Trends in Analytical Scale Separations

James W. Jorgenson

Summary. Although gas chromatography has been called a mature technology, advances are still being made in the development of faster columns as well as detectors which yield more information on solutes. In liquid chromatography short columns packed with 3-micrometer particles are already popular for fast analyses, while long narrow-bore columns are being developed for high-resolution separations. An increasing range of detection modes is enhancing the problem-solving capabilities of liquid chromatography. Electrophoresis continues to be of central importance to molecular biology. Computer-aided analysis and display of electropherogram patterns is helping researchers to better comprehend the wealth of data from two-dimensional electrophoresis. A new mode of two-dimensional electrophoresis is permitting separation of whole chromosomes containing as many as 3 million base pairs.

This article is not intended to be an exhaustive survey of the field of separation science, but rather to discuss some of the recent important developments in the instrumentation and practice of analytical scale separations. In view of the vital role detection plays in determining the success of a separation system as a whole, detection devices and procedures will receive considerable attention here. Certain topics reviewed in the instrumentation issue last year will be covered only briefly or not at all; this applies to the topics of high-performance liquid chromatography (HPLC) of biopolymers (*1*), supercritical fluid chromatography (*2*), ion chromatography, countercurrent chromatography, and field-flow fractionation (*3*), in particular.

Gas Chromatography

Gas chromatography (GC) is a well-developed technique for analysis of substances which are volatile or can be rendered volatile, either with high temperatures or by formation of volatile chemical derivatives. The relative merits of gas chromatography and liquid chromatography, with respect to separating power and detector sensitivity and selectivity, are such that GC is still the method of choice when applicable. Use of high-resolution capillary open-tubular columns is now widespread. This is due primarily to the development of convenient-to-use columns fabricated from flexible fused silica. Also, the manufacture of instruments designed for or compatible with the more demanding capillary columns has made their successful use much easier. The versatility of these capillary columns has been further enhanced by the development of polymer stationary phases which are cross-linked after being coated on the capillaries (*4*). These stationary phases are quite stable, being nonextractable with solvents and exhibiting low bleed. This often permits old columns to be regenerated by washing out nonvolatile contaminants with a series of solvents of various polarities. It also permits more abuse of the columns, such as injection of larger volumes of liquid samples, which would strip off an ordinary stationary phase.

There have been interesting developments in capillary columns of smaller diameter. The normal inside diameter of a capillary column is approximately 250 μm, but recently columns with diameters of 50 μm and below have been prepared. Work by Schutjes *et al.* (*5*) has demonstrated separation of roughly 100 hydrocarbon components from a plant extract in only 6 minutes. These columns require somewhat higher carrier gas pressures than ordinary capillaries and place greater demands on sample injections, system dead volumes, and detector sensitivity and speed. However, the combination of high resolution and speed is quite remarkable. This development makes the analysis of complex volatile mixtures an order of magnitude faster, permitting its application to more routine quality-control and screening tasks, among other things.

A more extreme development in the direction of high-speed analysis is the work of Jonker *et al.* (*6*) with short columns packed with micrometer-sized particles. Figure 1 shows results obtained from a column 1.19 mm in diameter and 32 mm long packed with 10-μm particles and operated at a head pressure of 930 pounds per square inch. The entire separation was completed within 150 msec. This, of course, puts great demands on design of the chromatographic system as well as detector speed. But the work expands chromatography into virtually another domain of measuring instruments, that of the high-speed chemical "sensor." The authors further demonstrate that this high speed creates the possibility for realistic use of ensemble averaging to enhance detectability in chromatography. Potential applications in process control are obvious. An interesting possibility is the use of a high-speed chromatograph acting as a computer's "nose." Lightweight, portable GC systems about the size of a matchbox have been pioneered at Stanford University by Angell and Terry (*7*). Capillary columns and injection valves are fabricated on silicon wafers by using the etching technology developed in the semiconductor industry. These mini-GC's could be suitable for remote monitoring and could even function as personal monitors carried relatively unobtrusively on a person's body.

Interest in "two-dimensional" gas chromatography is enjoying a revival, especially when involving use of capillary columns (*8*). In this technique, a mixture is separated on one kind of column, perhaps a nonpolar stationary phase. A particular portion of the chromatogram which is to be further resolved is then trapped from the effluent of the first column and subsequently introduced into a second column, which is coated with a stationary phase of different polarity. This process is somewhat analogous to the two-dimensional techniques used in thin-layer chromatography, hence the name two-dimensional gas chromatography. The potential resolving power of this approach is extremely high. Introduction of commercial instruments designed specifically to

James W. Jorgenson is an assistant professor in the Department of Chemistry, University of North Carolina, Chapel Hill 27514.

accommodate two-dimensional GC with capillary columns has greatly expanded interest in this technique.

Headway is being made in understanding retention on a molecular level in GC. This will eventually yield benefits of two kinds. First, it will permit rational selection of separation parameters, such as choice of stationary phase and column temperature, for a given separation problem. Second, it will enhance the qualitative information which can be extracted from GC retention data. Precise knowledge of the retention times of a solute on two or more different stationary phases will permit tentative chemical identification of the solute a priori (*9*).

