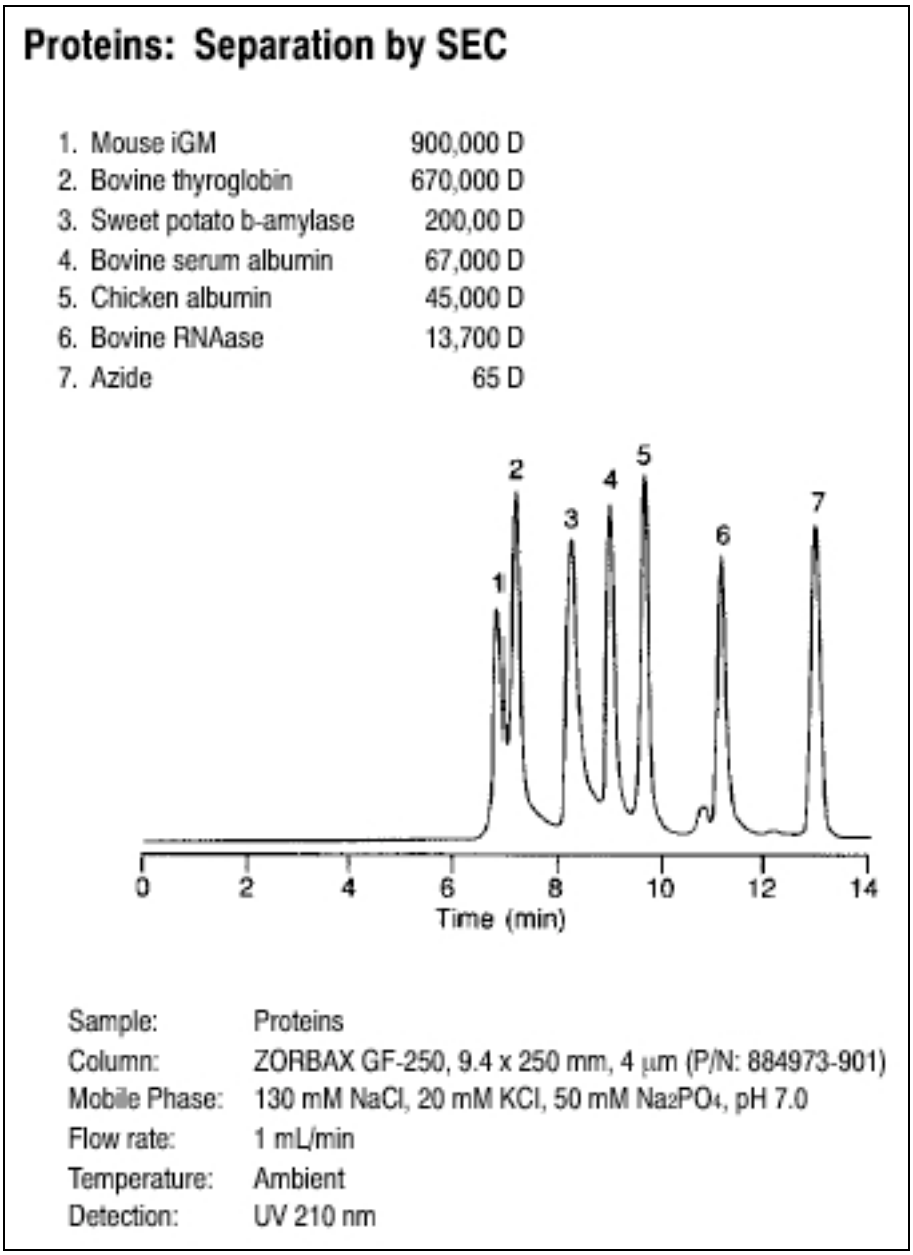


Figure 3.12 The separation of proteins by HPLC. Source: Copyright 2006 Agilent Technologies, Inc. Reproduced with permission.

