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Instrumental Methods in Bioprospecting: High Pressure Liquid Chromatography

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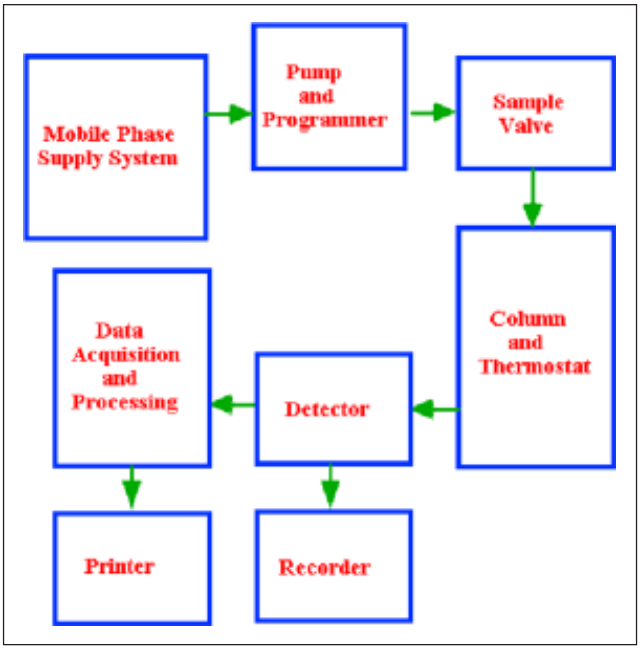
Liquid chromatography (LC) was the first type of chromatography to be discovered and, in the form of liquid-solid chromatography (LSC) was originally used in the late 1890s by the Russian botanist, Tswett to separate and isolate various plant pigments. The colored bands he produced on the adsorbent bed evoked the term chromatography (color writing) for this type of separation. In the late 1930s and early 1940s Martin and Synge introduced a form of liquid-liquid chromatography by supporting the stationary phase, in this case water, on silica gel in the form of a packed bed and used it to separate some acetyl amino acids. Martin and Synge suggested the use of small particles and high pressures in LC to improve the separation which proved to the critical factors that initiated the development of high performance liquid chromatography (HPLC). The statement made by Martin in 1941 contains all the necessary conditions to realize both the high efficiencies and the high resolution achieved by modern LC columns. Despite his recommendations, however, it has taken nearly fifty years to bring his concepts to fruition. The major impediment to the development of LC was the lack of a high sensitive detector and it was not until the refractive index detector was developed by Tiselius and Claesson in 1942 could the technique being effectively developed. The contemporary chromatograph, however, is a very complex instrument operating at pressures up to 10,000 PSI providing flow rates ranging from a few microliters per minute to 10-20 ml/minute depending on the type of LC that is carried out. Modern detectors can detect solutes at concentration levels of $1x10^{-9}$ g/ml and an analysis can be completed in a few minutes with just a few micrograms of sample.

Modern High Pressure Liquid Chromatography (HPLC)

HPLC is liquid chromatography which has been optimized to provide rapid high resolution separations. The basic liquid chromatograph consists of five basic units as follows. A block diagram of the basic liquid chromatograph is shown in the following figure.

1. Mobile phase supply system and gradient mixers.
2. HPLC high pressure pumps and sample valves.
3. HPLC columns with inert packing materials.
4. High sensitivity low dispersion HPLC detectors.
5. High speed data acquisition systems.