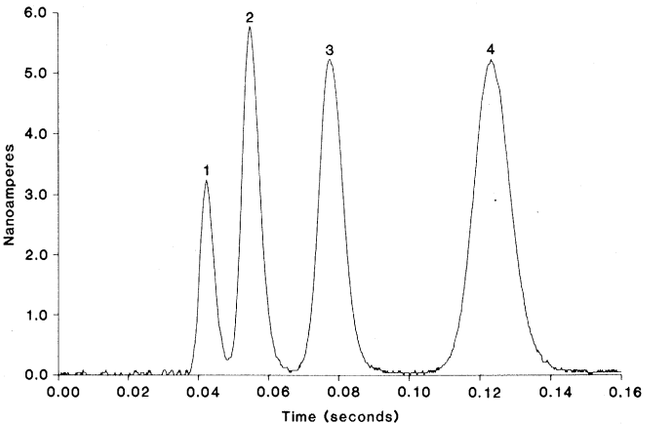

Fig. 1. High-speed gas chromatographic separation of: 1, methane; 2, ethane; 3, propane; 4, *n*-butane. Conditions are listed in the text. [From (*6*)]

The variety of dedicated GC detectors continues to expand. The microwave-induced plasma detector is an element-selective detector that works by monitoring the optical emissions from elements excited in a plasma (*10*). Elements which can be detected selectively include carbon, hydrogen, nitrogen, oxygen, phosphorus, sulfur, fluorine, chlorine, bromine, and iodine. In some designs, several elements can be detected simultaneously, permitting calculation of empirical formulas for solutes based on the relative intensities of the atomic emission signals. Detection limits are in the picogram range for some elements.

An optical absorption detector working in the far-ultraviolet region is also available commercially. Virtually all compounds have strong absorptions in this spectral region, and this detector may become a powerful complement to the array of detectors now available (*11*). Ion mobility spectrometers are proving to be sensitive and selective detectors for GC (*12*). Ion mobility spectrometry is a sort of gas-phase electrophoresis, where ions drift in an electric field in a gas at atmospheric pressure. This technique generates information on the size and charge of ions from their migration times (mobilities). By only monitoring ions of a particular mobility, quite effective selective detection can be accomplished. Although ion mobility spectrometry is at present not capable of as high a mass resolution as mass spectrometry, the ion mobility spectrometer is somewhat simpler and less expensive than a mass spectrometer since it does not require a high-vacuum system. The capabilities of combined gas chromatography–mass spectrometry (GC-MS) and gas chromatography–infrared spectrometry (GC-IR) continue to improve. One of the more striking developments in GC-MS is the introduction of relatively inexpensive quadrupole and ion trap mass spectrometers (*11*). These mass spectrometers are designed as dedicated GC "detectors" but can generate complete mass spectra as well as perform selected ion monitoring. The advantage is that the price has been reduced to the point where it is possible for many more laboratories to afford a GC-MS.

Gas chromatography–infrared spectrometry was made truly feasible by the development of the Fourier transform infrared (FTIR) spectrometer. In this area, too, significantly less expensive models are becoming available, increasing the attractiveness of FTIR spectrometers to a wider number of researchers. An interesting recent development is GC–matrix isolation–FTIR (*13*). In this technique, solute and carrier gas are frozen together on a "cold stage" at liquid helium temperatures, with each chromatographic peak being deposited separately on a different part of the stage. Because the solute molecules cannot rotate in the frozen carrier gas matrix, the resulting infrared spectra have exceptionally sharp and simple spectral lines, free of rotational structure. Spectra can be obtained at leisure long after the chromatogram is through, permitting ensemble averaging and yielding detection limits in the low picogram range. The matrix isolation technique offers a powerful alternative to "on-the-fly" techniques now in use in GC-IR. One problem with infrared spectra obtained in either a gas or frozen-matrix phase is that the spectra do not match the solution-phase spectra that were collected in the past. Large computer-accessible spectral data bases must be developed for either approach to realize its ultimate potential.

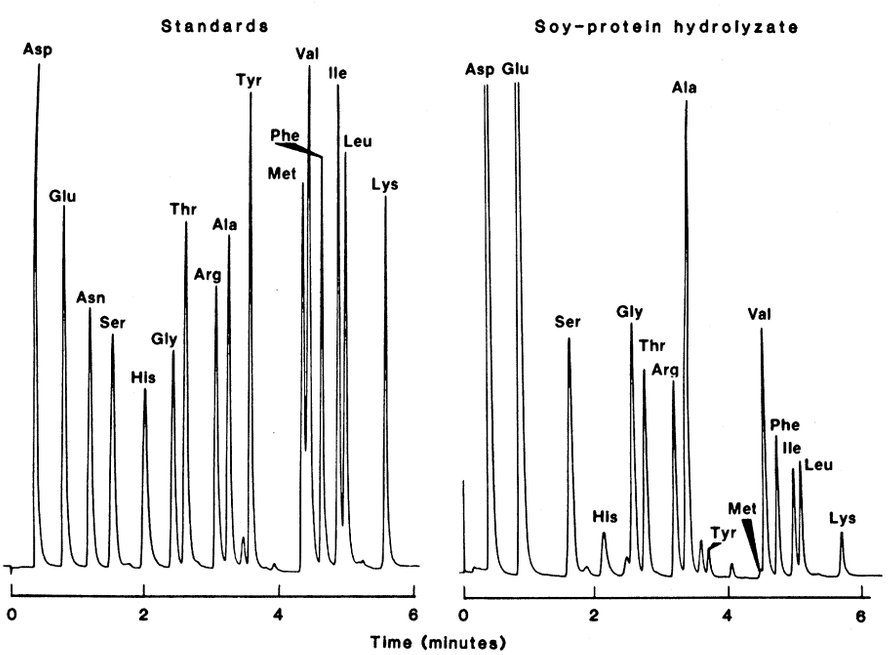

Fig. 2. High-speed liquid chromatographic separation of amino acids as fluorescent orthophthalaldehyde derivatives. Column: Perkin-Elmer, 3 cm long packed with 3-μm reversed-phase particles; mobile phase: sodium acetate buffer to 100 percent methanol, linear gradient in 5 minutes; flow rate: 2.5 ml/min; detector: fluorescence. [From (*14*)]

