A USER GUIDE

ON

HIGH PERFORMANCE LIQUID CHROMATOGRAPHY [HPLC]

By: www.pharmaguideline.com

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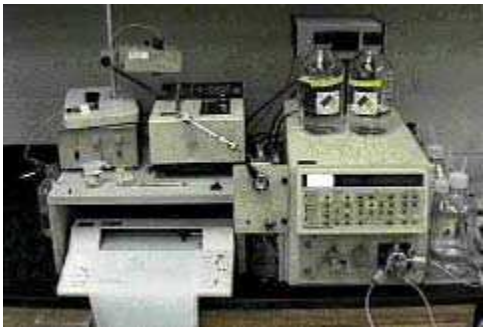
High Performance Liquid Chromatography (HPLC):

Introduction:



HPLC is a popular method of analysis because it is easy to learn and use and is not limited by the volatility or stability of the sample compound. The history section illustrates the HPLC's evolution from the 1970's to the 1990's. Modern HPLC has many applications including separation, identification, purification, and quantification of various compounds. It is important for those using HPLC to understand the theory of operation in order to receive the optimum analysis of their compounds. For those interested in purchasing or using an HPLC we have included a list of manufacturers, a troubleshooting guide, technical assistance, and a bibliography to help reduce your personal research and referencing time. Once you have completed the theory of operation, you will be qualified to take a quick quiz to test your understanding of HPLC systems.
High-performance liquid chromatography (HPLC) is a form of liquid chromatography to separate compounds that are dissolved in solution. HPLC instruments consist of a reservoir of mobile phase, a pump, an injector, a separation column, and a detector. Compounds are separated by injecting a plug of the sample mixture onto the column. The different components in the mixture pass through the column at different rates due to differences in their partitioning behavior between the mobile liquid phase and the stationary phase.

History of HPLC

Prior to the 1970's, few reliable chromatographic methods were commercially available to the laboratory scientist. During 1970's, most chemical separations were carried out using a variety of techniques including open-column chromatography, paper chromatography, and thin-layer chromatography. However, these chromatographic techniques were inadequate for quantification of compounds and resolution between Similar compounds. During this time, pressure liquid chromatography began to be used to decrease flow through time, thus reducing purification times of compounds being isolated by column chromatogaphy. However, flow rates were in consistant, and the question of whether it was better to have constant flow rate or constant pressure was debated.

High pressure liquid chromatography was developed in the mid-1970's and quickly improved with the development of column packing materials and the additional convenience of on-line detectors. In the late 1970's, new methods including reverse phase liquid chromatography allowed for improved separation between very similar compounds.
By the 1980's HPLC was commonly used for the separation of chemical compounds. New techniques improved separation, identification, purification and quantification far above the previous techniques. Computers and automation added to the convenience of HPLC. Improvements in type of columns and thus reproducibility were made as such terms as micro-column, affinity columns, and Fast HPLC began to immerge.
The past decade has seen a vast undertaking in the development of the micro-columns, and other specialized columns. The dimensions of the typical HPLC column are: XXX mm in length with an internal diameter between 3-5 mm. The usual diameter of micro-columns, or capillary columns, ranges from 3 µm to 200 µm. Fast HPLC utilizes a column that is shorter than the typical column, with a length of about 3 mm long, and they are packed with smaller particles.
Currently, one has the option of considering over x# types of columns for the separation of compounds, as well as a variety of detectors to interface with the HPLC in order to get optimal analysis of the compound. We hope this review will provide a reference which all levels of HPLC users will be able to find quick answers to their HPLC problems.
Although HPLC is widely considered to be a technique mainly for biotechnological, biomedical, and biochemical research as well as for the pharmaceutical industry, these fields currently comprise only about 50% of HPLC users. Currently HPLC is used by a variety of fields including cosmetics, energy, food, and environmental industries.

Chromatography:

Chromatography is a separations method that relies on differences in partitioning behavior between a flowing mobile phase and a stationary phase to separate the the components in a mixture.

A column (or other support for TLC, see below) holds the stationary phase and the mobile phase carries the sample through it. Sample components that partition strongly into the stationary phase spend a greater amount of time in the column and are separated from components that stay predominantly in the mobile phase and pass through the column faster.

As the components elute from the column they can be quantified by a detector and/or collected for further analysis. An analytical instrument can be combined with a separation method for on-line analysis.
Examples of such "hyphenated techniques" include gas and liquid

chromatography with mass spectrometry (GC-MS and LC-MS), Fouriertransform infrared spectroscopy (GC-FTIR), and diode-array UV-VIS absorption spectroscopy (HPLC-UV-VIS).

Specific chromatographic methods:

Gas chromatography (GC)
Applied to volatile organic compounds. The mobile phase is a gas and the stationary phase is usually a liquid on a solid support or sometimes a solid adsorbent.
High-performance liquid chromatography (HPLC)
A variation of liquid chromatography that utilizes high-pressure pumps to increase the efficiency of the separation.
Liquid chromatography (LC)
Used to separate analytes in solution including metal ions and organic compounds. The mobile phase is a solvent and the stationary phase is a liquid on a solid support, a solid, or an ion-exchange resin.
Size-exclusion chromatography (SEC)
Also called gel-permeation chromatography (GPC), the mobile phase is a solvent and the stationary phase is a packing of porous particles.
Thin-layer chromatography (TLC)
A simple and rapid method to monitor the extent of a reaction or to check the purity of organic compounds. The mobile phase is a solvent and the stationary phase is a solid adsorbent on a flat support.

SEPARATION MECHANISMS

A useful classification of the various LC techniques is based on the type of distribution (or equilibrium) that is responsible for the separation. The common interaction mechanisms encountered in LC are classified as adsorption, partition, ion-exchange, gel permeation or size exclusion, and chiral interaction. In practice, most LC separations are the result of mixed mechanisms. A brief description of the separation mechanisms is presented below.

Adsorption: When the stationary phase in HPLC is a solid, the type of equilibrium between this phase and the liquid mobile phase is termed 'adsorption'. All of the pioneering work in chromatography was based upon adsorption methods, in which the stationary phase is a finely divided polar solid that contains surface sites for retention of analytes. The composition of the mobile phase is the main variable that affects the partitioning of analytes. Silica and alumina are the only stationary phases used, the former being preferred for most applications. Applications of adsorption chromatography include the separation of relatively non-polar water-insoluble organic compounds. Because of the

polar nature of the stationary phase and the impact of subtle variations in mobile phase composition on the retention time, adsorption chromatography is very useful for the separation of isomers in a mixture.

Partition: The equilibrium between the mobile phase and a stationary phase comprising of either a liquid adsorbed on a solid or an organic species bonded to a solid is described as 'partition'. The predominant type of separation in HPLC today is based on partition using bonded stationary phases. Bonded stationary phases are prepared by reaction of organochlorosilane with the reactive hydroxyl groups on silica. The organic functional group is often a straight chain octyl (C-8) or octyldecyl (C-18); in some cases a polar functional group such as cyano, diol, or amino may be part of the siloxane structure. Two types of partition chromatography may be distinguished, based on the relative polarities of the phases.
When the stationary phase is polar and the mobile phase relatively less polar (n-hexane, ethyl ether, chloroform), this type of chromatography is referred to as 'normal-phase chromatography'. For this reason, the use of silica as the stationary phase (as in adsorption chromatography) is also considered to be a normal phase separation method.
When the mobile phase is more polar than the stationary phase (which may be a C-8 or C-18 bonded phase), this type of chromatography is called 'reversed-phase chromatography'. Reversed-phase chromatography separations are carried out using a polar aqueous-based mobile phase mixture that contains an organic polar solvent such as methanol or acetonitrile. Because of its versatility and wide range of applicability, reversed-phased chromatography is the most frequently used HPLC method. Applications include non-ionic compounds, polar compounds, and in certain cases ionic compounds.

Ion-exchange: Ion-exchange separations are carried out using a stationary phase that is an ion-exchange resin. Packing materials are based either on chemically modified silica or on styrene-divinylbenzene copolymers, onto which ionic side groups are introduced. Examples of the ionic groups include (a) sulfonic acid (strong cation exchanger), (b) carboxylic acid (weak cation exchanger), (c) quaternary ammonium groups (strong anion exchanger), and (d) tertiary amine group (weak anion exchanger). The most important parameters that govern the retention are the type of counter-ion, the ionic strength, pH of the mobile phase, and temperature. Ion chromatography is the term applied for the chromatographic separation of inorganic anions/cations, low molecular weight organic acids, drugs, serums, preservatives, vitamins, sugars, ionic chelates, and certain organometallic compounds.
The separation can be based on ion-exchange, ion-exclusion effects, or ion pairing. Conductivity detectors in ion chromatography provide universal and sensitive detection of charged species. The employment of

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some form of ion-suppression immediately after the analytical column eliminates the limitation of high background signal from the mobile phase in conductivity detection.

Size Exclusion: High molecular weight solutes (>10,000) are typically separated using size exclusion chromatography - gel filtration or gel permeation. In size-exclusion LC, the components of a mixture are separated according to their ability to penetrate into the pores of the stationary phase material. Packing materials used are wide-pore silica gel, polysaccharides, and synthetic polymers like polyacrylamide or styrene-divinylbenzene copolymer. In gel filtration the mobile phase is aqueous and the packing material is hydrophilic, while in gel permeation an organic mobile phase is used and the stationary phase is hydrophobic. Size-exclusion applications include the separation of large molecular weight biomolecules, and molecular weight distribution studies of large polymers and natural products. For a homologous series of oligomers, the retention time (volume) can be related to the logarithm of the molecular mass.

Chiral Interaction: Chiral compounds or enantiomers have identical molecular structures that are mirror images of each other. Rapid and accurate stereochemical resolution of enantiomers is a challenge in the field of pharmaceuticals and drug discovery. A chiral stationary phase contains one form of an enantiomeric compound immobilized on the surface of the support material. Typically, derivatives of optically active polysaccharides that are chemically bonded to silica form the packing material. A chiral separation is based on differing degrees of stereochemical interaction between the components of an enantiomeric sample mixture and the stationary phase.

METHOD DEVEOPMENT IN PARTITION CHROMATOGRAPHY

Successful chromatography requires a proper balance of the intermolecular forces between the solute, the mobile phase, and the stationary phase. Method development tends to be more complex in HPLC relative to GC because in the latter the mobile phase is inert and makes no contribution to the separation process. The important criteria to consider for method development are resolution, sensitivity, precision, accuracy, limit of detection, limit of quantitation, linearity, reproducibility, and time of analysis and robustness of the method. In all of these, the column quality plays an important role since the peak shape affects all criteria required for optimum separation. The factors that affect the column efficiency have already been described above. Column dimensions and particle size affect the speed of analysis, resolution, column backpressure, detection limit, and solvent consumption. HPLC methods have traditionally been developed using

columns measuring 10, 15 or 25 cm in length and 4.6 mm ID. Short columns of 5 cm or less in length and 1 or 2 mm ID are now available; when packed with particles of size 5 micron or less, very high efficiency columns are obtained. The advantages of using shorter columns are lower backpressures, dramatic solvent savings, greater sensitivity, reduced analysis time, and applicability to small sample quantities - all achieved without compromising resolution. Using these columns, gradient methods may be used to achieve very rapid analyses of samples that contain a wide polarity range of analytes. The future of reversed-phase HPLC method development will involve a significant increase in the use of use narrow-bore and micro-bore columns.

