HPLC: The New King of Analytical Chemistry?

More often than not, the identification of the constituent components of a mixture requires that they be separated from one another, a task well suited to the various chromatographic techniques: gas chromatography, liquid chromatography, and thin-layer chromatography. Of these, a modern variant of liquid chromatography called high-pressure (or, more often, high-performance) liquid chromatography (HPLC) is far and away the fastest growing. Numerous market surveys reveal that, as measured by instrument sales, HPLC use has been increasing by approximately 30 percent per year for the last few years and will continue to grow at the same rate for some time to come. An informal reader survey by the editors of *Industrial Research/Development* indicates, moreover, that the analytical instrument most readers will buy next is a liquid chromatograph.

A reporter traipsing the floors of the instrument exhibition at the Pittsburgh Conference found no cause to doubt these and other glowing forecasts of the future of HPLC. Including those companies offering accessories such as valves and fittings, solvents, column packings, and detectors, as well as those showing complete HPLC systems, nearly one in every ten exhibitors had some tie-in with liquid chromatography. And salesmen were more than ready to help. Said one, "We are really pushing HPLC this year."

The reason for the enthusiasm is not hard to find. A widely circulated estimate is that 80 to 85 percent of all chemical compounds are amenable to analysis by HPLC. By contrast, the most popular chromatographic technique, gas chromatography (which accounted for about three times the value of instrument sales in 1978 as liquid chromatography), is restricted to those substances that can be vaporized without tearing the compounds of interest apart. Thus, gas chromatography is best reserved for compounds with masses of 200 daltons or less, whereas HPLC is applicable to compounds with masses ranging from less than 100 to several million daltons. It was only historical difficulties in devising practical instrumentation that prevented earlier popularization of liquid chromatography and allowed gas chromatography to steal the march on its more versatile cousin.

Whereas the growth of gas chromatography followed immediately on its development in the early 1950's, complete liquid chromatography systems were not described at the Pittsburgh Conference until 1963, and commercial HPLC instruments did not make their debut until 1969. Since then, use of the technique has skyrocketed to the point that world sales of instruments and accessories totaled $125 million in 1978, according to James Little of Waters Associates, the leading HPLC manufacturer. Figures from another source divided this market as follows: Waters is the front-runner with about 45 percent of sales; Hewlett-Packard is second with about 15 percent; and clustered just behind Hewlett-Packard are Varian, Perkin-Elmer, and Spectra-Physics. If all this sounds too much like a Wall Street report, it is no accident; one veteran observer of trends at the Pittsburgh Conference not jokingly suggested that the local stockbroker might just be the best source of information on who is hot in HPLC.

Liquid chromatography actually subsumes several distinct techniques, but they all have one thing in common: a narrow column packed with a material that restricts the flow of the components of a sample dissolved in a liquid passing through the column. If the packing material has a different affinity for each component, it holds up passage of each for a different length of time and a separation of components is achieved. The trick in liquid chromatography is to pass all the components through the column as rapidly as possible, so that the analysis does not take too long a time and, at the same time, slowly enough to achieve a complete separation.

In the early days of liquid chromatography, which extend even farther back than the 1906 reports of the Russian botanist Mikhail Tswett, the technique was not much more complicated than placing the solution on the open top of a column, waiting, removing the packing, and examining each component where it rested in a well-defined band at a particular height in the packing. Things changed in 1938 when Tadeus Reichstein of the University of Basel in Switzerland put forth the idea of letting the solution pass all the way through the column and then catching each component as it eluted from the end. But the whole process was too slow, a matter of many hours, to be of widespread interest.

The secret in speeding up (the high-performance part) liquid chromatography was the use of small packing particles (now, spheres 5 to 10 micrometers in diameter are used) in small columns (10 to 25 centimeters long and with an inside diameter of 5 millimeters). In this way, the distances traveled by components on their travels through the column are much reduced and, hence, so are their travel times. But accomplishing this requires the application of high pressures (1000 to 5000 or more pounds per square inch) and specially designed pumps to provide a constant flow of solution through the column, usually at the rate of a few milliliters per minute.