Liquid Chromatography

Modern instrumental liquid chromatography (LC) has developed into a versatile and powerful technique for separation and characterization of a variety of substances. One of the most important characteristics of HPLC has been its ability to achieve good separation efficiencies in relatively short amounts of time. Five years ago a conventional HPLC column would be 25 cm long with an inside diameter of 4.6 mm and would be packed with 5- or 10-μm particles. Today virtually no column dimension can be considered typical. With the advent of packing materials nominally 3 μm in diameter, a trend toward shorter, "faster" columns has developed. This has culminated in columns as short as 3 cm packed with 3-μm particles, which permit analyses to be completed in a period as short as 1 minute. Figure 2 shows separations of amino acids as orthophthalaldehyde derivatives completed in 6 minutes (*14*). Although complete resolution is not achieved, very good separation is demonstrated in an exceptionally short time, and this is typical of what will become routine over the next few years. It is likely that the days of the dedicated amino acid analyzer are numbered, as rapid and effective single-column HPLC techniques for amino acid analyses are perfected.

Another notable trend in LC involves various columns of reduced diameter known collectively as microcolumns. Packed columns with inside diameters of 0.1 to 1.0 mm are generally referred to as "microbore" columns, although "minibore" would probably be a more appropriate name. Smaller diameter packed columns are usually referred to as packed capillaries. The third class of microcolumns consists of those in which the stationary phase is attached to the wall of a capillary, usually less than 0.1 mm in diameter; these are called open-tubular capillary columns.

Several reasons exist for the interest in microcolumns. First, microcolumns consume significantly less mobile phase than traditional columns. As an example, a column 1.0 mm in diameter will consume only 5 percent of the mobile phase of a column 4.6 mm in diameter when both are operated at the same mobile-phase linear velocity. Not only can this represent a significant savings in the cost of ordinary solvents, but it also makes it possible to employ more exotic mobile phases, such as perdeuterated solvents for use with infrared detection and chiral solvents for separation of optical isomers. The lower flow rates of microcolumns can also be an advantage when connecting a liquid chromatograph to a mass spectrometer, although this depends on the actual LC-MS interface used.

Another advantage of microcolumns is that a very small volume of sample will undergo less dilution when injected into a microcolumn than when it is injected on a traditional column, and thus sample components can be detected with greater sensitivity. The sensitivity advantage of microcolumns applies only in terms of absolute mass sensitivity in the "sample volume limited" case just described. When the volume of sample is not extremely limited (that is, there is enough sample for a normal-sized injection onto a traditional column, approximately 10 μl), there is usually no advantage in terms of sensitivity with microcolumns. In fact, in this case it appears likely that conventional columns and associated equipment offer greater sensitivity in terms of minimum detectable concentrations. Arguments are likely to continue over which approach provides more sensitive detection. The answer to this question is complex and depends on the specific detector used. In general, when the sample volume is quite limited (less than a few microliters), microcolumns will tend to have a sensitivity advantage. But when the sample is not this limited, traditional columns and equipment may offer greater sensitivity.

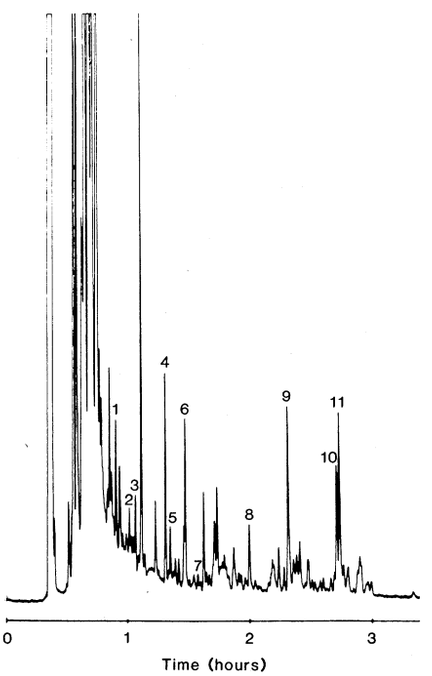

Fig. 3. High-resolution separation of blood plasma steroids (as fluorescent derivatives) by microcolumn liquid chromatography. Conditions are listed in the text. Tentatively identified components: 1, 11β-hydroxyetiocholanolone; 2, tetrahydrocortisol; 3, β-cortolone; 4, androsterone; 5, dehydroepiandrosterone; 7, pregnanetriol; and 8, pregnanediol. Peaks 6, 9, 10, and 11 appear to be polyderivatized androstane derivatives. [From (*15*)]

A third reason for interest in microcolumns is their achievement of high separation efficiencies. In the 1970's column-packing technology produced columns with consistently increasing separation efficiency. However, in the 1980's, with the introduction of 3-μm particles, column development has been directed toward increased speed, while improvements in column efficiency have stagnated. This can be traced in part to the high pressure required to pump liquids through beds of 3-μm particles. When 25-cm-long columns are packed with 3-μm particles, pressures approaching the 6000-pound-per-square-inch limit of most commercial HPLC equipment are required to pump the mobile phase through the columns. As these columns deteriorate, even higher pressures are required. In order to avoid being near the pressure limit, 3-μm particles are usually packed in columns shorter than 15 cm. Although the height equivalent of a theoretical plate is smaller in 3-μm than in 5-μm columns, the 5-μm column can be made longer, and the actual numbers of theoretical plates are comparable. The advantage of 3-μm packings in shorter columns is principally one of speed, and this has been a dominant trend in "conventional" column technology for the past several years. The separation efficiency of conventional columns is still on the order of 10,000 to 20,000 theoretical plates for a 25-cm-long column, virtually unchanged over the past 8 years.