Often in choosing a column for partition chromatography, the polarity of the stationary phase is matched roughly to that of the analytes in the sample; a mobile phase of different polarity is used for elution. The analytes must be soluble in the mobile phase and the solvent must be compatible with the analytical method. As a general guide, use normal phase chromatography for the separation of polar compounds and reversed-phase chromatography for components that are in the moderately polar to non-polar range.

Normal phase chromatography commonly involves the use of silica, aminopropyl, diol, and cyanopropyl stationary phases. These columns may be used to separate polar compounds such as amines, anilines, nitroaromatics, phenols, and pesticides.

Isocratic elution in reversed-phase chromatography is typically accomplished using a mobile phase mixture of water and another solvent of lower eluting strength (acetonitrile, methanol). In cases where the time of analysis is compromised or when the resolution is poor, gradient elution using 2 or 3 different solvents is recommended.

The relative polarity of a solvent is a useful guide to solvent selection in partition chromatography. The relative polarities of the listed solvents may differ slightly depending on the literature source, since the scale used to measure polarity may be different. The following should suffice as a general reference for relative solvent polarity.

Fluoroalkanes (least polar)
Hexane
Isooctane
Carbon tetrachloride
Toluene
Diethyl ether (ether)
Chloroform
Methylene chloride
Tetrahydrofuran (THF)
Acetone
Ethyl acetate
Dioxane

Isopropanol
Ethanol
Acetic acid
Methanol
Acetonitrile
Water (most polar)

There is a strong dependence of the retention time on the mobile phase composition, and the retention parameter may be easily altered by variation of solvent polarity. This is the easiest way to improve chromatographic resolution of two overlapping species or to decrease overall separation time for components with widely differing retention values. A good starting point is a mixture of water and a polar organic solvent (methanol or acetonitrile). The effect of mobile phase polarity on elution time can be tested at a few different solvent proportions. If greater selectivity is required, a mobile phase comprising of 3-4 solvents may be used. Theoretical calculations have indicated that a mobile phase mixture of water, THF, methanol, and acetonitrile may be used to resolve most reversed-phase applications within a reasonable length of time.

The various analytes to be separated may also be arranged based on the polarities of their functional groups. A general guide to relative solute polarity going from non-polar to the most polar group is as follows:

Hydocarbons (least polar)
Ethers
Esters
Ketones
Aldehydes
Amides
Amines
Alcohols
Water (most polar)

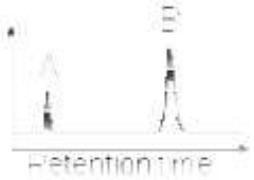
Unfortunately, theoretical predictions of mobile phase and stationary phase interactions with a given set of sample components are not always accurate, but they do help to narrow down the choices for method development. The separation scientist must usually perform a series of trial-and-error experiments with different mobile phase compositions until a satisfactory separation is achieved.

Basis of the separation process:

As explained in the introductory section, chromatographic separation process based on the difference in the surface interactions of the analyte and eluent molecules.

Let us consider a separation of a two component mixture dissolved in the eluent. Assume that component **A** has the same interaction with the adsorbent surface as an eluent, and component **B** has strong excessive interaction. Being injected into the column, these components will be

forced through by eluent flow. Molecules of the component **A** will interact with the adsorbent surface and retard on it by the same way as an eluent molecules. Thus, as an average result, component **A** will move through the column with the same speed as an eluent.
Molecules of the component **B** being adsorbed on the surface (due to their strong excessive interactions) will sit on it much longer. Thus, it will move through the column slower than the eluent flow.
Figure below represents the general shape of the chromatogram for this mixture.



Usually a relatively narrow band is injected (5 - 20 ul injection volume). During the run, the original chromatographic band will be spread due to the noneven flows around and inside the porous particles, slow adsorption kinetics, longitudinal diffusion, and other factors. These processes together produce so called band broadening of the chromatographic zone. In general, the longer the component retained on the column, the more broad its zone (peak on the chromatogram). Separation performance depend on both component retention and band broadening. Band broadening is, in general, a kinetic parameter, dependent on the adsorbent particle size, porosity, pore size, column size, shape, and packing performance. On the other hand, retention does not depend on the above mentioned parameters, but it reflects molecular surface interactions and depends on the total adsorbent surface.

High Performance Liquid Chromatography (HPLC) is one mode of chromatography; the most widely used analytical technique. Chromatographic processes can be defined as separation techniques involving mass-transfer between stationary and mobile phases.
HPLC utilizes a liquid mobile phase to separate the components of a mixture. These components (or analytes) are first dissolved in a solvent, and then forced to flow through a chromatographic column under a high pressure. In the column, the mixture is resolved into its components. The amount of resolution is important, and is dependent upon the extent of interaction between the solute components and the stationary phase. The stationary phase is defined as the immobile packing material in the column. The interaction of the solute with mobile and stationary phases can be manipulated through different choices of both solvents and stationary phases. As a result, HPLC acquires a high degree of versatility not found in other chromatographic systems and it has the ability to easily separate a wide variety of chemical mixtures.

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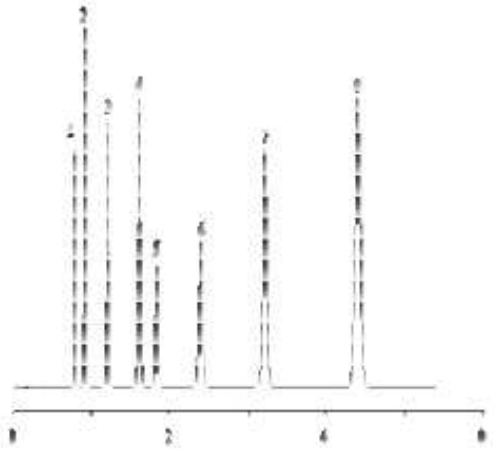

Fast and high-efficient separation of some aromatics. Hypersil-C8 (100x2) 3 mm, 60% MeOH in Water, 1.5 ml/min., 1 - Benzamide, 2 - Benzil Alcohol, 3 - Acetophenone, 4 - Methyl Benzoate, 5 - Phenetole, 6 - Naphthalene, 7 - Benzophenone 8 - Biphenyl.

Liquid Chromatography was first discovered in 1903 by M.S.Tswett, who used a chalk column to separate the pigments of green leaves. Only in 1960's the more and more emphasis was placed on the development

Some Basic HPLC Theory:

The following is a very simplified and general overview of HPLC theory. For another excellent source of background reading, try the on-line HPLC textbook written by Prof. Yuri Kazakevich and Prof. H.M.McNair of Seton Hall University. You can access it by the link in his homepage for Analytical Chemistry.
The goal of any chemical analysis is to separate a sample (blood, urine, water from a well, etc.) into its individual components in order to evaluate each component free from interference from the other components. Chromatography is a general technique that separates a mixture into its individual components. Those components are referred to as analytes--the chemical compounds of interest to the analyst. Chromatography is then coupled with a detection system that can characterize each type of analyte appropriately. High performance liquid chromatography (HPLC) is one such method. It is used to analyze liquid samples or the liquid extract of a sample.
The fundamental basis for HPLC consists of passing a sample (analyte mixture) in a high pressure solvent (called the mobile phase) through a steel tube (called a column) packed with sorbents (called the stationary phase). As the analytes pass through the column they interact between the two phases--mobile and stationary--at different rates. The difference in rates is primarily due to different polarities for the analytes. The analytes that have the least amount of interaction with the stationary

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phase or the most amount of interaction with the mobile phase will exit the column faster. Repeated interactions along the length of the column effect a separation of the analytes. Various mixtures of analytes can be analyzed by changing the polarities of the stationary phase and the mobile phase.

The many types of columns on the market today can help refine your HPLC method. (Drop by SRIF to look at our catalogs!) Choosing the right column is essential in obtaining good HPLC results. Obviously, the polarity of the stationary phase can be altered significantly. The stationary phase is typically bonded to a support phase, usually consisting of porous beads. The pore sizes can be varied to allow certain sized analytes to pass through at different rates. Furthermore, the dimensions of the column can be varied to allow different sample sizes to be analyzed.

Changes in the polarity of the mobile phase is another variable that can effect the efficiency of your HPLC separation. The mobile phase polarity is generally the opposite of your stationary phase. Our multisolvent delivery system allows the polarity of the mobile phase to be changed during the course of the HPLC run. The rate at which the polarity is changed defines the "gradient." This gradient technique helps to further seperate mixtures of variously polar analytes.

As the analytes exit the column, they can be detected by various means. Refractive index, electrochemical, or ultraviolet-absorbance changes in the mobile phase can indicate the presence of an analyte. The amount of analyte leaving the column will determine the intensity of the signal produced in the detector. The detector measures a signal peak as each analyte leaves the column. By comparing the time it takes for the peak to show up (called the retention time) with the retention times for a mixture of known compounds, the components of unknown sample mixtures can be identified. By measuring the signal intensity (response) and comparing it to the response of a known amount of that particular analyte, the amount of analyte in the mixture.

Our most popular detector at SRIF is the photodiode array detector. The PDA can continuously scan various wavelengths of the UV spectrum. As an analyte peak is detected, the UV spectrum is recorded. This 3rd dimension is useful in identifying compounds and determining if the peak consists of an individual analyte or a mixture of analytes that wasn't effectively separated.

All aspects of the SRIF HPLC system are controlled by a PC using Millenium Windows-based software. This software controls an autosampler which injects samples at proper intervals. It controls the mobile phase gradient, the solvent flow rate, mobile phase pressure, and it measures the signals produced by the detector. The results of your sample run can then be interpreted and printed in a variety of report formats.

See our section on qualtitative analysis for more information!

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Principle:

It is known that the resolving power of a chromatographic column increases with column length and the number of theoretical plates per unit length, although there are limits to the length of a column due to the problem of peak broadening. As the number of theoretical plates is related to the surface area of the stationary phase it follows that the smaller the particle size of the stationary phase, the better the resolution.Unfortunetly, the smaller the particle size, the greater the resistance to eluant flow. All of the forms of column chromatography so far discussed rely on gravity or lower pressure pumping systems for the supply of eluant to the column. The consequences of this is that the flow rates achieved are relatively low and this gives greater time for band broadening by simple diffusion phenomena. The use of faster flow rates is not possible because it creates a back-pressure which is sufficient to damage the matrix structure of the stationary phase, thereby actually reducing eluant flow and imparting resolution. In the past decade there has been a dramatic development in column chromatography technology which has resulted in the availability of new particle size stationary phase which can with stand these pressures and of pumping systems which can give reliable flow rates. These developments, which have occurred in adsorption, partion, ion-exchnage, exclusion and affinity chromatography, have resulted in faster and better resolution and explain why HPLC has emerged as the most popular, powerful and versatile form of chromatography.