The Basic Liquid Chromatograph

Mobile Phase Supply System and HPLC Gradient Mixers

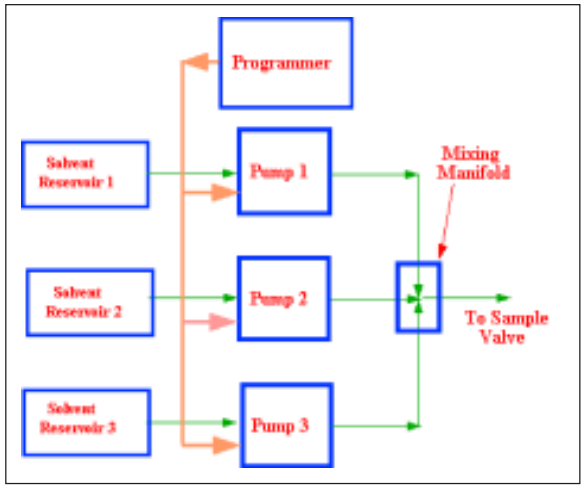
HPLC gradient mixers provide a very precise control of solvent composition to maintain a reproducible gradient profile. The mobile phase supply system consists of number of reservoirs (200-1,000 ml). At least two reservoirs would be necessary and are usually constructed of glass or stainless steel and contain an exit port open to air. Each reservoir is usually fitted with a gas diffuser through which helium can be bubbled. Many solvents and solvent mixtures (particularly aqueous mixtures) contain significant amounts of dissolved nitrogen and oxygen from the air. These gasses can form bubbles in the chromatographic system that cause both serious detector noise and loss of column efficiency. As helium is very insoluble in most solvents, it purges the oxygen and nitrogen from the solvent but does not produce bubbles in the system itself. Applying a vacuum to the reservoir is not a permanent solution to dissolved air as, on releasing the vacuum to allow the solvent to pass to the pump, air again dissolves in the solvent. The solvent is filtered through a stainless steel or sintered glass filter to remove any solid contaminants. Depending on the type of solvent programmer that is employed, the supply from each reservoir may pass either to a pump or to a valve blending device. Solvent reservoirs are not usually thermostatted but, when necessary, the solvent can be brought to the column temperature by the use of an appropriate heat exchanger.

The Gradient Programmer

High Pressure Gradient Programmer

There are two basic types of solvent programmer. In the first, the solvent mixing occurs at high pressure and in the second the solvents are premixed at low pressure and then passed to the pump. Theoretically, there can be any number of solvents involved in a mobile phase program, however, most LC analyses require only two solvents, nevertheless, up to four solvents can be accommodated.

The layout of a high pressure gradient system is shown in the following figure and includes, as an example, provision for three solvents to be mixed by appropriate programming.



High Pressure Gradient Programmer

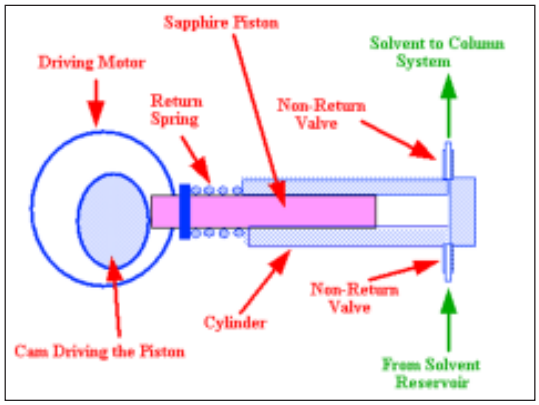
Solvent passes from each reservoir directly to a pump and then to a mixing manifold from which it passes to the sample valve and column. The pumps control the actual program and are usually driven by stepping motors. The volume delivery of each solvent is controlled by the speed of the respective pump which is precisely determined by the frequency of its power supply. The controlling frequency can be generated either by external oscillators or, if the chromatograph is computer controlled, directly from the computer itself.

HPLC Pumps

Because of the small particles used in modern HPLC, LC pumps need to operate reliably and precisely at pressures of 10,000 PSI or at least 6,000 PSI. To operate at these pressures and remain sensibly inert to the wide variety of solvents used HPLC pumps usually have sapphire pistons, SS cylinders and return valves fitted with sapphire balls and stainless steel seats. For analytical proposes HPLC pumps should have flow rates that range from 0-10 ml/min., but for preparative HPLC, flow rates in excess of 100 ml/min may be required. There are a number of different types of pumps that can provide the necessary pressures and flow-rates required by the modern liquid chromatograph. In the early years of the LC renaissance, there were two types of pump in common use; they were the pneumatic pump, where the necessary high pressures were achieved by pneumatic amplification, and the syringe pump, which was simply a large, strongly constructed syringe with a plunger that was driven by a motor. Today the majority of modern HPLCs are fitted with reciprocating pumps fitted with either pistons or diaphragms.