Difficult separations, involving mixtures of exceedingly similar substances or mixtures of great complexity, require more separation efficiency than is available from conventional columns. Efficiencies in excess of 100,000 theoretical plates would certainly be desirable. Connecting several conventional columns in series has proved a marginally effective process. It is an expensive approach and one that requires high pump pressures. Furthermore, results in generating high plate numbers by this method have not been encouraging. Among other factors, the art of connecting columns without introducing significant band spreading in the process is difficult. However, efficient columns a few meters long can be made by slurry packing particles into capillary tubes, particularly tubes of smooth-walled fused silica. Pioneering efforts in this direction have been carried out by the research groups of M. Novotny (*15*) and D. Ishii (*16*). Figure 3 shows a separation of fluorescent deriva-

tives of blood plasma steroids. The fused silica column used in this separation was 1.5 m long with an inner diameter of 240 μm and was packed with 3-μm octadecylsilane reversed-phase silica particles. The required pumping pressure is in the neighborhood of 4000 pounds per square inch and the analysis time is 3 hours. Although the pressure required is fairly high and the analysis time is a bit long, the separation efficiency of this column is roughly 200,000 theoretical plates, and its great separating power is evident in Fig. 3. Although these microbore columns do not achieve high efficiencies on a rapid time scale, they do produce remarkably high efficiencies that are difficult to attain with conventional columns.

Open-tubular columns with internal diameters less than 10 μm offer the potential of separation efficiencies in excess of 1 million theoretical plates within analysis times of about 1 hour. Development of this approach is at a more preliminary stage, as difficult problems in column fabrication and detection must be overcome. However, significant strides have been made in this direction. The extremely small sample volumes (picoliters) required for these open-tubular columns create the prospect for such applications as the high-resolution separation of hundreds of constituents from an individual cell.

Microbore columns have also opened the way for temperature programming in liquid chromatography. Columns of larger diameter have too large a thermal capacity and too slow thermal conduction to permit effective use of temperature programming. However, Bowermaster and McNair (*17*) recently demonstrated the utility of temperature programming with microbore columns, and obtained results similar to those achieved in temperature-programmed GC. This provides a useful and in some instances more convenient alternative to solvent programming in LC.

An alternative approach to microcolumns for high resolution in LC is two-dimensional LC. This can be accomplished in a manner analogous to that described for two-dimensional GC. Two separate columns can be arranged with switching valves to allow a portion of the effluent from the first column to be directed into the second column for further separation. However, Guiochon *et al.* (*18*) have proposed a bolder scheme more nearly analogous to two-dimensional thin-layer chromatography. Their approach is to use a flat rectangular "column" packed with high-performance packing material through which

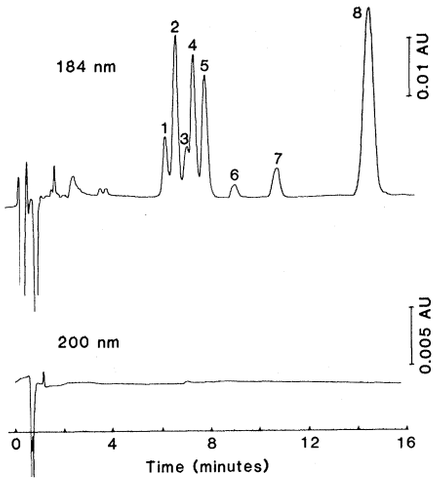

Fig. 4. Analysis of a water extract of an aged sample of *n*-hexanol (100 μl 1 percent *n*-hexanol). The upper trace was recorded at 184 nm, the lower trace at 200 nm. Peaks 1 through 7 are presumably degradation products or impurities in the *n*-hexanol. Peak 8 is *n*-hexanol. Column: 7-μm octylsilane reversed-phase; mobile phase: 75 percent aqueous 1 m*M* phosphoric acid containing 5 ppm HNO_3 and 25 percent acetonitrile; flow rate: 2 ml/min. The cluster of peaks at 6 to 10 minutes has retention of the isomeric pentanols. AU, absorbance units. [From (*25*)]

mobile phase is pumped in one direction. Later, a second mobile phase is pumped in a direction perpendicular to the first. Detection is carried out on-line by using diode-array or vidicon imaging. Preliminary results with this system are promising, but further refinement is needed.

Preparation of new packing materials for HPLC is an active and productive area of research. This is particularly true of HPLC for separation of biopolymers where problems of protein denaturation are to be avoided. As described in the review by Regnier (*1*), much has been learned about the HPLC behavior of biopolymers, and some general conclusions about how biomolecules should be handled in HPLC are emerging.

An interesting material for reversed-phase chromatography is a rigid and porous form of polystyrene/divinylbenzene available as 10-μm beads capable of withstanding pressures to at least 4000 pounds per square inch (*19*). One advantage of this material over traditional silylated silica materials is its ability to be used with a mobile phase *p*H as high as 13, unlike silica, which has an upper limit of about 8. Polystyrene also lacks the troublesome surface silanol groups which often lead to undesirable peak tailing. Furthermore, it is likely that polystyrene-based packings will be much more stable toward exposure to aqueous mobile phases over extended periods of time. The present disadvantages of polystyrene are its lower pressure tolerance, its tendency to swell in organic solvents, and also the fact that lower column efficiencies are observed in comparison to silica-based packings.

Considerable progress is being made in developing systems for separating enantiomers by HPLC (*20*). Of particular note is the work of Pirkle *et al.* (*21, 22*) on the rational design of chiral stationary phases. This group has had exceptional success in separating a wide range of enantiomers, and their stationary phases are now commercially available (*21, 22*). Systems with mobile phases containing chiral molecules are also being used with good results. These techniques are of particular interest to those making synthetic peptides, as a check on the extent of racemization occurring during synthesis.