Originally , HPLC was referred to as high pressure liquid chromatography but nowadays the term high performance liquid chromatography is preferred since it better describes the characteristics of the chromatography and avoids creating the impression that high pressure are an inevitable pre-requisite for high performance. This is now known not to be the case and the term *medium pressure liquid chromatography (MPLC)* has been coined for some separations.

The new technology in stationary phases has been applied to thin-layer chromatography giving rise to high performance thin layer chromatography (HPTLC). In general, however, the impact of this new technology has not been quite so great as it has been in column chromatography.

All factors affecting separations on liquid column chromatography apply to this technique also e.g. plate height, sample distribution between the stationary and liquid phases, and the selection of the stationary and liquid phases. Various methods of development of the chromatography (elution, gradient elution etc.) can be used with this technique.

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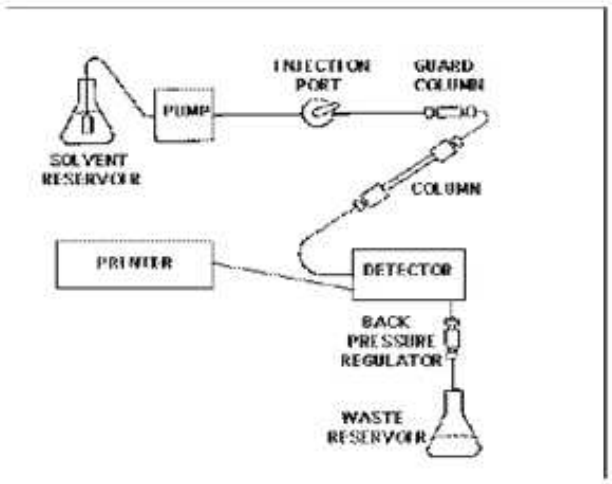
Instrumentation:

Solvents must be degassed to eliminate formation of bubbles. The pumps provide a steady high pressure with no pulsating, and can be programmed to vary the composition of the solvent during the course of the separation. Detectors rely on a change in refractive index, UV-VIS absorption, or fluorescence after excitation with a suitable wavelength. The different types of HPLC columns are described in a separate document.

A schematic diagram of a typical HPLC unit is shown in Figure. The system consists of:

1. A solvent reservoir and mixing system.
2. A high pressure pump.
3. A sample inlet pump.
4. A column.
5. A detector and recording unit.



The appropriate solvents (mobile liquid phase) from the reservoirs are allowed to enter the mixing chamber where a homogenous mixture is obtained.

A pump capable of maintaining high pressure draws the solvent from the mixing chambers and pushes it through the column.

The sample is injected through a port into the high pressure liquid carrier stream between the pumps and the column. The separation takes place on the column which vary from 50-100 cm in length and 2-3 mm in i.d. Typical flow rates are 1-2 ml/min with pressure up to several thousands psi. The column effluent passes through a non-destructive detector where a property such a ultraviolet absorbance, refractive index or molecular fluorescence is monitered, amplified and recorded as a typical detector response vs retention time chromatography. The effluent

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may be either discarded, recyled or saved for the further studies in a fraction collector which is synchronized with the detector.

Apparatus and Materials:

The Column:

The columns used for HPLC are generally made of stainless steel and are manufactured so that they can withstand pressures of 5.5 X 10^7 Pa (8000 p.s.i).Straight columns of 20 to 50 cm in length and 1 to 4 mm in diameter are generally used thought smaller capillary columns are available. The best columns are precision bored with an internal mirror finish which allows efficient packing of the column. Porous plugs of stainless steel or Teflon are used in the ends of the columns to retain the packing materials. The plugs must be homogenous to ensure uniform flow of solvents through the column. It is important in some separation involving liquid partition and ion-exchange that the column temperature is thermostatically controlled during the analysis.

Column Packing: Three forms of column packing materials are available based on a rigid solid structure. These are:

(i) *Microporous supports*: Where microporus ramify through the particles which are generally 5 to 10 µm in diameter.

(ii) *Pellicular (Superficial porous)*: supports where porous particle are coated onto an inert solid core such as a glass bead of about 40 µm in diameter.

(iii) *Bonded phases:* where the stationary phase is chemically bonded onto an inert support.

Column Efficiency:

Column efficiency refers to the performance of the stationary phase to accomplish particular separations. This entails how well the column is packed and its kinetic performance (Bidlingmeyer, 1984). The efficiency of a column can be measured by several methods which may or may not be affected by chromatographic anomalies, such as "tailing" or appearance of a "front." This is important because many chromatographic peaks do not appear in the preferred shape of normal Gaussian distribution. For this reason efficiency can be an enigmatic value since manufacturers may use different methods in determining the efficiency of their columns (Bidlingmeyer, 1984).
Calculation of column efficiency value:

All the following methods use this formula that measures N, or number of theoretical plates:

$$N = a \frac{t_r^2}{W^2}$$

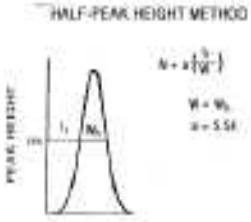
a = constant dependent on height where peak width measured

t_r = retention time

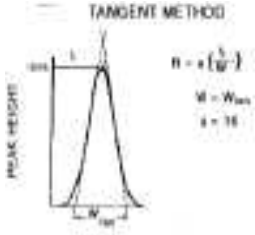
W = peak width

Inflection Method- Calculation is based upon inflection point which appears at 60.7% of the peak height for a normal Gaussian peak. At this point the width of the peak is equivalent to two standard deviation units. Any asymmetrical aspect of a peak should not affect this calculation since the width is measured above the anomalous occurance (i.e., tailing or fronting).

Half-peak height Method- As the name suggests, the measurement is based upon the width at 50% of peak height. For the same reason as inflection method, this measurement is not affected by asymmetry; however, this method is more reproducible from person to person since width at 50% peak height is less prone to be varied.



Tangent Method- Tangent lines are drawn on each side of the peak and the width is the distance between the two lines at the base of the peak. Therefore, it is more sensitive to asymmetrical peaks and variation in efficiency values is usually seen from user to user.



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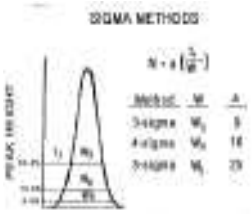
Sigma Methods- These methods measure peak width at decreasing levels of peak height. Thus, the three sigma method measures width at 32.4% of peak height, the four sigma method measures at 13.4%, and the five sigma method measures at 4.4%. The five sigma method is most sensitive to asymmetry because the width is measured at the lowest point.



Height/Area Method- This method utilizes the fact that the area of a peak is a function of its height and standard deviation. To determine efficiency, values for peak height and area are used in a different formula:

$$N = \frac{2\pi(ht_r)^2}{A^2}$$

h = peak height
A = area

A computer is usually necessary to use this method in order to calculate the area and height.

Moment Method- This method entails disregarding peak shape and expresses parameters of the peak in statistical moments. The zero moment, μ0, is the peak area. The first moment, μ1, is the mean and occurs at the center of the peak (which is the maximum peak height in normal Gaussian peaks). The second moment, μ2, is the variance of the peak. This is a detailed method where appropriate data systems are needed. For a more detailed discussion, a reference is provided.

These methods were evaluated by computer simulation based on efficiency values obtained on a series of synthetically modified Gaussian peaks (i.e., increasing the 'tailing') and compared to the actual value based on the moment method (which was determined to be the most accurate). Briefly, the results were as follows:

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CALCULATION METHOD—ACCURACY (Bidlingmeyer, 1984).

Inflection	Low
Half-peak height	Low
Tangent	Low
Height: Area ratio	Medium
Four sigma	Medium
Five sigma	High
Asymmetry	High

There are various columns that are secondary to the separating column or stationary phase. They are: Guard, Derivatizing, Capillary, Fast, and Preparatory Columns.

Guard Columns are placed anterior to the separating column. This serves as a protective factor that prolongs the life and usefulness of the separation column. They are dependable columns designed to filter or remove: 1) particles that clog the separation column; 2) compounds and ions that could ultimately cause "baseline drift", decreased resolution, decreased sensitivity, and create false peaks; 3) compounds that may cause precipitation upon contact with the stationary or mobile phase; and 4) compounds that might co-elute and cause extraneous peaks and interfere with detection and/or quantification. These columns must be changed on a regular basis in order to optimize their protective function. Size of the packing varies with the type of protection needed.

Derivatizing Columns- Pre- or post-primary column derivatization can be an important aspect of the sample analysis. Reducing or altering the parent compound to a chemically related daughter molecule or fragment elicits potentially tangible data which may complement other results or prior analysis. In few cases, the derivatization step can serve to cause data to become questionable, which is one reason why HPLC was advantageous over gas chromatography, or GC. Because GC requires volatile, thermally stabile, or nonpolar analytes, derivatization was usually required for those samples which did not contain these properties. Acetylation, silylation, or concentrated acid hydrolysis are a few derivatization techniques.

Capillary Columns- Advances in HPLC led to smaller analytical columns. Also known as microcolumns, capillary columns have a diameter much less than a millimeter and there are three types: open-tubular, partially packed, and tightly packed. They allow the user to work with nanoliter sample volumes, decreased flow rate, and decreased solvent volume usage which may lead to cost effectiveness. However, most conditions and instrumentation must be miniaturized, flow rate can be difficult to

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reproduce, gradient elution is not as efficient, and care must be taken when loading minute sample volumes.

Microbore and small-bore columns are also used for analytical and small volumes assays. A typical diameter for a small-bore column is 1-2 mm. Like capillary columns, instruments must usually be modified to accommodate these smaller capacity columns (i.e., decreased flow rate). However, besides the advantage of smaller sample and mobile phase volume, there is a noted increase in mass sensitivity without significant loss in resolution.--*Capillary Electrophoresis*.

Fast Columns- One of the primary reasons for using these columns is to obtain improved sample throughput (amount of compound per unit time). For many columns, increasing the flow or migration rate through the stationary phase will adversely affect the resolution and separation. Therefore, fast columns are designed to decrease time of the chromatographic analysis without forsaking significant deviations in results. These columns have the same internal diameter but much shorter length than most other columns, and they are packed with smaller particles that are typically 3 µm in diameter. Advantages include increased sensitivity, decreased analysis time, decreased mobile phase usage, and increased reproducibility.

Preparatory Columns- These columns are utilized when the objective is to prepare bulk (milligrams) of sample for laboratory preparatory applications. A preparatory column usually has a large column diameter which is designed to facilitate large volume injections into the HPLC system.