Single Piston Reciprocating Pump

The single piston reciprocating pump was the first of its type to be used with high efficiency LC columns (columns packed with small particles) and is still very popular today. It is simple in design and relatively inexpensive. A diagram of the single piston pump is shown in the figure.



Single Piston Reciprocating Pump

Most pistons of modern LC pumps are made of synthetic sapphire to reduce wear and extend the working life of the pump. The cylinder is usually made of stainless steel and is attached to two non-return valves in line with the inlet and outlet connections to the pump. The piston is driven by a

stainless steel cam which forces the piston into the cylinder expressing the solvent through the exit non-return valve. After reaching the maximum movement, the piston follows the cam and returns as a result of the pressure exerted by the return spring. During this movement the cylinder is loaded with more solvent through the inlet non-return valve. The shape of the cam is cut to provide a linear movement of the piston during expression of the solvent but a sudden return movement on the refill stroke. In this way the pulse effect that results from the refill action is reduced.

Rapid Refill Pump

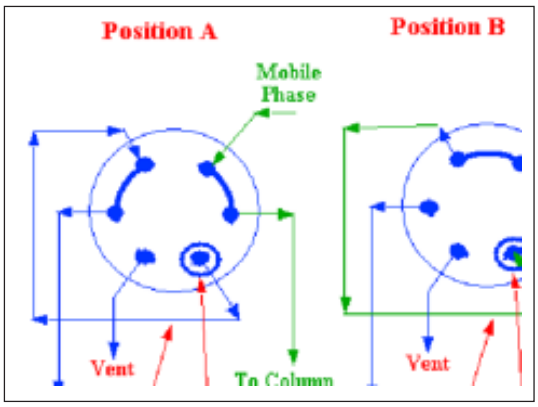
In order to avoid the refill pulses resulting from a single piston pump, a number of rapid refill systems have been developed. The designs have ranged from cleverly designed actuating cams to drive the piston rapidly in the refill mode to electronically operated piston movements.

Diaphragm Pump

The unique property of the reciprocating diaphragm pump is that the actuating piston does not come into direct contact with the mobile phase and thus, the demands on the piston-cylinder seal are not so great. The diaphragm has a relatively high surface area and thus, the movement of the diaphragm is relatively small and consequently the pump can be operated at a fairly high frequency.

HPLC Sample Valves

Since sample valves come between the pump and the column it follows that HPLC sample valves must also tolerate pressures up to 10,000 PSI. For analytical HPLC, the sample volume should be selectable from sub- micro liter to a few micro liters, whereas in preparative HPLC the sample volume may be even greater than 10 ml. The higher the operating pressure the tighter the valve seating surfaces must be forced together to eliminate any leak. It follows that any abrasive material, however fine, that passes into the valve can cause the valve seating to become scored each time it is rotated which will ultimately lead to leaks. This will cause the sample size to vary between samples and eventually affect the accuracy of the analysis. In LC, the sample valve contains an extra loading port and behaves like an internal loop valve. The basic difference between this type of valve and the normal external loop sample valve is the introduction of an extra port at the front of the valve. This port allows the injection of a sample by a syringe directly into the front of the sample loop. Position (A) shows the inject position. Injection in the front port causes the sample to flow into the sample loop. The tip of the needle passes through the rotor seal and, on injection, is in direct contact with the ceramic stator face. After injection, the valve is rotated to position (B) and the mobile phase flushes the sample directly onto the column. The sample is actually forced out of the beginning of the loop so it does not have to flow through the entire length of the loop. This type of injection system is ideally suited for quantitative LC, and is probably by far the most popular injection system in use.



External Loop Sample Valve

HPLC Columns

HPLC columns are packed with very fine particles (usually a few microns in diameter). The very fine particles are required to attain the low dispersion that give the high plate counts expected of modern HPLC. Plate counts in excess of 25,000 plates per column are possible with modern columns, however, these very high efficiencies are very rarely found with real samples because of the dispersion associated with injection valves, detectors, data acquisition systems and the dispersion due to the higher molecular weight of real samples as opposed to the common test samples. LC columns, in general, achieve their separation by exploiting the different intermolecular forces between the solute and the stationary pahse and those between the solute and the mobile phase. The column will retain those substances that interact more strongly with the stationary pahse than those that interact more strongly with the mobile phase. In particular optically pure compounds can be used to make Chiral HPLC stationary phases.