Selection of mobile phases for optimal separations is now on a more rational footing, thanks in particular to the work of J. L. Glajch, J. J. Kirkland, and L. R. Snyder. They have developed efficient methods for the experimental selection of mobile phases which maximize separation of solutes. Their methods apply not only to isocratic elution, but also to mobile phase gradients, and involve manipulation of both solvent strength and selectivity in a quaternary mobile phase system (*23*).

Detection is an aspect which has seen some important developments in the past few years. Ultraviolet absorption detectors based on photodiode arrays are now commercially available. These detectors permit acquisition of entire ultraviolet and/or visible spectra of chromatographic effluent at a rate of approximately one spectrum every second. These spectra not only are useful for determining the identity of unknown peaks, they can also provide an additional "dimension" for resolution of peaks which are not completely separated chromatographically. Sophisticated computer-based systems for data display are available to enhance ease of comprehension and use of the large amount of data generated by HPLC with photodiode array detection (*24*). Another interesting development in ultraviolet absorption detection is the use of very short wavelengths (below 200 nm) for detection. Van der Wal and Snyder (*25*) have reported on detection of a variety of substances at a wavelength of 184 nm. Figure 4 shows the separation of a variety of saturated alcohols. While detection at 200 nm shows no evidence of solutes, detection at 184 nm is dramatically superior, with solutes very easily detected. The authors were also able to demonstrate the compatibil-

ity of detection at 184 nm with mobile-phase gradients, running from 100 percent water to 35 percent acetonitrile-water, without encountering baseline drift. Although short wavelengths do place strict limitations on the choice and purity of mobile phases, this approach should be useful for the detection of substances otherwise difficult to detect, such as alcohols, sugars, ethers (compounds without conjugated double bonds in general), and, in particular, the majority of polymers.

Electrochemical detection continues to grow rapidly in importance, with high sensitivity and selectivity being its greatest assets. Reports of use of electrochemical detection for amino acids and sugars without prior derivatization are appearing in the literature, and in general electrochemical detection is proving applicable to an ever increasing list of substances. The use of electroactive "tags" to label compounds before electrochemical detection is also receiving increased attention. Dual series and dual parallel electrode configurations have been introduced. By applying different potentials to the two electrodes a variety of approaches to achieve further selectivity in electrochemical detection are possible (*26*).

Fluorescence is an important mode of detection which is capable of high sensitivity. It has an expanding range of application through the increasing number of fluorescent derivatives that can be made from nonfluorescent compounds. A related newcomer to the detection scene is chemiluminescence (CL) (*27*). In CL, energy from a chemical reaction is used to excite a luminescent compound, whose optical emissions are then monitored. Probably the greatest potential advantage of CL over fluorescence is its prospect for detection limits several orders of magnitude lower than those in fluorescence. This is because in CL there is no input beam of light and thus, in principle, the only light detected must originate from luminescing solute molecules (a "zero-background" measurement). Chemiluminescence for HPLC detection is a relatively young technique and so its range of applicability is more limited than that of fluorescence at present. But this situation will probably change quickly as reagents and luminescent "tags" are designed with CL in mind.

Post-column reaction detection is of growing importance. This technique involves post-column on-line chemical reaction of solutes to form more easily detected substances. It is most commonly used with ultraviolet-visible absorption detection or fluorescence detection and has been most widely used in amino acid analyzers (post-column reaction with ninhydrin, fluorescamine, orthophthalaldehyde, and so on). Reaction may be accomplished by mixing the column effluent with liquid reagent streams and then allowing sufficient time and temperature for the reaction to proceed. This necessitates a "mixing coil," a length of tubing between column and detector, where the liquid streams mix thoroughly and the reaction takes place. For slow reactions, excessive peak spreading will occur in this mixing coil, and the technique of flow segmentation with gas bubbles (as used in the Technicon autoanalyzer) has been applied as a remedy. Alternatively, reagent can be immobilized on a solid support, packed as a porous bed, and column effluent allowed to flow over it. This approach minimizes many of the problems associated with post-column mixing of liquid streams (*28*).

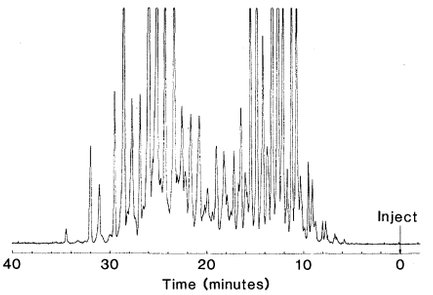

Fig. 5. Separation of triglycerides of butterfat. A 5-μm octadecylsilane reversed-phase column was used. Mobile phase linear gradient: 60 percent acetone, 40 percent acetonitrile at time 0, to 99 percent acetone, 1 percent acetonitrile at 35 minutes. Flow rate: 0.8 ml/min. [From (*29*)]

A long-term problem in LC has been the lack of any completely general detector whose response is proportional to the mass of eluting solute. As an example, with ultraviolet absorption detection the largest peak in a chromatogram may be due to a minor component in the sample, while the major constituents may remain undetected. A new approach to this problem involves nebulization of column effluent in a flowing gas stream, followed by evaporation of the mobile phase, leaving small particles of nonvolatile solute. These solute particles are detected by light scattering while still entrained in the gas flow. Stolyhwo *et al.* (*29*) have demonstrated a version of this detector which has an approximate detection limit of 1 ng of solute injected into an HPLC column. The detector response is roughly proportional to the square of the mass of solute injected, but the response factors for all compounds are very nearly the same. Of course, the detector can only detect relatively nonvolatile solutes, but these are the ones likely to be encountered in LC, since volatile solutes can be analyzed by GC. A further advantage of this detector is that it is compatible with mobile phase gradients, unlike the other "general" LC detection mode, refractive index. Figure 5 shows a separation of butterfat triglycerides on a reversed-phase column with a gradient in acetonitrile and acetone. One definite limit of the light-scattering detector is that it cannot be used with mobile phases containing nonvolatile salts. However, the relatively good sensitivity and nondiscriminatory response make this detection principle extremely attractive where it is applicable.