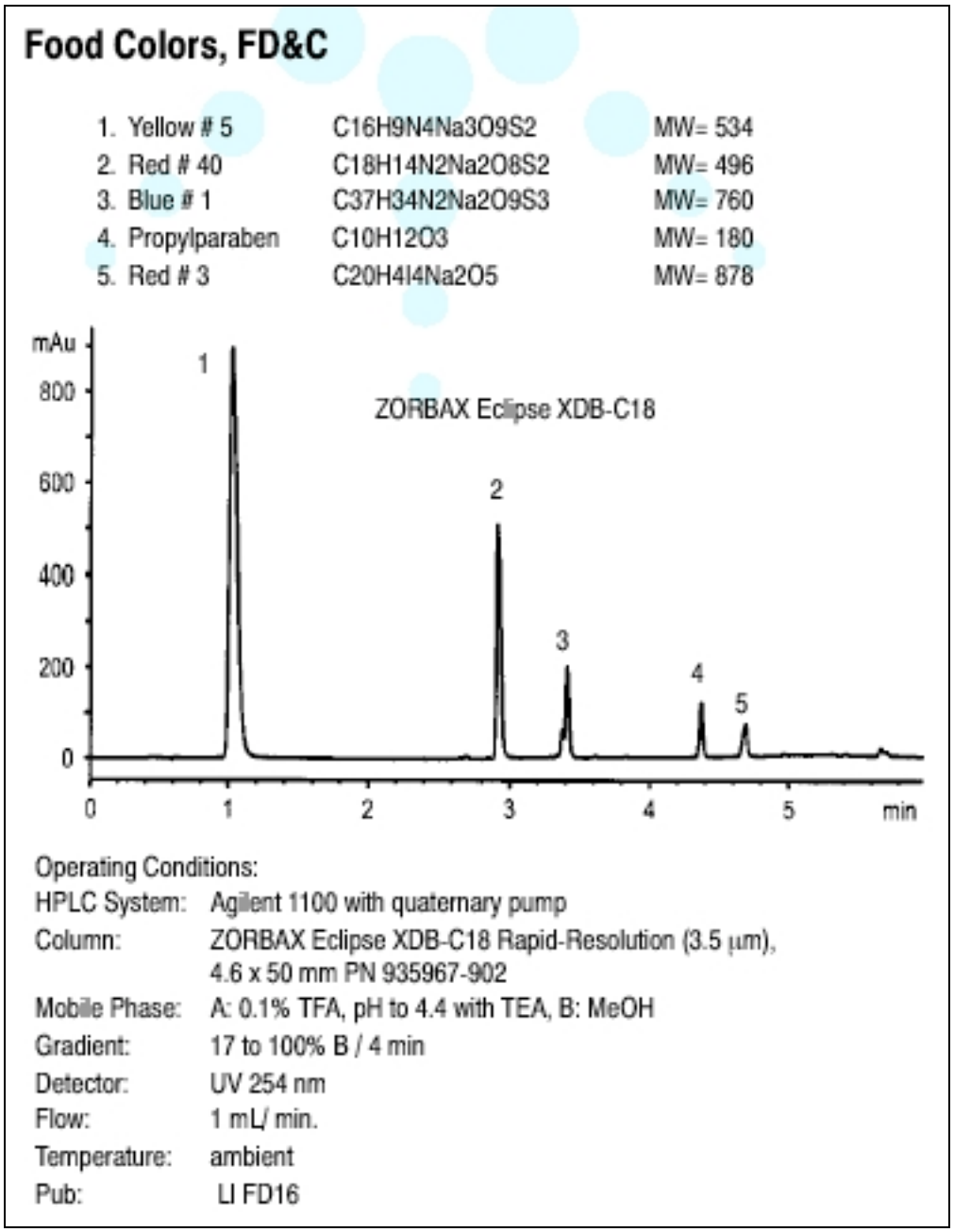


Figure 3.13 The separation of food coloring agents by HPLC. Source: Copyright 2006 Agilent Technologies, Inc. Reproduced with permission.

A separation of anions in drinking water by IC is shown in Figure 3.14.