The different types of HPLC are classified by the nature of the affinity between the packing material and the components of the sample. One speaks of adsorption chromatography when the components stick to the surface of a porous solid material, such as silica gel, that has a high ratio of surface area to volume. If the solid is coated with a liquid layer in which the components are differentially soluble, one refers to partition chromatography. Archer Martin and Richard Synge, who were then at the Wool Industries Research Association, Leeds, England, received a Nobel Prize for their 1941 exposition of this concept. One of the later developments that made HPLC systems practical was finding a way to chemically link the thin liquid layer to the solid packing particle so that the liquid would not continually be washed off and a chemical equilibrium could be established between the fractions of the analyzed species dissolved in the two liquid phases. For this reason, the term bonded-phase chromatography has replaced partition chromatography in HPLC.

> HPLC use has been increasing by about 30 percent per year.

In the parlance of chromatography, the flowing solvent is called the mobile phase, whereas the solid packing and the bonded liquid layer are called the stationary phase. In normal partition chromatography, the mobile phase is a nonpolar liquid and the stationary phase is polar. In many situations it has proved possible to achieve better separations by reversing the polarities of the two liquid phases. When this change is made, one refers to reverse phase chromatography. As it turns out, reverse phase chromatography is now the most widely used variation of HPLC.

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Two other forms of HPLC are ion exchange chromatography and size exclusion chromatography. In ion exchange chromatography, ion exchange resins in the form of small or porous particles serve as the column packing material. This technique is generally restricted to such specialized uses as amino acid analysis where the materials to be separated are ionic species. In size exclusion chromatography, the dimensions of the pores in the packing material are small enough to physically prevent the passage of large molecules such as polymers, and the technique is primarily used for polymer analyses, such as determinations of molecular weights.

In principle, any HPLC instrument could accomplish, with the appropriate column and detector, any of these forms of liquid chromatography. In practice, not all can, and, in particular, size exclusion chromatography is usually done by a special chromatograph designed specifically for this purpose; part of the reason for this is that the column must often be kept at a high temperature for this type of chromatography.

Besides a column and a pump, the other essential part of a liquid chromatography system is the detector. The three most common detectors measure refractive index, ultraviolet absorption, or fluorescence emission. Detectors are therefore essentially conventional optical instruments modified to incorporate special flow cells through which the solution passes continuously. Since the volume of a flow cell is quite small (a few microliters), the optics must be adjusted to handle samples much smaller than normal.

Of the three types of detectors, the refractive index instrument—which detects the presence of a component if its refractive index is different from that of the solvent—is the closest thing to a universal detector for all substances, but it is not as sensitive as the other detectors (minimum detectable sample is 5×10^{-7} gram per milliliter). The ultraviolet absorption detectors are a thousand times more sensitive, but they record absorption at only one wavelength (usually 254 nanometers) and not all substances have absorption bands in this region of the spectrum. Recently, the use of variable wavelength ultraviolet spectrophotometers has become popular. But because the intensity of light from sources that emit light over a broad range of wavelengths is much lower than that from sources that emit at only a few discrete wavelengths, the sensitivity of these instruments is lower. Fluorescence detectors are more sensitive yet, but they are usable only if the analyzed substance is intrinsically fluorescent or if it can be made to emit light by the addition of fluorescent moities or fluorophores.

Instruments that incorporate these three ingredients (pump, column, and detector) along with the now almost obligatory microcomputer to control their operation and a second computer for data handling come in a wide variety of sizes, capabilities, and costs. The cost, in fact, is rather modest—at least when compared to some other types of instrumentation, and it is difficult to pay more than about $40,000 for a "Cadillac" model HPLC. According to David Banks of Hewlett-Packard, the most rapidly growing segment of the liquid chromatography market is that devoted to more or less routine separations, as opposed to frontier research. Much of the motivation for this trend appears to lie with government environmental and safety regulations that have driven companies to monitor their chemical processes more closely. Another motivation stems from the need to control the properties of chemical products. As simple a thing as the tartness of grapefruit juice can be fine-tuned by adjusting the concentration of a certain acid. In any case, the HPLC instruments shown at the Pittsburgh Conference seemed to be oriented toward users interested in testing large numbers of samples with minimum operator attention.

Several manufacturers thus exhibited instruments with automatic sampling devices. In most cases, these are carousels holding (depending on the manufacturer) approximately 60 test tubes or vials. Usually three or more sample injections could be taken from each vial, so that about 200 samples could be taken automatically. (One company, Altex Scientific, demonstrated an autosampler in which the vials were stacked vertically rather than in a carousel.)