Accessories important to mention are the **back-pressure regulator** and the **fraction collector**. The back-pressure regulator is placed immediately posterior to the HPLC detector. It is designed to apply constant pressure to the detector outlet which prevents the formation of air bubbles within the system. This, in turn, improves chromatographic baseline stability. It is usually devised to operate regardless of flow rate, mobile phase, or viscosity. The fraction collector is an automated device that collects uniform increments of the HPLC output. Vials are placed in the carousel and the user programs the time interval in which the machine is to collect each fraction. Each vial contains mobile phase and sample fractions at the corresponding time of elution. Packings for columns are diverse since there are many modes of HPLC. They are available in different sizes, diameters, pore sizes, or they can have special materials attached (such as an antigen or antibody for immunoaffinity chromatography). Packings available range from those needed for specific applications (affinity, immunoaffinity, chiral, biological, etc.) to those for all-purpose applications. The packings are attached to the internal

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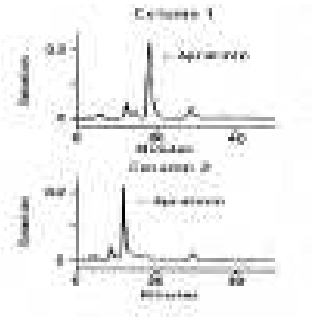
column hull by resins or supports, which include oxides, polymers, carbon, hydroxyapatite beads, agarose, or silica, the most common type.

COLUMN WARNING:

Essential to any scientist's experimentation is reproducibility of results in order to further support or reject data or hypotheses. For a chromatographer, a separation that has been accomplished or optimized must be able to be repeated in subsequent HPLC analyses. Any anomaly that occurs can usually be attributed to either 1) the age of the column, 2) the chromatographer or 3) the manufacturer. Certain measures can limit the effects of age and the user. For example, proper column storage with the appropriate liquid interface, such as methanol for certain reverse-phase columns, with each end capped tightly assures no drying of the resin and increases "life" of the column. Use of a guard column can prevent the user from potentially contaminating or introducing unwanted particles into the column. Identifying the manufacturer as the chief culprit is many times unwarranted among peers or supervisors. However, there are certain instances where the irregularities can be aimed at the manufacturers. In these cases, column-to- column or batch-to-batch variations in the commercially available products must be addressed. Recently, a disparity in results originated from separation of proteins using reverse-phase HPLC. Aprotinin, a 58-amino acid polypeptide, was the protein separated using a C4 column (Column 1) at pH 5 with sodium phosphate/ammonium sulfate buffer and an acetonitrile gradient. A new column (Column 2) from the same batch was acquired from the manufacturer and the resultant separation was characterized by different retention time, "tailing", and some loss of efficiency:



The manufacturer admitted to changing the column packing procedure (using distilled water instead of tap water) upon inquiry. A similar study was performed using a C4 column produced by another manufacturer. In this case, biosynthetic human growth hormone (bhGH), a 191-amino acid protein, and TS-bhGH, a derivative of bhGH containing a tri-sulfide bridge (instead of a di-sulfide bridge), were separated at neutral pH with high resolution and efficiency. However, when another column (Column

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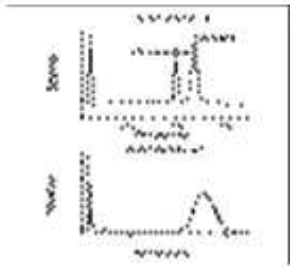
was purchased and tested, the separation of hGH and bhGH was totally absent:



The company had no explanation for the occurance. However, it was determined that the original company had been taken over by another company since the first column was acquired, and the parent company could not trace the difference in batches. A scientist, more than any other user, should not assume a simplified situation where consistent results will be attained from the same model unless proven. In the case of chromatography, stationary phases should constantly be tested, calibrated, or analyzed. By no means should unwanted results be immediately attributed to faults of the manufacturer. On the other hand, the manufacturer should not be immune to reproach regarding divergent columns. In either case, the onus is placed on the user.

Mobile Phase:

The mobile phase in HPLC refers to the solvent being continuously applied to the column, or stationary phase. The mobile phase acts as a carrier for the sample solution. A sample solution is injected into the mobile phase of an assay through the injector port. As a sample solution flows through a column with the mobile phase, the components of that solution migrate according to the non-covalent interactions of the compound with the column. The chemical interactions of the mobile phase and sample, with the column, determine the degree of migration and separation of components contained in the sample. For example, those samples which have stronger interactions with the mobile phase than with the stationary phase will elute from the column faster and thus have a shorter retention time, while the reverse is also true. The mobile phase can be altered in order to manipulate the interactions of the sample and the stationary phase. There are several types of mobile phases, these include: Isocratic, gradient, and polytyptic.
In isocratic elution compounds are eluted using constant mobile phase composition. The separation of compounds can be described using several equations:

EQUATIONS FOR ISOCRATIC ELUTION	
RETENTION TIME (s)	(1) $t_R = t_0 k' + t_0$
BANDWIDTH (ml)	(2) $\sigma = V_m(1 + k')N^{-1/2}$
RESOLUTION ($\Delta t_R/4\sigma$ or $\Delta t_g/4\sigma_g$)	(3) $R_S = [(1/4)(\alpha - 1)N^{1/2}][k'/(1 + k')]$
CAPACITY FACTOR	(4) $k' = (t_R - t_0)/t_0$

<> (Snyder, 1983)

All compounds begin migration through the column at onset. However, each migrates at a different rate, resulting in faster or slower elution rate. This type of elution is both simple and inexpensive, but resolution of some compounds is questionable and elution may not be obtained in a reasonable amount of time <>(Snyder, 1983).

In gradient elution different compounds are eluted by increasing the strength of the organic solvent. The sample is injected while a weaker mobile phase is being applied to the system. The strength of the mobile phase is later increased in increments by raising the organic solvent fraction, which subsequently results in elution of retained components. This is usually done in a stepwise or linear fashion. There are several equations that describe gradient elution:

EQUATIONS FOR GRADIENT ELUTION	
RETENTION TIME (s)	(1) $t_g = t_0\bar{k}\ \log(2.3k_0/\bar{k})$
BANDWIDTH (ml)	(2) $\sigma_g = V_m(1 + \bar{k})N^{-1/2}/2$
RESOLUTION ($\Delta t_R/4\sigma$ or $\Delta t_g/4\sigma_g$)	(3) $R_S = [(1/4)(\alpha - 1)N^{1/2}][\bar{k}/(1 + \bar{k})]$
CAPACITY FACTOR	(4) $\bar{k} = t_G/\Delta\phi S t_0$ $= t_G F/\Delta\phi S\ V_m$

<> (Snyder, 1983)At the onset of sample introduction, the compounds are initially retained at the inlet of the column. As the solute capacity, or k', for the compound decreases, the compound begins to migrate through the stationary phase. Each of the other compounds in the sample subsequently migrate as their k' values decrease. Compared with isocratic elution, resolution and separation are improved, and bandwidths are nearly equal:

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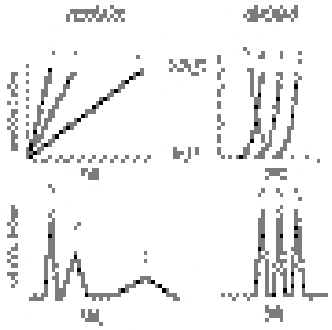

Isocratic vs. Gradient Elution:

The **Knox** equation describes column efficiency or plate number N in relation to certain experimental conditions, such as column length, column diameter, temperature, flow-rate, molecular weight, etc. (Equation). Plate number N is equal to plate height value H divided by particle diameter (dp). Plate height value H is in turn equal to column length L divided by N. Two of the Knox coefficients, B and C, depend on k' and size of the compound. In the equations above, k' in the isocratic equations is replaced with average k' in the gradient equations. In fact, this is the only difference in the bandwidth and resolution equations between the two. Thus, separation and height of the peak are dictated by the exact same conditions for both isocratic and gradient elution <>(Snyder, 1983).
>From the equation for capacity factor in gradient elution, it can be seen that average k' value depends on flow-rate, gradient time, and column dead volume. This differs in isocratic elution where k' is not dependent on time of separation, flow- rate, or column dimensions.
A special feature in gradient elution is **linear-solvent strength (LSS) gradients**. These give approximately equal values of average k' for samples eluting at different times during separation. This is the reason why gradient elution can yield constant bandwidths for different compounds and equal resolution for pairs of compounds which have similar alpha or separation factor values.

Polytyptic Mobile Phase, sometimes referred to as mixed-mode chromatography, is a versatile method in which several types of chromatographic techniques, or modes, can be employed using the same column. These columns contain rigid macro porous hydrophobic resins covalently bonded to a hydrophilic organic layer. SEC, IEC, hydrophobic or affinity chromatography are some of the methods that may be utilized. By changing the mobile phase, the mode of separation is thereby changed which allows the chromatographer to achieve the desired selectivity in the separations.

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Stationary Phase:

The stationary phase in HPLC refers to the solid support contained within the column over which the mobile phase continuously flows. The sample solution is injected into the mobile phase of the assay through the injector port. As the sample solution flows with the mobile phase through the stationary phase, the components of that solution will migrate according to the non-covalent interactions of the compounds with the stationary phase. The chemical interactions of the stationary phase and the sample with the mobile phase, determines the degree of migration and separation of the components contained in the sample. For example, those samples which have stronger interactions with the stationary phase than with the mobile phase will elute from the column less quickly, and thus have a longer retention time, while the reverse is also true. Columns containing various types of stationary phases are commercially available. Some of the more common stationary phases include: Liquid-Liquid, Liquid-Solid (Adsorption), Size Exclusion, Normal Phase, Reverse Phase, Ion Exchange, and Affinity.
Liquid-Solid operates on the basis of polarity. Compounds that possess functional groups cabable of strong hydrogen bonding will adhere more tightly to the stationary phase than less polar compoounds. Thus, less polar compounds will elute from the column faster than compounds that are highly polar.

Liquid-Liquid operates on the same basis as liquid-solid. However, this technique is better suited for samples of medium polarity that are soluble in weakly polar to polar organic solvents. The separation of non-electrolytes is achieved by matching the polarities of the sample and the stationary phase and using a mobile phase which possesses a markedly different polarity.

Size-Exclusion operates on the basis of the molecular size of compounds being analyzed. The stationary phase consists of porous beads. The larger compounds will be excluded from the interior of the bead and thus will elute first. The smaller compounds will be allowed to enter the beads and will elute according to their ability to exit from the same sized pores they were internalized through. The column can be either silica or non-silica based. However, there are some size-exclusion that are weakly anionic and slightly hydrophobic which give rise to non-ideal size-exclusion behavior.

Normal Phase operates on the basis of hydrophilicity and lipophilicity by using a polar stationary phase and a less polar mobile phase. Thus hydrophobic compounds elute more quickly than do hydrophilic compounds.

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Reverse Phase operates on the basis of hydrophilicity and lipophilicity. The stationary phase consists of silica based packings with n-alkyl chains covalently bound. For example, C-8 signifies an octyl chain and C-18 an octadecyl ligand in the matrix. The more hydrophobic the matrix on each ligand, the greater is the tendancy of the column to retain hydrophobic moieties. Thus hydrophilic compounds elute more quickly than do hydrophobic compounds.