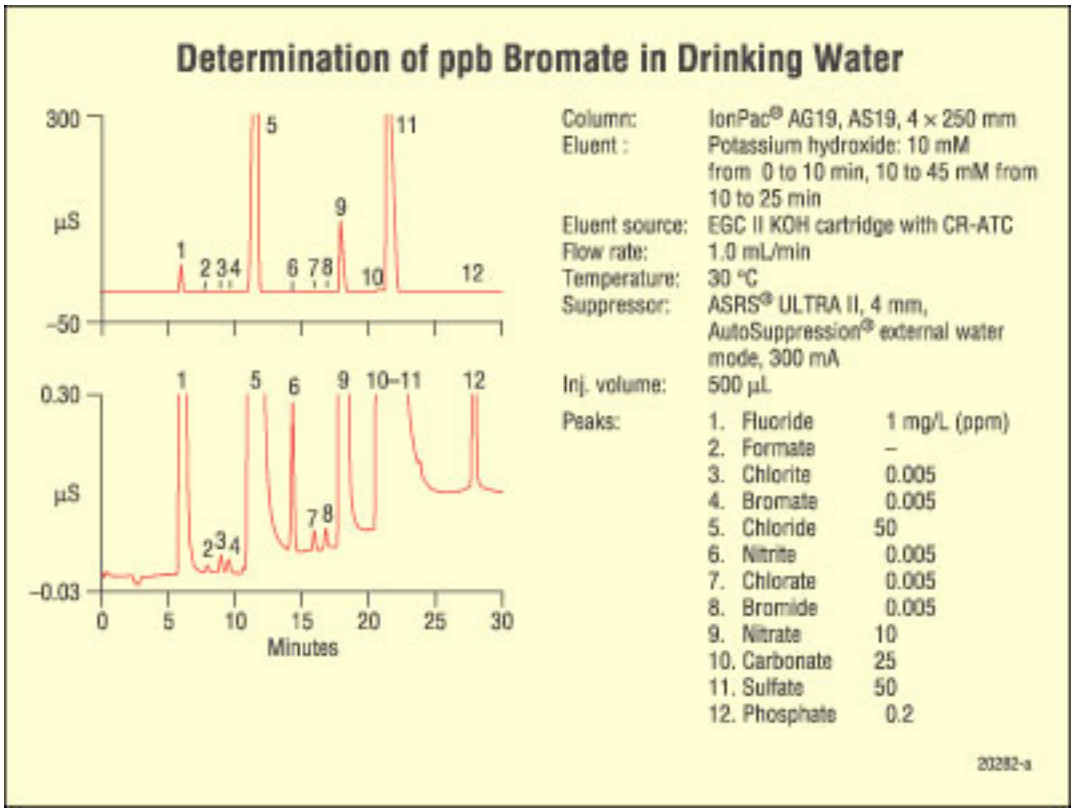


Figure 3.14 Separation of anions by IC. Source: Dionex Corporation, Inc. Reprinted with permission, courtesy of Dionex Corporation, Sunnyvale, California.

3.6 Summary

This chapter described the basic applications and operation of an HPLC system. Modern HPLC systems significantly expand the capabilities of chromatography to include the relatively delicate biomolecules that are so abundant in nature. Current research in ultra high pressure systems may result in HPLC performances that match those of capillary column GC in the near future. Basic non-confirmatory detectors systems were described in this chapter

and confirmatory identification by mass spectrometer techniques that are applicable to all forms of chromatography will be covered in Chapter 4.

3.7 Questions

1. Contrast the advantages and disadvantages of thin layer chromatography (TLC) versus modern HPLC.

2. What does HPLC stand for?

3. What are the advantages of dual reciprocating pumps have over syringe pumps?

4. How much does a basic HPLC system cost?

5. What are the sub-categories of liquid chromatography?

6. What is the difference between normal phase HPLC and reverse phase HPLC? Which is most commonly used today?

7. What chemical factors determine if a chemical will be analyzed in a GC or LC?

8. Can moderately volatile, thermally stable chemical be analyzed on an LC?

9. Why do we filter analyte solutions before injection into an HPLC?

10. Draw a basic HPLC system and label all of the components.

11. Why are pressurized gases used in HPLC?

12. What two preparatory steps must be taken before a solvent can be used as an HPLC mobile phase?

In general, what is the maximum pressure limit of standard HPLC systems?

13. What is the purpose of the proportioning valve? How does this reduce the cost of an HPLC?

14. What is the difference in isocratic and gradient programming? Why is gradient programming sometimes necessary?

15. Why are dual piston pumps preferred over single piston pumps?

16. What is the purpose of a pulse damper?

17. Why are six-port valves used for injecting samples in HPLC?

18. Draw and explain how a six-port valve works.

19. Why are in-line filters used in HPLC systems?

20. What is the composition of the stationary phase and purpose of the guard column?

21. What are common stationary phases used in reverse phase HPLC?

22. Why do chromatographers purchase their analytical columns instead of self-packing their own?

23. How will a poorly packed column affect performance?

24. What is the relationship between performance (resolution) and stationary phase particle size?

25. Compile a list of HPLC detectors and provide a list of chemicals each can be used to analyze.

26. Name three advanced types of LC.

27. Why is U-HPLC superior to standard HPLC?

28. How does IC differ from standard HPLC?

29. What is the purpose of the suppressor column in IC?

30. Draw a suppressor column for cation analysis in IC. Explain how it works. Write all requisite chemical reactions.

31. Although not shown in this textbook, attempt to draw a suppressor column for anion analysis in IC. Explain how it works. Write all requisite chemical reactions.

32. What is a super critical fluid?

33. What types of compounds are usually separated by SCF chromatography?

34. What type of gradient is used in SCF chromatography?

35. Review each of the chromatograms in Section 3.5 and determine what intermolecular force is involved in these attractions.

3.8 References

Agilent Technologies Internet site at http://www.home.agilent.com

Dionex Corporations Internet site at http://www.dionex.com

Supelco-Aldrich Internet site at http://www.sigmaaldrich.com

Waters Corporation Internet site at http://www.waters.com