Liquid Chromatography Stationary Phases

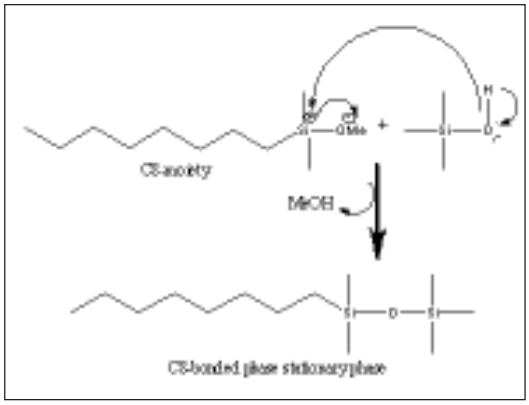
Traditionally the stationary phase used in LC has been silica gel which separates solutes largely on the basis of polarity, although, due to its unique structure, silica gel also exhibits strong exclusion characteristics. The bonded phases were introduced to provide a material that would separate solutes by dispersive interactions and also to provide some semie polar stationary phases. The bonded phases were also based on silica gel. More recently, polymeric stationary phases were introduced to provide materials that were insoluble in water and that were stable at extremes of pH.

The Structure of Silica Gel

The matrix of the primary silica gel particle consists of a core of silicon atoms joined together with oxygen atoms by siloxane bonds (silicon-oxygen-silicon bonds). On the surface of each primary particle some residual, uncondensed hydroxyl groups from the original polymeric silicic acid remain. There are three types of hydroxyl group. The first is a single hydroxyl group attached to a silicon atom which has three siloxane bonds joining it to the gel matrix. The second is one of two hydroxyl groups attached to the same silicon atom which, in turn, is joined to the matrix by only two siloxane bonds. These twin hydroxyl groups are called Geminal hydroxyl groups. The third is one of three hydroxyl groups attached to a silicon atom which is now only joined to the silica matrix by only a single siloxane bond.

Bonded Phases

Bonded phases are formed by reacting the surface hydroxyl groups with an appropriate reagent to chemical link an organic moiety to the silica surface. The nature of the organic moiety will determine the type of interaction that will take place between the solute and the surface. The most efficient bonded phase has the maximum surface coverage. It is understood, that due to stearic hindrance from the bonded moiety itself, only a proportion of the silanol groups can be bonded and there is little that can be done to avoid this problem. However, there are other reasons for incomplete silanization of the silica. Incomplete silanization can result from the reagent molecule being excluded from the smaller pores of the silica. Exclusion can be a particular problem when bonding relatively large molecular weight materials such as long chain hydrocarbons onto the silica surface. It is therefore, important to choose a silica gel that has a relatively large pore size (*e.g.*, a mean pore diameter of



150Å) which may limit the surface area to between 150 and 250 sq.m per gram and thus, reduce the retentive capacity of the stationary phase. The solvents normally used in bonded phase synthesis are aromatic hydrocarbons *e.g.,* toluene that boils at 110ÚC or mixed xylenes that boil 138-140^{Ú}C. The procedure varies a little depending on the size of the batch and the type of silanizing reagent. A method of synthesis of bonded phase for the alkoxysilane reagents is illustrated below. The most reactive alkoxy reagents are the methoxy and ethoxysilanes and their reaction with a hydroxyl group is accompanied by the release of methanol or ethanol.

The final capping process is also the same as that employed in the method using the chlorosilanes reagents, utilizing hexamethyldisilazane as the capping reagent. The alkoxy-silanes are almost as readily available as the chlorosilanes and are easier and more pleasant to handle.

LC Mobile Phases

The choice of phase system can be very complex, particularly if multicomponent mixtures are to be separated. In the first instance the type of stationary phase needs to be chosen and this choice must be based on the interactive character of the solutes to be separated. If the solutes are predominantly dispersive then the stationary phase must also be dispersive (a reversed phase) to promote dispersive interaction with the solutes and provide adequate retention and selectivity. If the solutes are strongly polar then a polarizable stationary phase (one containing aromatic rings or cyano groups) would be appropriate to separate the solutes by polar and induced polar interactions. If the solutes are weakly polar then a strong polar stationary phase would be required (such as silica gel) to separate the solute by polar interactions.