A very subtle development in quantitation by LC has come from the group of E. S. Yeung at Iowa State University. They are developing approaches which permit accurate quantitation without requiring any knowledge of a solute's identity. This somewhat surprising result is made possible by measuring the detector's response to a solute and mobile phase in two consecutive chromatographic runs, using two different mobile phases. Identical amounts of sample are injected in each run. Since the solute dilutes the mobile phase, a quantitative measure of the amount of solute present is possible. This quantitation principle works with any detector which measures a property of the mobile phase, such as refractive index and conductivity detectors, and yields quantitative information in the form of the volume fraction or mole fraction of solute. It is also applicable to the thermal conductivity detector in GC (*30, 31*).

On-line LC-MS is developing rapidly and is reviewed by Vestal (*32*). Combined liquid chromatography–infrared spectrometry has received some attention, but solvent interferences create obvious limitations. Single-wavelength infrared detection has proved especially useful in detecting polymers in gel permeation chromatography.

Considerable interest in the use of lasers in LC detection has been evident. Use of lasers in chemical analysis is reviewed by Zare (*33*), so only a few comments on lasers and detection in HPLC will be made here. Lasers have been used with good to excellent results for detection by such principles as fluorescence, photoionization, thermal lens calorimetry, photoacoustic spectroscopy, and Raman spectroscopy. Although the marriage of HPLC and lasers is promising, the routine adoption of such

devices is hindered by the current state of the art in lasers. The most useful lasers, those which have high power and tunability in the ultraviolet spectral region, are expensive, complex, and not as reliable as necessary. A few lasers with more limited power and wavelength coverage are exceptions to this rule (that is, helium-neon and helium-cadmium lasers). The ultimate impact of lasers on LC detection hinges mainly on the development of less expensive, more reliable, tunable, simple to operate lasers.

Electrophoresis

Electrophoresis has been developed principally by biochemists for the separation of biologically derived molecules, especially biopolymers. It has evolved as a sophisticated and powerful group of techniques, yet one that involves a great many hand operations. Its development has been rapid, and its importance in biochemistry and the life sciences rivals that of chromatographic techniques. Gel density gradient electrophoresis is at the heart of the new techniques for nucleotide sequencing. And the startling work of O'Farrell (*34*) in two-dimensional electrophoresis (isoelectric focusing followed by gel gradient electrophoresis) has spawned many important applications in the analysis of complex protein mixtures (*35*).

An interesting recent trend is the use of very thin gels (as thin as 50 μm) for electrophoresis (*36*). Such gels promote much more favorable heat transfer, permitting use of higher electric field gradients and resulting in faster analyses. The rate of staining and destaining steps is also enhanced, further reducing the time required for an analysis. Of course, there is a trade-off in lower sample capacity. In the field of isoelectric focusing, the development of immobilized *p*H gradients is of interest (*37*). Immobilization prevents drift of the gradient during running and eliminates conductivity gaps.

In the area of zone detection several recent developments should be mentioned. In addition to the traditional organic dye stains, there are now metal-based "stains" for localization of protein bands in gels. Silver stain is significantly more sensitive than Coomassie blue for protein detection, and the technique is much faster (*38*). The "Kodavue" stain, developed by Eastman Kodak, is said to have faster processing and a more linear rate of development than the silver stain (*39*). The Kodavue system is based on reduction of a nickel complex to metallic nickel. The chemistry is such that staining kits are claimed to have an indefinite shelf life, and prepared working solutions have a shelf life of several months. Detection limits for both the silver stain and the Kodavue stain are in the low nanogram range for proteins, although those who have worked with both have found the silver stain to be roughly ten times more sensitive.

Complex electropherogram patterns, especially those from two-dimensional electrophoresis, have created new challenges for data collection and manipulation. The combination of imaging with vidicon or diode array cameras, followed by computer analysis and display of the images, is under intensive development (*40–43*). Problems with reproducibility of spot positions and staining intensity must be overcome for computer-assisted detection to be more useful. A variety of image analysis and display techniques are possible, including color-coded "topographical maps" and "three-dimensional" perspective plots. Computer techniques for enhancing band detection, highlighting spot boundaries, and matching spots on two different plates also exist. Scatterplots and correlation plots are convenient display techniques for comparing quantities of proteins present on two separate plates, and permit rapid comparison of the rate of synthesis of hundreds of proteins from different sources.