A complication that arises for multicomponent samples is that the best solvent (mobile phase)—that is, the solvent that will provide evenly spaced peaks separated sufficiently so that complete resolution is achieved but not so widely that too much time is wasted—can vary from one pair of components to another. A partial solution to this problem is to continuously vary the composition of the solvent as the chromatogram is being run. Generally two, but in some cases three, solvents can be mixed in any desired proportion that changes with time. This procedure is called gradient elution.

Thus, the microcomputer that controls the operation of the liquid chromatograph must be able to set the basic operating parameters of each separation, such as pressure limits, flow rate, and column temperature, and at the same time manage the gradient program appropriate to each sample. For a hundred or more samples, it is well beyond the microcomputer's capability to remember

Three-Dimensional Fluorescence Spectroscopy

The first commercial instrument designed to record automatically three-dimensional spectra of fluorescence intensity as a function of both excitation and emission wavelengths made its debut at this year's Pittsburgh Conference. The Fluorocomp total luminescence spectroscopy (TLS) system introduced by Baird Corporation combines a high-resolution ratio spectrofluorometer with a minicomputer, a floppy disk file system, a digital plotter, and a software package, all at a cost of about $45,000.

Fluorescence spectroscopy is an established method for qualitative and quantitative analyses of substances, such as aromatic organic compounds, that fluoresce—that is, absorb light at some wavelengths and reemit it at others. The traditional approach is to pick a wavelength of light that excites the sample, and then to measure the intensity of light emitted by the sample as a function of wavelength (an "emission" spectrum). Alternatively, the intensity of emitted light at a given wavelength can be measured while varying the wavelength of light in the exciting light beam (an "excitation" spectrum). One problem with these approaches is that a sample containing more than one fluorescent compound produces composite spectra, and it may be difficult to determine how much of a given peak is due to each of the compounds.

With a "total luminescence" (TL) spectrum, fluorescence intensity is measured as a function of both excitation and emission wavelengths. Just as a topographic map of the earth's surface shows elevation as a function of latitude and longitude, a contour plot of the TL spectrum can be made showing fluorescence in-

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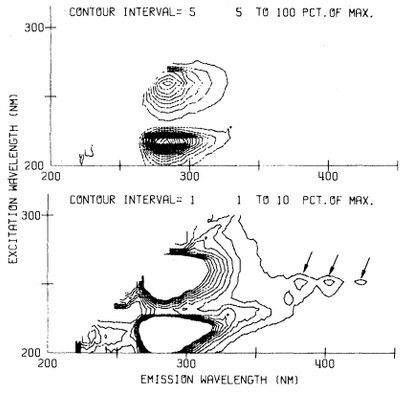
a separate program for each sample, but samples that will be treated alike can be grouped; microcomputer-controlled chromatographs can generally store about ten programs. And instruments can, if necessary, be connected to mini- or larger computers for further direction.

At the other end of the chromatography process are the data, usually in the form of a spectrum giving the response of the optical detector as a function of time. Peaks in the output at specific times provide the identities of the components of the sample, whereas the areas under the peaks are a measure of the quantity of each component present. At a minimum then, data systems report the elution time of each peak and the peak area. Often there are mathematical routines stored and these enable overlapping peaks to be deconvoluted and permit the average peak parameters and the variances for multiple runs to be calculated. And, if errors are detected that

tensity over a range of excitation and emission wavelengths. Compounds with overlapping spectral maxima for a given excitation wavelength may be readily distinguishable at other excitation wavelengths. Also, nondescript bumps and wiggles in an emission spectrum may suggest trace amounts of impurities when the TL spectrum is examined. In the figure, two different contour intervals are used in plots of the TL spectrum of undecylbenzene. With the 1 percent contour interval, three small peaks (indicated by arrows) are suggestive of an anthracene-like contaminant.

Almost any spectrofluorometer can produce TL spectra under manual



control, but the process is rather tedious. With Baird's system, the operator converses with the computer initially, telling it how to produce the desired spectrum. Then the computer takes over. It takes about $1^1/_2$ hours to record a complete TL spectrum, much less time than would be needed with an ordinary spectrofluorometer under manual control. One of the floppy disks is dedicated to system software, the second is used to store TL spectra. Typically, between 10 and 20 spectra will fit on a single diskette. If desired, data stored on the disk can be processed to produce TL contour plots, as well as detailed excitation and emission spectra. More sophisticated routines for processing TL spectra are currently under development.

Baird's TLS system is expected to be a practical addition to research and industrial labs engaged in hazardous materials searches or chemical quality assurance programs. In addition, pharmacologists and clinical and forensic chemists might find such an instrument a valuable asset.—F.F.H.