Ion-Exchange operates on the basis of selective exchange of ions in the sample with counterions in the stationary phase. IE is performed with columns containing charge-bearing functional groups attached to a polymer matrix. The functional ions are permanently bonded to the column and each has a counterion attached. The sample is retained by replacing the counterions of the stationary phase with its own ions. The sample is eluted from the column by changing the properties of the mobile phase do that the mobile phase will now displace the sample ions from the stationary phase, (ie. changing the pH).
Affinity operates by using immobilized biochemicals that have a specific affinity to the compound of interest. Separation occurs as the mobile phase and sample pass over the stationary phase. The sample compound or compounds of interest are retained as the rest of the impurities and mobile phase pass through. The compounds are then eluted by changing the mobile phase conditions.

Injectors for HPLC:

Samples are injected into the HPLC via an injection port. The injection port of an HPLC commonly consists of an injection valve and the sample loop. The sample is typically dissolved in the mobile phase before injection into the sample loop. The sample is then drawn into a syringe and injected into the loop via the injection valve. A rotation of the valve rotor closes the valve and opens the loop in order to inject the sample into the stream of the mobile phase. Loop volumes can range between 10 µl to over 500 µl. In modern HPLC systems, the sample injection is typically automated.
Stopped-flow Injection is a method whereby the pump is turned off allowing the injection port to attain atmospheric pressure. The syringe containing the sample is then injected into the valve in the usual manner, and the pump is turned on. For syringe type and reciprocation pumps, flow in the column can be brought to zero and rapidly resumed by diverting the mobile phase by means of a three-way valve placed in front of the injector. This method can be used up to very high pressures.

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HPLC Pumps:

There are several types of pumps available for use with HPLC analysis, they are: Reciprocating Piston Pumps, Syringe Type Pumps, and Constant Pressure Pumps.
Reciprocating Piston Pumps consist of a small motor driven piston which moves rapidly back and forth in a hydraulic chamber that may vary from 35-400 µL in volume. On the back stroke, the separation column valve is closed, and the piston pulls in solvent from the mobile phase reservoir. On the forward stroke, the pump pushes solvent out to the column from the reservoir. A wide range of flow rates can be attained by altering the piston stroke volume during each cycle, or by altering the stroke frequency. Dual and triple head pumps consist of identical piston-chamber units which operate at 180 or 120 degrees out of phase. This type of pump system is significantly smoother because one pump is filling while the other is in the delivery cycle.
Syringe Type Pumps are most suitable for small bore columns because this pump delivers only a finite volume of mobile phase before it has to be refilled. These pumps have a volume between 250 to 500 mL. The pump operates by a motorized lead screw that delivers mobile phase to the column at a constant rate. The rate of solvent delivery is controlled by changing the voltage on the motor.
In Constant Pressure Pumps the mobile phase is driven through the column with the use of pressure from a gas cylinder. A low-pressure gas source is needed to generate high liquid pressures. The valving arrangement allows the rapid refill of the solvent chamber whose capacity is about 70mL. This provides continuous mobile phase flow rates.

Detectors and Detection Limits:

The **detector** for an HPLC is the component that emits a response due to the eluting sample compound and subsequently signals a peak on the chromatogram. It is positioned immediately posterior to the stationary phase in order to detect the compounds as they elute from the column. The bandwidth and height of the peaks may usually be adjusted using the coarse and fine tuning controls, and the detection and sensitivity parameters may also be controlled (in most cases). There are many types of detectors that can be used with HPLC. Some of the more common detectors include: Refractive Index (RI), Ultra-Violet (UV), Fluorescent, Radiochemical, Electrochemical, Near-Infra Red (Near-IR), Mass Spectroscopy (MS), Nuclear Magnetic Resonance (NMR), and Light Scattering (LS).

Refractive Index (RI) detectors measure the ability of sample molecules to bend or refract light. This property for each molecule or compound is

called its refractive index. For most RI detectors, light proceeds through a bi-modular flow-cell to a photodetector. One channel of the flow-cell directs the mobile phase passing through the column while the other directs only the mobile phase. Detection occurs when the light is bent due to samples eluting from the column, and this is read as a disparity between the two channels.

Ultra-Violet (UV) detectors measure the ability of a sample to absorb light. This can be accomplished at one or several wavelengths:

A) *Fixed Wavelength* measures at one wavelength, usually 254 nm

B) *Variable Wavelength* measures at one wavelength at a time, but can detect over a wide range of wavelenths

C) *Diode Array* measures a spectrum of wavelengths simultaneously

UV detectors have a sensitivity to approximately 10^{-8} or 10^{-9} gm/ml.

Fluorescent detectors measure the ability of a compound to absorb then re-emit light at given wavelengths. Each compound has a characteristic fluorescence. The excitation source passes through the flow-cell to a photodetector while a monochromatic measures the emission wavelengths.
Has sensitivity limit of 10^{-9} to 10^{-11} gm/ml.

Radiochemical detection involves the use of radio labeled material, usually tritium ($_3H$) or carbon-14 ($_{14}C$). It operates by detection of fluorescence associated with beta-particle ionization, and it is most popular in metabolite research. Two detector types:

A) *Homogeneous*- Where addition of scintillation fluid to column effluent causes fluorescence.

B) *Heterogeneous*- Where lithium silicate and fluorescence caused by beta-particle emission interact with the detector cell.

Has sensitivity limit up to 10_{-9} to 10^{-10} gm/ml.

Electrochemical detectors measure compounds that undergo oxidation or reduction reactions. Usually accomplished by measuring gain or loss of electrons from migrating samples as they pass between electrodes at a given difference in electrical potential.
Has sensitivity of 10^{-12} to 10^{-13} gm/ml

Mass Spectroscopy (MS) Detectors- The sample compound or molecule is ionized, it is passed through a mass analyzer, and the ion current is detected. There are various methods for ionization:

A) *Electron Impact (EI)*- An electron current or beam created under high electric potential is used to ionize the sample migrating off the column.

B) *Chemical Ionization*- A less aggresive method which utilizes ionized gas to remove electrons from the compounds eluting from the column.

C) *Fast Atom Bombarbment (FAB)*- Xenon atoms are propelled at high speed in order to ionize the eluents from the column.

Has detection limit of 10^{-8} to 10^{-10} gm/ml.

Nuclear Magnetic Resonance (NMR) Detectors- Certain nuclei with odd-numbered masses, including H and ^{13}C, spin about an axis in a random fashion. However, when placed between poles of a strong magnet, the spins are aligned either parallel or anti-parallel to the magnetic field, with the parallel orientation favored since it is slightly lower in energy. The nuclei are then irradiated with electromagnetic radiation which is absorbed and places the parallel nuclei into a higher energy state; consequently, they are now in "resonance" with the radiation. Each H or C will produce different spectra depending on their location and adjacent molecules, or elements in the compound, because all nuclei in molecules are surrounded by electron clouds which change the encompassing magnetic field and thereby alter the absorption frequency.

Light-Scattering (LS) Detectors- When a source emits a parallel beam of light which strikes particles in solution, some light is reflected, absorbed, transmitted, or scattered. Two forms of LS detection may be used to measure the two latter occurrences:

Nephelometry- This is defined as the measurement of light scattered by a particulate solution. This method enables the detection of the portion of light scattered at a multitude of angles. The sensitivity depends on the absence of background light or scatter since the detection occurs at a black or null background.

Turbidimetry- This is defined as the measure of the reduction of light transmitted due to particles in solution. It measures the light scatter as a decrease in the light that is transmitted through the particulate solution. Therefore, it quantifies the residual light transmitted. Sensitivity of this method depends on the sensitivity of the machine employed, which can range from a simple spectrophotometer to a sophisticated discrete analyzer. Thus, the measurement of a decrease in transmitted light from a large signal of transmitted light is limited to the photometric accuracy and limitations of the instrument employed.

Near-Infrared Detectors- Operates by scanning compounds in a spectrum from 700 to 1100 nm. Stretching and bending vibrations of particular chemical bonds in each molecule are detected at certain

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wavelengths. This is a fast growing method which offers several advantages: speed (sometimes less than 1 second), simplicity of preparation of sample, multiple analyses from single spectrum, and no consumption of the sample.

Applications for HPLC

Preparative HPLC refers to the process of isolation and purification of compounds. Important is the degree of solute purity and the throughput, which is the amount of compound produced per unit time. This differs from analytical HPLC, where the focus is to obtain information about the sample compound. The information that can be obtained includes identification, quantification, and resolution of a compound.

Chemical Separations can be accomplished using HPLC by utilizing the fact that certain compounds have different migration rates given a particular column and mobile phase. Thus, the chromatographer can separate compounds (more on chiral separations) from each other using HPLC; the extent or degree of separation is mostly determined by the choice of stationary phase and mobile phase.

Purification refers to the process of separating or extracting the target compound from other (possibly structurally related) compounds or contaminants. Each compound should have a characteristic peak under certain chromatographic conditions. Depending on what needs to be separated and how closely related the samples are, the chromatographer may choose the conditions, such as the proper mobile phase, to allow adequate separation in order to collect or extract the desired compound as it elutes from the stationary phase. The migration of the compounds and contaminants through the column need to differ enough so that the pure desired compound can be collected or extracted without incurring any other undesired compound.

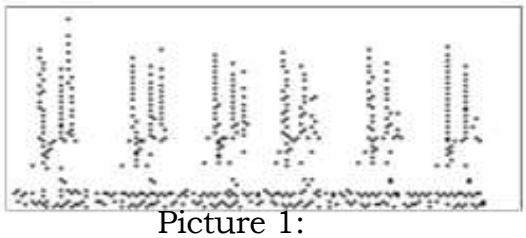
HPLC of Proteins and Polynucleotides.

Identification of compounds by HPLC is a crucial part of any HPLC assay. In order to identify any compound by HPLC a detector must first be selected. Once the detector is selected and is set to optimal detection settings, a separation assay must be developed. The parameters of this assay should be such that a clean peak of the known sample is observed from the chromatograph. The identifying peak should have a reasonable retention time and should be well separated from extraneous peaks at the detection levels which the assay will be performed. To alter the retention time of a compound, several parameters can be manipulated. The first is the choice of column, another is the choice of mobile phase,

and last is the choice in flow rate. All of these topics are reviewed in detail in this document.

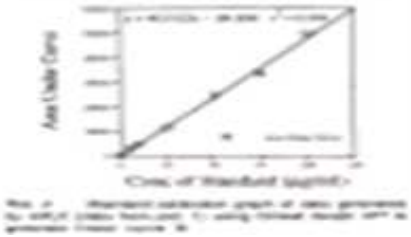
Identifying a compound by HPLC is accomplished by researching the literature and by trial and error. A sample of a known compound must be utilized in order to assure identification of the unknown compound. Identification of compounds can be assured by combining two or more detection methods.

Quantification of compounds by HPLC is the process of determining the unknown concentration of a compound in a known solution. It involves injecting a series of known concentrations of the standard compound solution onto the HPLC for detection. The chromatograph of these known concentrations will give a series of peaks that correlate to the concentration of the compound injected.