As already mentioned, electrophoresis is pivotal in the separation of nucleotide polymers. This applies to the separation of extremely large DNA polymers as well. Recently, a new type of two-dimensional electrophoresis was described by Schwartz and Cantor (*44*) for the separation of chromosome-sized DNA's. Traditional electrophoretic methods for separation of polynucleotides rely on the gel sieving effect. This approach is useful only with DNA polymers shorter than about 50,000 base pairs (50 kb). Larger DNA's migrate through gels with anomalously high mobilities, presumably because of their ability to rearrange their conformation in any way necessary to permit them to continue migrating through the gel network. This difficulty can be alleviated somewhat by reducing the gel concentration. At agarose concentrations as low as 0.15 percent, DNA's as large as 750 kb can be handled, but the gels are quite fragile. The technique of Schwartz and Cantor makes use of inhomogeneous electric fields (electric field gradients). In an electric field gradient DNA molecules are elongated along the field lines. After sufficient time for the molecule to be fully elongated, the first inhomogeneous field

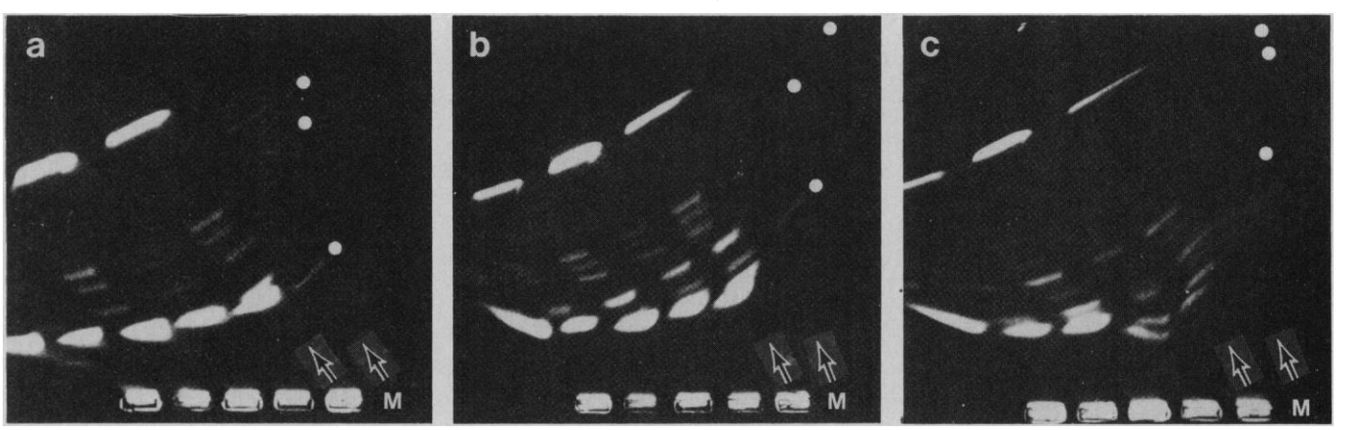

Fig. 6. Separation of yeast chromosomal DNA's as a function of pulse time. Pulse time: (a) 25 seconds; (b) 40 seconds; (c) 45 seconds. Sample application wells are at the bottom of each plate, with the direction of migration indicated by the arrows. Lane M contains the molecular weight markers G, T2, and T7 bacteriophage (758, 166, and 40 kb, respectively, bottom to top). The bands for these markers are so faint that the white dots indicate their positions. The lanes starting from the unlabeled arrow contain alternatively, from right to left, DNA from yeast strains D273-10B/A1 and DBY782 and DNA stained with ethidium bromide. [From (*44*)]

is turned off, and a second field (homogeneous or inhomogeneous) is applied in a direction perpendicular to the first. Initially, the molecule finds it impossible to migrate in response to the new field because it is elongated perpendicular to its intended direction of travel, and in this conformation it cannot penetrate the gel. However, the molecule eventually relaxes back to a more compact configuration, from which it can then migrate through the gel. This relaxation time depends on molecular weight; the longer the DNA, the longer the relaxation time. Shortly after the molecule has relaxed and is migrating through the gel, the original inhomogeneous field is reapplied. This entire sequence is repeated thousands of times. The net effect is that the DNA follows a diagonal path across the gel, and the smaller the DNA, the faster it migrates. Since relaxation times of large DNA's are on the order of tens of seconds, the appropriate duration for each applied field before switching to the perpendicular field (the pulse time) varies from a few seconds to a hundred seconds. An agarose gel concentration of 1.5 percent has been used in the work so far with exceptional results. Figure 6 shows electropherograms of yeast chromosomal DNA's separated by this method with three different pulse times. The middle case (40-second pulse time) shows the best overall resolution of zones; seven separate zones are apparent. Separations require on the order of 10 hours, and molecules as large as 3000 kb (roughly 2×10^9 daltons) have been separated so far. The authors state that larger chromosomes from more complex eukaryotes will probably require somewhat lower agarose concentrations, but should be amenable to separation by this technique.

Free zone electrophoresis (electrophoresis without a stabilizer such as a gel) has received some attention recently. It has the advantage of being applicable to the separation of particles, such as cells, which are too large to migrate through the network of pores in gel media. However, the lack of gels can lead to troublesome convective flows, resulting in a serious loss in resolution. Elaborate schemes have been developed to counteract the zone-spreading effects of convection. Such techniques as rotating the electrophoresis tube about its longitudinal axis, developed by Hjerten (*45*), and subjecting solutes to helical "orbital" motions, as developed by Kolin (*46*), have been successfully demonstrated. The fact that convective flow should be absent in the "gravity-free" environment of an earth-orbiting satellite has led McDonnell Douglas and NASA to conduct electrophoresis experiments involving separations of serum components in the space shuttle (*47*). Such testing may lead to large-scale preparative electrophoresis of sophisticated pharmaceutical products in space. Free zone electrophoresis in capillaries of 75-μm inner diameter in the earth's gravity is an alternative approach (*48*). The small diameter and high surface-to-volume ratio of such capillaries diminish the magnitude of convective flow. Isotachophoresis, particularly in capillaries, continues to grow in importance (*49*). This variant of electrophoresis can be a powerful method for separating substances of similar mobility, and commercial equipment has been available for many years.