Column Ovens

The effect of temperature on LC separations is often not nearly so profound as its effect in GC separations, but can be critical when closely similar substances are being separated. In LC a change in temperature will change the free energy of the solute in both phases, (generally in a commensurate manner) and so the net change in the free energy difference with temperature, which controls the magnitude of the absolute retention, can be relatively small. Its effect on relative retention, however, can be very significant and, in fact, be the determining factor in achieving a satisfactory resolution. An increase in temperature will increase the diffusivity of the solute in both phases and thus increase the dispersion due to longitudinal diffusion and decrease dispersion due to resistance to mass transfer.

HPLC Detectors

A large number of LC detectors have been developed over the past thirty years based on a variety of different sensing principles. However, only about twelve of them can be used effectively for LC analyses and, of those twelve, only five are in common use. The dominant detectors used in LC analysis are the UV detector (fixed and variable wavelength), photo diode array detector, the

electrical conductivity detector, the fluorescence detector and the refractive index detector. These detectors are employed in over 95% of all LC analytical applications.

The UV Detector

The UV detector is by far the most popular and useful LC detector that is available to the analyst at this time. Although the UV detector has some definite limitations (particularly for the detection of non polar solutes that do not possess a UV chromaphores) it has the best combination of sensitivity, linearity, versatility and reliability of all the LC detectors so far developed. Multi-Wavelength UV detectors utilize a single of wavelengths to detect the solute. Most multi wavelength UV detectors can also provide a UV spectrum of the eluted solute if appropriately arranged.

Electrical Conductivity Detectors

The electrical conductivity detector can only detect those substances that ionize and consequently, are frequently used in the analysis of inorganic acids, bases and salts. It has also found particular use in the detection of organic acids and bases that are frequently required in environmental studies and in biotechnology applications. The sensor is the simplest of all the detectors consisting of only two electrodes situated in a suitable flow cell.

Fluorescence Detector

The fluorescence detector is one of the most sensitive LC detectors and for this reason is often used for trace analysis. Unfortunately, although the detector is very sensitive, its response is only linear over a relatively limited concentration range. In fact, the response of the detector can only be assumed to be linear over a concentration range of two orders of magnitude. Unfortunately, the majority of substances do not naturally fluoresce which is a serious disadvantage to this type of detector. It follows, that in many instances fluorescent derivatives must be synthesized to render the substances of interest detectable.

Refractive Index Detector

The refractive index detector is one of the least sensitive LC detectors. It is very sensitive to changes in ambient temperature, pressure changes, flow-rate changes and can not be used for gradient elution. Despite these many disadvantages, this detector is extremely useful for detecting those compounds that are nonionic, do not adsorb in the UV, and do not fluoresce.

HPLC Data Acquisition

The output from the detector, usually in millivolts, is passed to a scaling amplifier that converts the signal to a voltage that is acceptable to the analog to digital (A/D) converter The A/D converter changes the voltage output to a binary number which is temporarily stored in a register. This process is continuously repeated at a defined rate, called the 'sampling rate'. The current binary number, stored in the register is regularly sampled by the computer and stored (usually on hard disk). On completion of the analysis the computer accesses all the data from store, calculates the retention report, compares peak heights or peak areas to provide the quantitative analysis according to the processing program that is used and finally prints out the results in tabulated form. Modern data processing software often includes routines that can process chromatograms where the components of the sample are incompletely resolved. The routines deconvolute the individual peaks from the

composite envelope and calculate the area of the individual de-convoluted peaks. Such algorithms can be used very effectively on peaks that are entrained in the tail of a major peak but are not so accurate for composite envelopes containing many unresolved peaks.

Conclusions

HPLC is probably the most universal type of analytical procedure; its application areas include quality control, food component analyses, process control, forensic analysis, environmental monitoring, and clinical testing. In addition HPLC also ranks as one of the most sensitive analytical procedures and is unique in that it easily copes with multi-component mixtures. It has achieved this position as a result of the constant evolution of the equipment used in LC to provide higher and higher efficiencies at faster analysis times with a constant incorporation of new highly selective column packings.

Suggested Reading

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