Picture 1:

Using the area of a triangle equation (A=1/2b x h) to calculate the area under each peak, a set of data is generated to develop a calibration curve. This is done by graphing peak area vs. the concentration of the sample solution. Most graphs can be generated using a computer software program such as Excel or Cricketgraph. From this graphing software, a best-fit line can be derived, and the equation of that line can be determined. This equation of a line, y=mx + b, generated by the data, is the calibration curve equation.



Picture 2:

The equation of the line is then used in the following manner: A scientist injects a sample of unknown concentration x (x-axis of calibration curve) onto the HPLC; the chromatograph gives a peak output of area y (y-axis of the calibration curve). The area, y, is then in the equation of a line y=mx + b from the calibration curve, and the concentration is found by solving the equation for x.

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SEPARATION OF PHOSPHOLIPIDS BY HPLC

With UV detection, several solvent systems were proposed for the separation of phospholipid classes but they are generally based on acetonitrile or hexane/2-propanol. With the first, phosphatidylethanolamine elutes before phosphatidylcholine which is itself eluted before sphingomyelin. The acidic lipids, such as phosphatidic acid, cardiolipin and phosphatidylinositol, are eluted before phosphatidylethanolamine. Mobile phases containing hexane/2-propanol allow also phosphatidylethanolamine to be eluted before phosphatidylcholine, but the latter and sphingomyelin are not well separated. In contrast, phosphatidylinositol and phosphatidylserine are eluted between phosphatidylethanolamine and phosphatidylcholine. As phospholipids are ionic molecules, a counter-ion is required in the running solution. Sulfuric and phosphoric acids have been proposed but better results were obtained with the addition of 0.5 mM serine (*Christie WW, J Chromatogr 1986, 361, 396*).
With the light-scattering detection new eluting systems were designed to avoid the interferences from non volatile modifiers added to the solvents (acids, salts...).

All that applies to intact phospholipids and enables to determine their relative amount and possibly their composition after post-detector collection.
Sometimes, the direct HPLC separation is not enough efficient and a previous deacylation is needed. Thus, for the fine analysis of polyphosphoinositides, the total phospholipids or fractions purified by TLC are deacylated and the water soluble products (glycerophosphoinositol phosphates) are separated by HPLC with an anion exchange column.

ANALYSIS OF INTACT PHOSPHOLIPIDS

Two types of separation are selected:

Separation based on a silica column

First method:

The proposed method used a ternary gradient and was used to separate all lipid classes but can be easily adapted for the separation of only phospholipid classes

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Apparatus:

HPLC system, light scattering detector
3µm silica column (10 cm x 4.6 mm)

Reagents:

Iso-octane, tetrahydrofurane (THF), 2-propanol, chloroform.

Procedure:

Mobile phase A: iso-octane/THF, 99/1 (v/v)
Mobile phase B: 2-propanol/chloroform, 80/20 (v/v)
Mobile phase C: 2-propanol/water, 50/50 (v/v)
The gradient of solvents is complex and combined with a flow gradient for about 32 min.

The authors demonstrated that they could easily separate lipid classes from rat tissues in 30 min and detect 1 µg or less of any of the separated lipids. Cerebrosides and sulfatides co-migrated (elution time: about 12 min). However, sphingomyelin was not completely separated from phosphatidylcholine at the end of the run. The procedure was reproducible and allows the detection of low levels of phospholipids (200 ng) in a sample of no more than 80 µg of total heart lipids.

Second method:

The proposed method is based on a binary gradient of chloroform and methanol solutions and can be considered as one of the most efficient methods (*Becart J et al., J High Resolution Chromatogr 1990, 13, 126*). A diol column may be used instead of the proposed silica gel column.

Apparatus:

HPLC system, light-scattering detector
Lichrospher Si 60, 5 µm, 12 cm x 4 mm, Merck

Reagents:

Chloroform, methanol, ammonium hydroxide.

Procedure:

Mobile phase A: chloroform/methanol/ammonium hydroxide, 80/19.5/0.5 (v/v)

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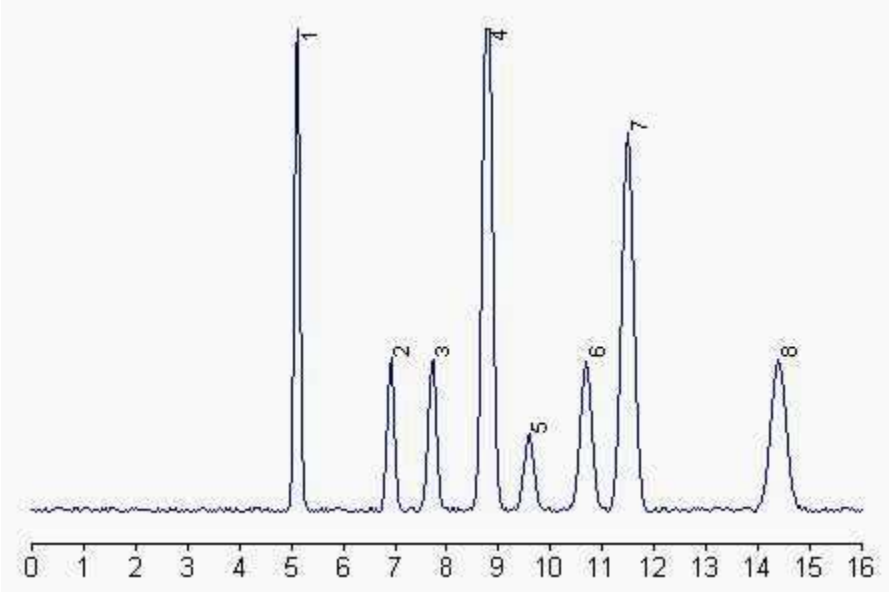
Mobile phase B: chloroform/methanol/water/ammonium hydroxide, 60/34/5.5/0.5 (v/v)

The gradient is: 0-14 min: linear from A/B, 50/50 to 100% B, 14-25 min: hold 100% B, 25-30 min: 100% B to A/B, 50/50 and 15 min regeneration before another injection. Samples are dissolved in the mixture chloroform/methanol/water (70/25/5) before injection. The flow rate is 1 ml/min.



1: phosphatidylethanolamine, 2: phosphatidylinositol, 3: phosphatidylserine, 4: phosphatidylcholine, 5: phosphatidic acid, 6 and 7: sphingomyelin, 8: lysophosphatidylcholine.

Separation based on a diol column

1 - Separation of the classical phospholipids
Olsson NJ at al., J Chromatogr, 1996, 681, 213

Apparatus:

HPLC system, light-scattering detector
Nucleosil 100-7 OH DIOL column (25 cm x 4 mm, 7 µm from Macherey-Nagel).

Reagents:

Hexane, 2-propanol, butanol, THF, iso-octane

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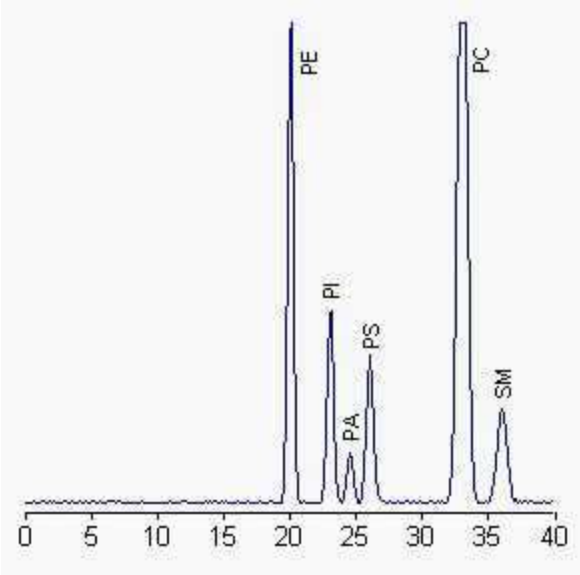
Procedure:

Mobile phase A: hexane/2-propanol/butanol/THF/iso-octane/water, 64.5/17.5/7/5/5/1 (v/v)
Mobile phase B: 2-propanol/butanol/THF/iso-octane/water, 73/7/5/5/10 (v/v)
ammonium acetate was added to both solvents (180 mg/l)
The binary gradient was linear over 50 min from 0% to a final composition of 100% B. The column was maintained at 65°C and the flow rate at 1 ml/min

The authors claimed that the DIOL phase column was notably resistant. Acidic phospholipids were adequately separated. Moreover, a partial resolution was obtained between alkenyl-PE and diacyl-PE. A similar method was proposed for plant lipids (*Arnoldsson KC et al., in "Phospholipids: characterization, metabolism, and novel biological applications". Cevc G, Paltauf F, Eds, AOCS Press, Champaign, Ill, 1995, 44*).



PE: diacyl-phosphatidylethanolamine, PI: phosphatidylinositol, PA: phosphatidic acid, PS: phosphatidylserine, PC: phosphatidylcholine, SM: sphingomyelin

When plasmalogens are present, the corresponding peak is ahead of diacyl-PE. Complex glycolipids can also be separated with this HPLC method, cerebrosides being eluted at about 5 min and sulfatides 2 min before PE. To maintain peaks within the calibrated range of phospholipid standards, the authors injected no more than 250 µg phospholipids. Further analysis may be run on fractions with a higher column sample load (about 0.5 mg lipids).

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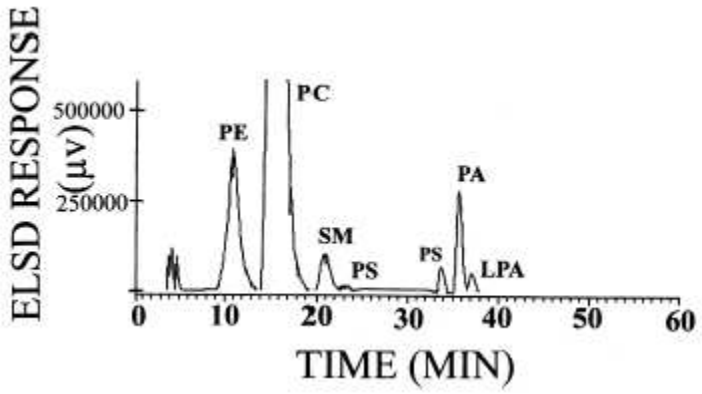
2 - Separation of phosphatidic acid and lysophosphatidic acid
Holland WL et al., J Lipid Res 2003, 44, 854-8

As phosphatidic acid and its lyso compounds are involved in the regulation of various cellular processes, there is a necessity to quantify accurately these bioactive lipids in cellular extracts. Below is described a method which allows their accurate quantification using a chloroform/methanol (1/2, v/v) extraction, a diol HPLC column (Supelcosil LC-318 diol column (25 cm long, 4.6 ID, 5 m particle size), a ternary basic gradient (*Stith BJ et al., J Lipid Res 2000, 41, 1448*) and a light scattering detector.