Other Separation Methods

Supercritical fluid chromatography (SFC) was reviewed by Gere (*2*) in last year's instrumentation issue. Interest is growing in this technique, which makes use of fluids beyond their critical points as mobile phases. Supercritical fluids have properties somewhere between those of classical liquids and gases. Their densities are high enough to offer some of the solvating power of liquids, while their viscosities are low enough to permit the fairly rapid solute diffusion more characteristic of gases. In general, the performance characteristics of SFC lie somewhere between those of GC and LC, and the technique is proposed to bridge the "gap" between GC and LC. The main question is, how significant is the gap between GC and LC? With GC already able to handle compounds of fairly low volatility, and with faster and higher resolution modes of LC on the horizon, the middle ground of analytical problems poorly addressed by GC or LC may actually be quite small. Future developments in LC will probably determine the ultimate impact of SFC. Supercritical fluids do have some unique advantages over ordinary liquid mobile phases when chromatographic columns are linked to spectral detectors. Their high volatility aids in coupling to mass spectrometers. Several chromatographically useful supercritical fluids such as carbon dioxide and xenon have extensive regions of transparency in the infrared spectral region, permitting effective coupling with infrared spectrometers (*50, 51*).

The separation methods known collectively as field-flow fractionation (FFF) were also discussed in last year's review (*3*). These techniques, conceived by J. C. Giddings in the mid-1960's, have been developed largely in his laboratories. The past 5 years has seen a significant growth of interest in these techniques in other laboratories as well. Du Pont is scheduled to begin marketing sophisticated sedimentation-FFF instrumentation, developed by J. J. Kirkland and W. Yau, in the near future. The FFF techniques in general probably provide the best known approach for separation of substances ranging in size from relatively large polymers to micrometer-sized particles. Although much development of instrumentation remains to be done, FFF will most likely turn out to be extremely valuable in separation and characterization of large polymers and particles, perhaps surpassing in importance the techniques of gel permeation chromatography and centrifugation. For the separation of more moderately sized polymers, gel permeation will probably remain dominant.

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Fourier Transform Mass Spectrometry

Michael L. Gross and Don L. Rempel

Summary. Fourier transform mass spectrometry will play an important role in the future because of its unique combination of high mass resolution, high upper mass limit, and multichannel advantage. These features have already found application in gas chromatography–mass spectrometry, multiphoton ionization, laser desorption, and secondary ion mass spectrometry. However, its most notable feature is the ability to store ions. This characteristic, when combined with the others, will allow expeditious study of the interaction of gas-phase ions with both photons (photodissociation) and neutral molecules, and the convenient application of this fundamental information for chemical analysis.

Considerable research activity is focused on instrumentation development in mass spectrometry today (*1*). One motivation for this activity is the increasing complexity of problems in application areas. In environmental chemistry and energy research, the mixtures become more complex and the required detection limits decrease. More polar, structurally intricate, and thermally sensitive compounds are now prepared in chemical synthesis and isolated in biochemical and medical research.

Desorption methods of ionization (*2, 3*) have been developed to handle compounds not amenable to traditional methods of vaporization and ionization. These methods include fast atom bombardment, laser desorption, secondary ion methods, ^{252}Cf plasma desorption, and field desorption. The mass analyzer of the future must deal with nonvolatile, highly polar or ionic, thermally sensitive materials with molecular masses approaching or exceeding 10,000 atomic mass units (amu), which will be introduced into the analyzer by using desorption ionization. Thus, the principal motivation for instrument development is the opportunity to solve new problems, an opportunity brought about by desorption ionization methods.

The Fourier transform mass spectrometer is a relatively new type of instrument which has its origins in the omegatron (*4*) and the ion cyclotron resonance (ICR) spectrometer of Wobschall (*5*). Its immediate progenitor is the ICR mass spectrometer of Baldeschwieler and students (*6*). The transition to Fourier transform mass spectrometry (FTMS) required development of a trapped ion cell, first introduced to the ICR community by McIver (*7*), and of methods to excite rapidly and detect simultaneously all the ions. These latter developments were made and the first FT mass spectrometer demonstrated by Comisarow and Marshall in 1974 (*8*).

The technique of FTMS has a number of features that make it a potentially important tool for attacking the difficult problems mentioned above. Some of these features are (i) simple mechanical design, (ii) high mass resolution, (iii) high upper mass limit, (iv) long ion storage, and (v) multichannel advantage.

Mechanical simplicity. The FT mass spectrometer is a relatively simple instrument in terms of mechanical design (*9*). The heart of the spectrometer to date has been a single cell (ion trap), usually a six-electrode 2.5-cm cube introduced by Comisarow in 1980 (*10*). This cell, like the mechanical components of all mass spectrometers, is mounted in a high-vacuum chamber. Its operation also requires a magnetic field, which has been provided by an electro- or a superconducting magnet. The cell serves as the ion source, mass analyzer, and detector, the three essential operating components of any mass spectrometer. Normally, these components are separated spatially; with FTMS the ionization and mass analysis–detection are separated temporally and computer-controlled.

High mass resolution. Improved mass resolution usually leads to better precision and accuracy of mass assignments, which add specificity to quantification and remove ambiguity from the identification of knowns and proof of structure of new substances. The highest resolution attainable with a commercially available sector mass spectrometer is 150,000 (Kratos MS-50), whereas the best resolution demonstrated for FTMS is 100,000,000 at mass-to-charge ratio m/q 18 (*11*). Another noteworthy example is the dramatic mass separation of Cl^+ and Cl^- obtained with FTMS (*11a*) (see Fig. 1).

High mass limit. A focus of mass spectrometry is the determination and

Michael L. Gross is professor of chemistry and director of the Midwest Center for Mass Spectrometry, University of Nebraska, Lincoln 68588. Don L. Rempel is a senior research associate at the Midwest Center for Mass Spectrometry.