New Tool for Mass Spectrometry

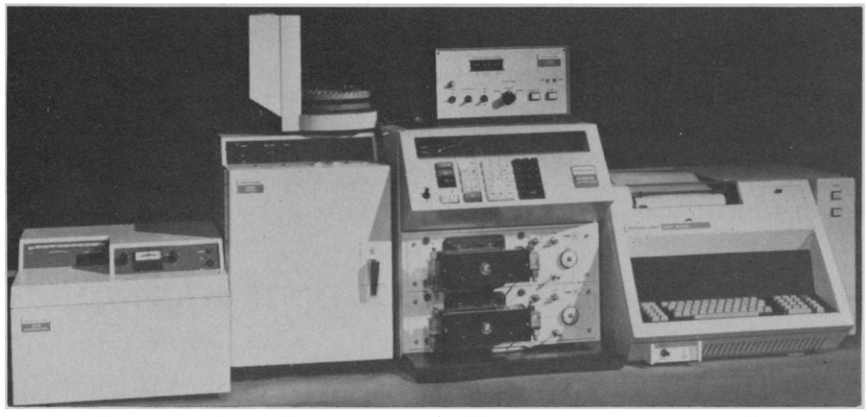
A new instrument that was not physically present at the Pittsburgh Conference, but that was nonetheless discussed with great interest was the Lamma 500, a *l*aser *m*icroprobe *m*ass *a*nalyzer developed by Leybold-Heraus GmbH of Cologne, Federal Republic of Germany. In simplest terms, the instrument is a mass spectrometer in which a laser beam is used to ionize the sample, but several refinements in design are claimed to give it much better spatial and analytical resolution than other types of commercial spectrometers and similar instruments constructed in research laboratories.

The basic components of the system are a laser, an optical microscope, and a time-of-flight mass spectrometer. Thinly sliced specimens similar to those used for microscopy are placed in the evacuated entry port of the spectrometer under a cover slide that functions both as an optical window for the microscope and as a vacuum seal for the port. The microscope serves both to image the specimen and to focus the laser on a small area (less than 1 micrometer in diameter) of the specimen chosen for analysis. A short pulse of the laser vaporizes about 10^{-13} gram of sample and simultaneously ionizes it. The microplasma thus created is drawn into the spectrometer by magnetic fields. Each laser pulse produces a complete mass spectrum of either positive or negative ions in about 1 second. Because more than half of the ionized sample is drawn into the spectrometer, detection limits are quite low, on the order of 10^{-18} to 10^{-20} gram. This is comparable to or better than any other commercial instrument, the company contends.

For analysis of elements, Lamma spectroscopy is thus quite similar to electron probe microanalysis (EPM) (*Science*, 22 July 1977, p. 356), with the obvious difference that the detection techniques are different. Lamma can identify the isotopic composition of a sample, whereas EPM cannot. Lamma can also be used for organic materials; EPM cannot. The new technique is particularly useful for identifying organic compounds, the company says, because the parent ion (from which the molecular formula of the compound can be obtained) is produced in abundance and because mass spectra produced by Lamma are generally much simpler than those produced by other techniques. Polymeric material generally breaks down into ionic species characteristic of its chemical structure.

Many of the applications are similar to those of EPM. Lamma spectrometry has been used, for example, to study the distribution of lithium in brain cells, of fluorine in teeth, and of calcium in muscle cells. It has also been used to detect individual manganese-containing bacteria in heterogeneous populations of bacteria, microdistribution of preservatives in wood, and uranium in carbon microspheres. The technique can also, however, presumably be extended beyond EPM to study the subcellular distribution of organic materials, such as drugs, metabolites, and so forth.

The Lamma 500 now sells for the equivalent of $200,000 in Germany, but will probably be somewhat more expensive in this country because of import duties.—T.H.M.



The price of Perkin-Elmer's automated HPLC system is just over $41,000.

make results meaningless, the computer can turn the chromatograph off or move on to another set of samples.

Manufacturers offering complete systems with most of these capabilities included Hewlett-Packard, Micromeritics, Perkin-Elmer, Spectra-Physics, Varian, and Waters. Companies offering complete systems, with the exception of data systems, included Altex and DuPont. For those willing to live without push-button keyboards and microprocessor control, liquid chromatographic systems were offered by Glenco Scientific, Instrumentation Specialties Company, Laboratory Data Control, Tracor Instruments, and the Schoeffel Instruments Division of Kratos. Finally, all these companies and Gow-Mac Instrument Company manufacture basic liquid chromatographs (column, pump, and detector) without the gradient elution capability. Nongradient systems (also called isocratic), it might be emphasized, are all that are needed for routine quality control–type applications and can be purchased for as little as $1750, although most range in price from $4000 to $8000.