The use of radio labelled molecules allowed the authors to estimate a total recovery rate of 48% for phosphatidic acid and 53% for its lyso compound. A polynomial third-order cubic regression equation was used to quantify the phospholipids.

A simple HPLC method was described for the simultaneous analysis of phosphatidylcholine and its lyso derivatives (*Adlercreutz D et al., JAOCS 2001, 78, 1007*). This method was proposed to be suitable for studying reactions on the phospholipid and acyl migration of its lyso derivatives.

A review by Van der Meeren P et al. of several HPLC separation techniques for phospholipid groups may be found in the book "*Food analysis by HPLC" (Nollet LML Ed., Marcel Dekker inc, 1992*).

ANALYSIS OF POLYPHOSPHOINOSITIDES (DEACYLATED FORMS)

The study of the various phosphoinositide isomers is done after removing the fatty acyl groups and separating the deacylated forms by HPLC with a salt gradient. Since the water-soluble derivatives have no absorption characteristics, the methodology involves the determination of radioactive products. Thus, an on-line continuous flow liquid scintillation detector is

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required. Nonradioactive forms may be determined using a conductivity detector (see below).

Procedure:

Deacylation of phospholipids: The lipids dried or adsorbed to silica gel are treated in a screw-capped glass tube with 1.8 ml of deacylating reagent (40% methylamine/water/methanol/butanol, 5.4/3.2/9.1/2.3, v/v) at 53°C for 50 min (water bath). The mixture (or the supernatant if the silica gel is present) is then evaporated under a nitrogen flow in a well ventilated hood.
Water (2 ml) is added to the dried residue, the solution is deeply frozen and then freeze-dried. The dried samples are resuspended in 2 ml of water, and extracted with an equal volume of butanol/petroleum ether/ethyl formate (20/4/1, v/v). The aqueous phase is again freezedried and stored at -70°C until analysis by HPLC.

HPLC analysis of deacylated phospholipids:
Column: Partisphere SAX from Whatman (5 m, 250 x 4 mm).
Elution: a discontinuous gradient obtained from mixing solution A (water) and solution B (1M (NH4)2HPO4 titrated at pH 8 with conc. phosphoric acid) according to the table below:

Time (min)	Flow (ml/min)	A (%)	B (%)
0	1	100	0
5	1	100	0
65	1	82	18
102	1	57	43
102.1	1	100	0
102.2	1	100	0
102.3	2	100	0
122	2	100	0
122.1	1	100	0
122.2	0	100	0

Non-radioactive nucleotides (AMP, ADP and ATP) are added to the aqueous sample (total volume: 0.1 up to 1 ml) prior filtration and injection to allow monitoring with a UV detector to evaluate the reproducibility of elution times. We add 20 1 of a mixture containing 200 M of each nucleotide to 1 ml sample.

The method of suppressed conductivity detection was shown to be efficient in determining the amounts of isoforms of phosphatidylinnositol monophosphates (PI2P et PI) and biphosphates (PI3,4P_2, PI4,5P_2 and PI3,5P_2) (*Nasuhoglu C et al., Anal Biochem 2002, 301, 243*). The major

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anionic head group can be identified down to a detection limit of about 100 pmol but that detection remains 1 to 2 log units less sensitive than for isotope techniques. That procedure was shown to be efficient in the study of the activation of phosphatidylinositide kinase.

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Alliance HPLC System

2695 Separations Module [W a t e r s]

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Alliance HPLC System

Introduction:

The Alliance HPLC System is built around the 2695 Separations Module, which offers integrated solvent and sample management.
The 2695 is designed to work with both Empower and MassLynx software, the complete range of Waters HPLC column chemistries and formats, including Intelligent Speed (IS), Symmetry, XBridge and XTerra columns, and a variety of detectors, from UV-visible to single- to triple-quadrupole mass spectrometers.

Alliance HPLC System

The Alliance HPLC System features:

* Integrated solvent and sample management functions that ensure consistent system-to-system performance and high reproducibility
* Alliance HPLC Systems can run with Empower Software for HPLC and LC/MS and with MassLynx for LC/MS or LC/MS/MS, enabling to you control operating parameters, and capture, process and store results data from a personal or networked computer
* A large, intuitive, LCD-based user interface on the 2695 Separations Module allows rapid system set-up through AutoStartPLUS routines that streamline daily startups
* Alliance Systems and columns are manufactured to a rigid set of performance specifications, allowing you to confidently transfer a method between instruments and still get consistent results, unit to unit and lab to lab
* Improved control of your column environment via a column heater or heater/cooler, allowing you to control temperatures whether you're using single or multiple columns
* Integrated column switching valves permit rapid column selection, enabling unattended method development
* Physical design that provides easy, tool-free access to pistons, seals, and the piston seal-wash as well as the lower needle-wash seal of the sample manager.

Alliance LC and LC/MS systems set the standard for reliability and performance in today's laboratories that are pressured to run samples, around the clock, with unsurpassed accuracy.

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The Alliance LC/MS System allows laboratories to perform HPLC and routine mass spectrometry (MS) for unsurpassed sensitivity and ruggedness. As part of the Alliance LC/MS System, the ZQ Mass Detector takes LC/MS speed, sensitivity and ruggedness to a new level.

Automated Method Development:

Manual method development and transfer can be a labor-intensive, timeconsuming and imprecise process—especially when an end user has to modify a transferred method.
The Waters Automated Method Development System combines the Alliance HPLC System with specialized method development features in Empower Software to evaluate a wide range of experimental conditions. The system helps you determine the effect of factors such as organic concentration, pH, temperature, and buffer concentration on your separation to ensure a method is robust and does not require re-optimization when transferred.

Data Management:

Alliance HPLC Systems are fully controlled by Waters Laboratory Informatics products. With Empower Software control of your Alliance HPLC System, you can acquire and store data, search for results and develop audit trail information. MassLynx Software provides seamless system integration and single keyboard control for easy method setup and processing of chromatographic and spectral data. A number of MassLynx Software application managers are available to help you control and manage data for specialized laboratory applications.

Overview:

2695 Separations Module:

The heart of the Alliance System is the 2695 Separations Module and its integrated solvent and sample management configuration.

Solvent Management:

The 2695 Separations Module's solvent management system degasses and blends up to four chromatographic solvents in precise proportions for smooth, pulse-free solvent delivery, while the latest Performance PLUS check-valve technology assures minimal downtime.

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Solvent management features:

* Serial flow path with only two inlet check valves, reducing complexity and enhancing overall ruggedness
* Vacuum degassing using ultra-high efficiency second-generation polymer membrane technology
* Precise low-pressure quaternary blending (first in-first out) for highly reproducible gradient profiles across the flow range
* Independently driven pistons produce pulse-free solvent flow without use of pulse dampeners
* Fixed delay volume (regardless of system back pressure) for consistent, predictable chromatography
* A programmable flow rate range that covers two orders of magnitude (from 50 microliters to five milliliters per minute) without the need for hardware modifications
* Tool-free routine maintenance on plungers/seals/seal-wash
An automated active plunger seal wash is standard on all Alliance HPLC Systems.

Sample Management:

Up to 120 industry-standard vials can be accommodated in the 2695 Separations Module's five individual sample carousels. Sample queues are quick to set up—whether it's just one sample or a sequence of multiple methods from different analysts.

Sample management features:

* Reproducible injections from one to hundreds of microliters—compatible with any analytical scale LC column chemistry
* Variable volume injections with no need to swap loops for analytical methods
* Carryover management via a programmable needle wash cycle and an active needle wash solution (without wash vials)
* Programmable inject-needle height that accommodates various vial geometries and takes into account vial bottom thickness
Programmable syringe draw rate for viscous samples/solvents
* Five independent carousels: Run samples in one carousel while prepping the next sequence in another, enabling new samples/carousels to be added into the system—without disturbing the ongoing sample queue
* Sampling routines for automated reference peak addition or automated pre-column derivitization procedures
* Tool-free lower seal-wash frit and injection syringe replacement, for easy routine maintenance

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The Flexible and Reliable Workhorse for all Your HPLC Requirements

Alliance HPLC Systems have been continually expanded and perfected to accommodate an enormous range of real world, problem-solving HPLC separation challenges.

Other instrument is an Agilent Technologies (formerly part of Hewlett Packard) 1100 Series HPLC system with quaternary pump, autosampler, and diode array, electrochemical and fluorescence detectors More information is available from the Agilent Technologies.

Agilent System- 1100 Series

Troubleshooting High Performance Liquid Chromatography

Do you have a problem with any of the following topics:

1. *Peaks*
2. *Leaks*
3. *Recovery*
4. *Sensitivity*
5. *Retention*
6. *Equilibration*
7. *Baseline*
8. *Pressure*

Broad Peak Causes and Solutions:

Cause	Solution
Analytes eluted early due to sample overload	Dilute sample 1:10 and reinject
Detector-cell volume too large	Use smallest possible cell volume consistent with sensitivity needs; use detector with no heat exchanger in system
Injection volume too large	Decrease solvent strength of injection solvent to focus solute; inject smaller volume
Large extra column volume	Use low- or zero-dead-volume endfittings and connectors; use smallest possible diameter of connecting tubing (<0.10 in. i.d.); connect tubing with matched fittings
Mobile-phase solvent viscosity too high	Increase column temperature; change to lower viscosity solvent
Peak dispersion in injector valve	Decrease injector sample loop size; introduce air bubble in

	front and back of sample in loop
Poor column efficiency	Use smaller-particle-diameter packing, lower-viscosity mobile phase, higher column temperature, or lower flow rate
Retention time too long	Use gradient elution or stronger isocratic mobile phase
Sampling rate of data system too low	Increase sampling frequency
Slow detector time constant	Adjust time constant to match peak width
Some peaks broad - late elution of analytes retained from previous injection	Flush column with strong solvent at end of run; end gradient at higher solvent concentration

Ghost Peaks: Causes and Solution:

Contamination	Flush column to remove contaminatint; use HPLC-grade solvent
Elution of analytes retained from previous injection	Flush column with strong solvent at end of run; end gradient at higher solvent concentration.
Ion-pair chromatography - upset equilibrium	Prepare sample in mobile phase; reduce injection volume
Oxidation of trifluoroacetic acid in peptide mapping	Prepare trifluoroacetic acid solutions fresh daily; use antioxidant
Reversed-phase chromatography - contaminated water	Check suitability of water by running different amounts through column and measure peak height of interferences as function of enrichment time; clean water by running it

	through old reversed-phase column; use HPLC-grade water.
Unknown interferences in sample	Use sample cleanup or prefractionation before injection.

