Trends in Reversed-Phase HPLC Column Practices: 1997

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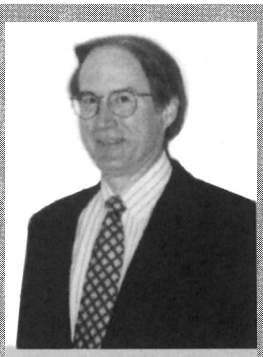

Dr. Brian Bidlingmeyer

An active scientist in the field of high-performance liquid chromatography (HPLC), Dr. Brian A. Bidlingmeyer has published over 60 technical papers and is the author of two books, one on practical HPLC and the other on preparative HPLC. As a result of his pioneering work and significant contributions to the practice and understanding of modern HPLC, Dr. Bidlingmeyer has received numerous awards in the separation science area, including the Heinrich Emmanual Merck Prize (1990) for contributions to analytical chemistry, the International Ion Chromatography Award (1988) for contributions to that area, and an IR 100 Award (1985) for a new method of amino acid analysis ("PicoTag" method). His current research interest is the elucidation of retention mechanisms in reversed-phase separations.

Dr. Bidlingmeyer is a graduate of Kenyon College and received his Ph.D. in chemistry from Purdue University under Professor L.B. Rogers. He serves and has served on numerous scientific councils, organization committees, and advisory boards including the prestigious journals Analytical Chemistry *and* Fresenius' Journal of Analytical Chemistry *(Germany). He is presently an associate editor and contributing editor to the* Journal of Chromatographic Science, *writing a monthly column on HPLC troubleshooting. He is also on the advisory board to LC–GC Magazine.*

Dr. Bidlingmeyer is employed by Hewlett-Packard at the Little Falls Newport site and is involved with the research, development, and manufacturing of the Zorbax brand of HPLC packings. In addition to having work experience in the chemical and the pharmaceutical industries, Dr. Bidlingmeyer is a previous Vice President/Technical Director of Waters.

Abstract

This work provides an overview of recent trends in the use of high-performance liquid chromatography columns. Both applied and investigative areas of the science are examined, and trends in technology, materials, techniques, and configuration are discussed.

Introduction

Modern liquid chromatography, commonly referred to as high-performance liquid chromatography (HPLC), is over 25 years old. Because of its age, many consider HPLC a mature technique, and as such, view the field as stagnant. However, nothing could be further from reality. The field of HPLC is changing rapidly, although more subtly than it did in its early years, and it is interesting to note that the trends today are in essence driven by the same application benefits that were prevalent in the very early years of LC (1): improved quantitation, rapid determination of purity, preconcentration of samples, and rapid determination of structure, to name just a few. The annual worldwide sales of HPLC instruments and consumables are estimated to be around $1.5 billion. This impressive number indicates that the technique has gained wide acceptance in the modern analytical laboratory as well as in most areas of science. With over 100,000 practitioners, this technique is one of the most popular tools in the modern analytical laboratory.

The purpose of this paper is not to give an all-inclusive, comprehensive report on the voluminous amount of literature that has appeared in past years, but instead to discuss the trends in HPLC column usage in the most general sense. In this context, there are two broad areas in which HPLC is utilized: the application focus for applied uses and the HPLC

use for investigative purposes. The trends in these two broad areas are driven by forces with different objectives. In the applied area, scientists are generally looking to accomplish tasks quicker and more productively. In addition to these goals, the investigative scientist wants the highest efficiencies available and increased capability to fill the need to handle the difficult task of analyzing increasingly small amounts of more complex and sometimes unknown samples. The investigative researcher will often trade off increased time for increased efficiencies.

Each of the two application areas is influenced by a main objective that guides the work. In the applied area, which is, in this author's opinion, the largest use of modern HPLC, methods are in place to cost-effectively generate information to verify or confirm the expectations of the analyst. In the investigative area, the HPLC activity is focused on providing definitive information. In the applied area, the desire is to confirm the expectations as fast as possible and, often times, effort is expended to develop the optimum method. In the investigative area, obtaining definitive information usually takes precedence over the cost-effectiveness of the method because the specific work will be done less frequently. For example, when a potential drug is discovered, the investigative work focuses on the isolation of a compound with the goal of identifying its structure. Replicate elucidation is seldom required. Once the compound is isolated, much of the applied work (the drug development process) confirms the compounds presence based on less rigorous information such as retention time and ultraviolet (UV) spectra. This confirmation is based on a consistency of information (retention time matches, UV spectra matches, etc.). If the compound is believed to be present in the sample, it is internally consistent that it would elute at a specific retention time and have a specific UV–visible spectrum.