Liquid chromatographs have a lot in common with stereo audio systems. In particular, one can assemble a system from components sold by one or several manufacturers according to one's tastes or buy an integrated system with all components unified in a single cabinet. Hewlett-Packard and Varian, for example, take the second approach, whereas Waters, Perkin-Elmer, and most others take the modular route.

For those who are intrinsically modular in their thinking or who may have older, noncomputer-controlled liquid chromatographs, a company called Sys-Tec of New Brighton, Minnesota, sells a controller system that can be used with most commercial HPLC units. Among other features, the Sys-Tec controller can store gradient elution procedures for 99 different samples, and it can monitor operation of the chromatograph and set off alarms or halt operation in case of malfunctions. Although far-fetched, said Mark Brenner of Sys-Tec, the controller could activate an automatic dialing telephone and play a prerecorded message to a researcher at home, if an emergency arose. The price of the controller is $4250.

One of the major difficulties in liquid chromatography is sample preparation. It is this facet of the overall sequence of operations that has, since the advent of automated instruments, become the most time-consuming and labor intensive. Thus, attention is now being given to automated sample preparation systems. Perhaps the most ambitious of these so far is the FAST-LC system developed by Technicon Industrial Systems. The FAST-LC is essentially a combination wet chemistry laboratory and HPLC. A solid or liquid sample introduced into the system can automatically be subjected to such operations as one or more solvent extractions, derivatization, filtering, and evaporation to dryness prior to being injected into the HPLC. If necessary, further chemistry can be done automatically on samples after elution, such as addition of fluorophores to nonfluorescent components of a sample. Complete FAST-LC systems range in price from $25,000 to $38,500, depending on options.

A second sample preparation instrument at the Pittsburgh Conference was shown by Instrument Specialties Company. An array of 210 test tubes can be moved so that any desired tube is placed in the sample processing station. At the station, virtually any wet chemistry process can be accomplished. Operation of the processor is by way of a keyboard that feeds instructions to a microcomputer. Up to 960 instructions can be entered. Costing $20,000, the sample processor is not by any means restricted to HPLC sample preparation. Among other capabilities are automation of titrations, protein determinations, tissue culture procedures, enzyme kinetic studies, and peptide syntheses.

A third sample preparation instrument, the PREP-1 by DuPont, is less universal. Able to handle up to 12 samples at once, the PREP-1 is a centrifuge that forces the starting solution through a resin bed that concentrates the desired component and passes the remainder. A second centrifuge operation together with the use of a washing solution collects the sample, which is then transferred manually to the HPLC. The cost of the PREP-1 is $7950.

One of the hallmarks of gas chromatography has been the mating of the chromatograph with instruments such as mass spectrometers or infrared spectrophotometers, thereby greatly expanding the sensitivity and usefulness of the technique. Gas chromatography/mass spectrometry (GC/MS) accounts for about a third of all gas chromatograph sales, for example. A similar future may be in store for liquid chromatography. Banks noted that the cost of mass spectrometers is continuing to decline rapidly and that therefore (a hint?) it would not be surprising to see an LC/MS instrument at a future Pittsburgh Conference.

The trend toward combination instruments is, in fact, already under way. The Nicolet Instrument Corporation introduced a combination HPLC-Fourier transform infrared spectrophotometer. Fourier transform instruments of any kind require minicomputers to accomplish the fast Fourier transform algorithm and thus are inherently expensive. Nicolet's LC/IR is priced at about $120,000. The system is so new that researchers do not yet know what its most useful applications may be. One that has been suggested is the examination of polymers in size exclusion chromatography. The infrared spectra would give information about the details of the polymer's functional groups.

A second combination instrument is being experimentally marketed by Varian, a liquid chromatograph–gas chromatograph. As envisioned by Varian, researchers could select any desired peak in the liquid chromatogram and automatically transfer the corresponding portion of the eluted solution to the gas chromatograph for a more detailed study. The liquid chromatograph thus acts as a rather high-powered sample clean-up device for its gas cousin. Varian sells the interface device needed to transfer samples between chromatographs for $3000.

—ARTHUR L. ROBINSON