Negative Peaks Causes and Solutions:

Refractive index detection - refractive index of solute less than that of mobile phase	Reverse polarity to make peak positive
UV-absorbance detection - absorbance of solute less than that of mobile phase	Use mobile phase with lower UV absorbance; if recycling solvent, stop recycling when recycled solvent affects detection

Peak Doubling Causes and Solutions:

Blocked frit	Replace or clean frit; install 0.5-um porosity in-line filter between pump and injector to eliminate mobile-phase contaminants or between injector and column to eliminate sample contaminants
Coelution of interfering compound	Use sample cleanup or prefractionation; adjust selectivity by changing mobile or stationary phase
Coelution of interfering compound from previous injection	Flush column with strong solvent at end of ran; end gradient at higher solvent concentration
Column overloaded	Use higher-capacity stationary phase; increase column diameter; decrease sample amount

Column void or channeling	Replace column, or, if possible, open top endfitting and clean and fill void with glass beads or same column packing; repack column
Injection solvent too strong	Use weaker injection solvent or stronger mobile phase
Sample volume too large	Use injection volume equal to one-sixth of column volume when sample prepared in mobile phase for injection
Unswept injector flow path	Replace injector rotor

Peak Fronting Causes and Solutions

Channeling in column	Replace or repack column
Column overloaded	Use higher-capacity stationary phase; increase column diameter; decrease sample amount

Peak Tailing Causes and Solutions:

Basic solutes - silanol interactions	Use competing base such as triethylamine; use a stronger mobile phase; use base-deactivated silica-based reversed-phase column; use polymeric column
Beginning of peak doubling	See peak doubling
Chelating solutes - trace metals in base silica	Use high purity silica-based column with low trace-metal content; add EDTA or chelating compound to mobile phase; use polymeric column
Silica-based column - degradation at high pH	Use polymeric, sterically protected, or high-coverage reversed-phase column; install

	silica gel saturatorcolumn between pump and injector
Silica-based column - degradation at high temperature	Reduce temperature to less than 50 C
Silica-based column - silanol interactions	Decrease mobile-phase pH to suppress silanol ionization; increase buffer concentration; derivatize solute to change polar interactions
Unswept dead volume	Minimize number of connections; ensure injector rotor seal is tight; ensure all compression fittings arecorrectly seated
Void formation at head of column	Replace column, or, if possible, open top endfitting and clean and fill in void with glass beads or samecolumn packing; rotate injection valve quickly; use injection valve with pressure bypass; avoid pressure shock

Spikes Causes and Solutions:

Bubbles in mobile phase	Degas mobile phase; use back-pressure restrictor at detector outlet; ensure that all fittings are tight
Column stored without caps	Store column tightly capped; flush reversed-phase columns with degassed methanol

Leak at column or fittings: Causes and Solution:

Catastrophic loose fitting	Tighten or replace fitting
Noncatastrophic white powder	Cut tubing and replace ferrule;

at loose fitting	disassemble fitting, rinse and reassemble.

Leak at Detector: Causes and Solution:

Catastrophic detector-seal failure	Replace detector seal or gaskets.

Leak at injection valve: Causes and Solution:

Catastrophic worn or scratched valve rotor	Replace valve rotor

Leak at Pump: Causes and Solution:

Catastrophic pump-seal failure	Replace pump seal; check piston for scratches and, if necessary, replace.

Poor sample recovery: Causes and Solution:

Absorption or adsorption of proteins	Change HPLC mode to reduce nonspecific interactions; add protein-solubilizing agent, strong acid or base (with polymeric columns only), or detergent such as SDS to mobile phase.
Adsorption on column packing	Increase mobile phase strength to minimize adsorption; for basic compounds add competing base or use base-deactivated packing
Adsorption on tubing and other hardware components	Use inert (PEEK), glass-lined, or titanium tubing and flow-path components
Chemisorption on column packing	Ensure no reactive groups are present; use polymeric packing; change column type and mode

Hydrophobic interactions between stationary	Use short-chain reversed-phase packing; use 300-A pore diameter packing; use hydrophilic packing or ton-exchange media; use hydrophobic interaction chromatography
Less than 99% yield for basic compounds irreversible adsorption on active sites	Use endcapped, base-deactivated, sterically protected, high coverage, or polymeric reversed-phase
Less than 90% yield for acidic compounds - irreversible adsorption on active sites	Use endcapped or polymeric packing; acidify mobile phase

Lack of Sensitivity: Causes and Solutions:

Autosampler flow lines blocked	Check flow and clear any blockages
Detector attenuation set too high	Reduce detector attenuation
First few sample injections - sample adsorption in injector sample loop or column	Condition loop and column with concentrated sample.
Injector sample loop underfilled	Overfill loop with sample
Not enough sample injected	Increase amount of sample inected
Peaks are outside detector's linear range	Dilute or concentrate sample to bring detector response into linear range
Sample losses during sample preparation	Use internal standard during sample prepartion; optimize sample preparation method
Sample losses on column	See problem: Recovery

Changing retention times: Causes and Solution:

Buffer retention times	Use buffer with concentration greater than 20 mM.
Contamination buildup	Flush column occasionally with strong solvent
Equilibration time insufficient for gradient run or changes in isocratic mobile phase	Pass at least 10 column volumes through the column for gradient regeneration or after solvent changes
First few injections - active sites	Condition column by injecting concentrated sample
Inconsistent on-line mobile-phase mixing	Ensure gradient system is delivering a constant composition; compare with manually prepared mobile phase; partially premix mobile phase
Selective evaporation of mobile-phase component	Cover solvent reservoirs; use less-vigorous helium purging; prepare fresh mobile phase
Varying column temperature	Thermostat or insulate column; ensure laboratory temperature is constant.

Decreasing Retention times: Causes and Solutions:

Active sites on column packing	Use mobil-phase modifier, competing base (basic compounds), or increase buffer strength; use higher coverage column packing.
Column overloaded with sample	Decrease sample amount or use larger-diameter column
Increasing flow rate	Check and reset pump flow rate.
Loss of bonded stationary phase or base silica	Use mobile-phase pH between pH 2 and pH 8

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Varying column temperature	Thermostat or insulate column; ensure laboratory temperature is constant.

Increasing Retention times: Causes and Solutions:

Decreasing flow rate	Check and reset pump flow rate; check for pump cavitation; check for leaking pump seals and other leaks in system.
Changing mobile-phase composition	Cover solvent reservoirs; ensure that gradient system is delivering correct composition.
Loss of bonded stationary phase	Use mobile-phase pH between pH 2 and pH 8

Slow column equilibration time: Causes and Solutions:

Reversed phase ion pairing - long chain ion pairing reagents require longer equilibration time	Use ion-pairing reagent with shorter alkyl chain length

Slow column equilibration time: Causes and Solutions:

Reversed phase ion pairing - long chain ion pairing reagents require longer equilibration time	Use ion-pairing reagent with shorter alkyl chain length

Varying retention times: Causes and Solutions:

Gradient - insufficient column-regeneration time	Increase equilibrating time in mobile-phase A to obtain constant retention times for early peaks
Ion pairing - insufficient equilibration time	Increase equilibration time; ion pairing may require as much as 50 column volumes for mobile-phase changeover
Isocratic - insufficient equilibration time	Pass 10-15 column volumes of

	mobile phase through column for equilibration

Disturbance at void time: Causes and Solution:

Air bubbles in mobile phase	Degas or use back pressure restricor on detector
Positive-negative - difference in refractive index of injection solvent and mobile phase	Normal with many samples; use mobile phase as sample solvent

Drifting baseline: Causes and Solutions:

Negative direction (gradient elution) - absorbance of mobile-phase A	Use non-UV absorbing mobile phase solvents; use HPLC grade mobile phase solvents; add UV absorbing compound to mobile phase B.
Positive direction (gradient elution) - absorbance of mobile phase B	Use higher UV absorbance detector wavelength; use non-UV absorbing mobile phase solvents; use HPLC grade mobile phase solvents; add UV absorbing compound to modile phase A.
Positive direction - contamination buildup and elution	Flush column with strong solvent; clean up sample; use HPLC grade solvents
Wavy or undulating - temperature changes in room	Monitor and control changes in room temperature; insulate column or use column oven; cover refractive index detector and keep it out of air currents.

Noise: Causes and Solutions:

Continous - detector lamp problem or dirty flow cell	Replace UV lamp(each should last 2000 h); clean and flush flow cell
Gradient or isocratic proportioning - lack of solvent mixing	Use proper mixing device; check proportioning precision by spiking one solvent with UV absorbing compound and mointor UV

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	absorbance detector output
Gradient or isocratic proportioning - malfunctioning proportioning valves	Clean or replace proportioning precision valves; partially remix solvents
Occasional sharp spikes - external electrical interference	Use voltage stabilizer for LC system; use independent electrical circuit
Periodic - pump pulses	Service or replace pulse damper; purge air from pump; clean or replace check valves.
Random - contamination buildup	Flush column with strong solvent; clean up sample; use HPLC grade solvent
Spikes - bubble in detector	Degas mobile phase; use back pressure restrictor at detector outlet
Spikes - column temperature higher than boiling point of solvent	Use lower column temperature

Decreasing Pressure: Causes and Solutions:

Insufficient flow from pump	Loosen cap on mobile phase reservior
Leak in hydralic lines from pump to column	Tighten or replace fittings; tighten rotor in injection valve
Leaking pump check valve or seals	Replace or clean check valves; replace pump seals.
Pump cavitation	Degas solvent; check for obstruction in line from solvent reservoir to pump; replace inlet-line frit

Fluctuating Pressure: Causes and Solutions:

Bubble in pump	Degas solvent; sparge solvent with helium
Leaking pump check valve or seals	Replace or clean check valves; replace pump seals

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High back pressure: Causes and Solutions:

Column blocked wth irreversibly adorbed sample	Improve sample cleanup; use guard column; reverse-flush column with strong solvent to dissolve blockage
Column particle size too small (for example 3 micrometers)	Use larger particle size (for example 5 micrometer)
Microbial growth on column	Use at least 10% organic modifier in mobile phase; use fresh buffer daily; add 0.02% sodium azide to aqueous mobile phase; store column in at least 25% organic solvent without buffer
Mobile phase viscosity too high	Use lower viscosity solvents or higher temperature
Plugged frit in in-line filter or guard column	Replace frit or guard column
Plugged inlet frit	Replace endfitting or frit assembly
Polymetric columns - solvent change causes swelling of packing	Use correct solvent with column; change to proper solvent compositionl consult manufacturer's solvent-compatibility chartl use a column with a higher percentage of cross-linking
Salt precipitation (especially in reversed-phase chromatography with high concentration of organic solvent in mobile phase)	Ensure mobile phase compatibility with buffer concentration; decrease ionic strength and water-organic solvent ratio; premix mobile phase
When injector disconnected from column - blockage in injector	Clean injector or replace rotor

Increasing pressure: Causes and Solutions:

Blocked flow lines	Systematically disconnect components from detector end to column end to find blockage; replace or clean blocked component
Particulate buildup at head of column	Filter sample; use .5 micrometer in-line filter; disconnect and backflush column; replace inlet frit
Water-organic solvent systems -	Ensure mobile phase compatibility

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buffer precipitation	with buffer concentration; decrease ionic strength or water organic solvent ratio

Models of HPLC:

Agilent -1100 Series

Agilent -1100 Series

Alliance -2695 (Waters)

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