In many settings, these two areas, applied and investigative, represent almost diametrically opposed views of what defines effective effort and results. It is as if they are two independent operations in terms of the utilization of HPLC. Occasionally one observes that the investigative effort is designed with a view to the eventual use of the chromatography in the applied area when the compound later progresses through the drug development cycle. However, in light of the differences in the main objectives of these two areas of effort, it is interesting to note that the trends in these two areas are similar. The trends are (*a*) using smaller size particles, (*b*) using the advantage of the column, and (*c*) using short columns with in-line mass spectrometers as detectors.

Trends in Applied HPLC

In the applied area of HPLC, a change in the practice will only occur if the change offers a clear advantage over the existing way. To that end, the overriding trend in the applied world is the need for increased productivity. This is in keeping with the general overtone in many businesses attempting to stay competitive, which is to do more with less. Thus, the market drivers in the applied area of HPLC that effect change are those that will increase throughput, increase sensitivity, maintain reliability, and be cost-effective. Regulatory, economic, and environmental pressures are placing demands on laboratories to produce large quantities of quality data so that projects can be completed on time. Such pressures refine the main goal and underline the need for multiple subgoals: reduced time and expense in the analysis activity, faster analyses with no sacrifice in separation resolution, rugged and easily reproduced methods using existing equipment, and reduced solvent consumption. Thus, in the applied area, trends are those implemented changes that meet or are intended to meet the increasing demands for developing faster, more rugged, and more reproducible HPLC methods.

With increased productivity as the main emphasis in a wide variety of industries, it is no surprise that the main trend in HPLC is focused on higher throughput using smaller particles and/or short columns, resulting in decreased retention times while maintaining efficiency. Because the column is required to function in an existing HPLC, this puts some constraints on the types of columns that can be used. Table I shows the summary of calculating the pertinent parameters necessary to make the decision as to what columns are appropriate for improving the speed of today's analyses when using reversed-phase columns. This calculation assumes that today's applied separations require no more than 13,000 theoretical plates. The various column lengths, particle sizes, and flow rates that will result in the desired efficiency of 13,000 plates are shown. As particle size is decreased, the column efficiency will increase as the square of the relative decrease in particle size. However, the operating pressure will increase by the square of the relative decrease in particle size. In addition, as the column is reduced in volume (shorter lengths), the band-broadening contribution of the instrument becomes important relative to the final volume of the eluted peak (V_p). The "best" column configuration is a trade-off between efficiency, pressure drop, and peak volumes compatible with current instrumentation.

Based on the data shown in Table I, a 3-µm particle in a column length of 10 cm is the best compromise for today's instrumentation. This conclusion is based on the fact that this particle size provides good efficiency, reasonable pressure drop (1850 psi), and is compatible with most instruments in use today that typically contribute a band-spreading volume of 70–100 µL. If the column can generate a peak volume (V_p) of less than the 70 µL value, there would not be a gain in the overall performance because the instrument would broaden the peak more than the column. Small particles (e.g., 1 or 2 µm) contained in well-packed, short columns installed in a conventional or "standard" HPLC instrument will not exceed the performance of the column packed with 3-µm packing and, in the applied areas, would likely only match the performance of the 3-µm material. Thus, the 3-µm packing results in the best match of column performance to instrument performance.

In 1991 only about 10% of HPLC separations were conducted with 3-µm particles, although it was well-known even then that these small particle–short column configurations would produce faster separations without sacrificing resolution (2). The main reason that the 3-µm columns were not rapidly embraced in 1991 was that these columns tended to plug faster and have shorter lifetimes than those using conventional 5-µm

particles. The other reasons were that the column efficiencies were lower than what was expected, and backpressure tended to be higher than what was expected (3). In the practical sense, the movement to 3-µm columns in the applied area has been slow because the columns packed with 3-µm particles have been viewed as less rugged than columns packed with 5-µm particles. This was probably due to the fact that using 3-µm particles required 0.5-µm end frits to hold the packing in the column, and these frits were such good filters that they were prone to plug in operation.

Therefore, there was a hesitation to move to shorter columns because of a perceived lack of ruggedness. However, the interest in increasing throughput using short columns has been strong and has resulted in the recent trend toward using 3.5-µm particles packed in columns with standard 2-µm column end frits that are not prone to plug. Additionally, the practical use of 3.5-µm particles requires that the particle size distribution be quite narrow. The absence of fines smaller than 2 µm in the particle size distribution of a nominal 3.5-µm packing (Figure 1) allows the use of 2-µm frits rather than the 0.5-µm frits used on columns containing 3-µm particles. Columns of 3.5-µm particles with no fines are operable at high flow rates with modest backpressures, thus permitting faster analyses without loss of resolution and with the ruggedness of the popular 5-µm particle columns. Column configurations containing 3.5-µm particles are available from a number of suppliers, and in 1996–97, use of these 3.5-µm columns represents a trend in the applied area of HPLC.

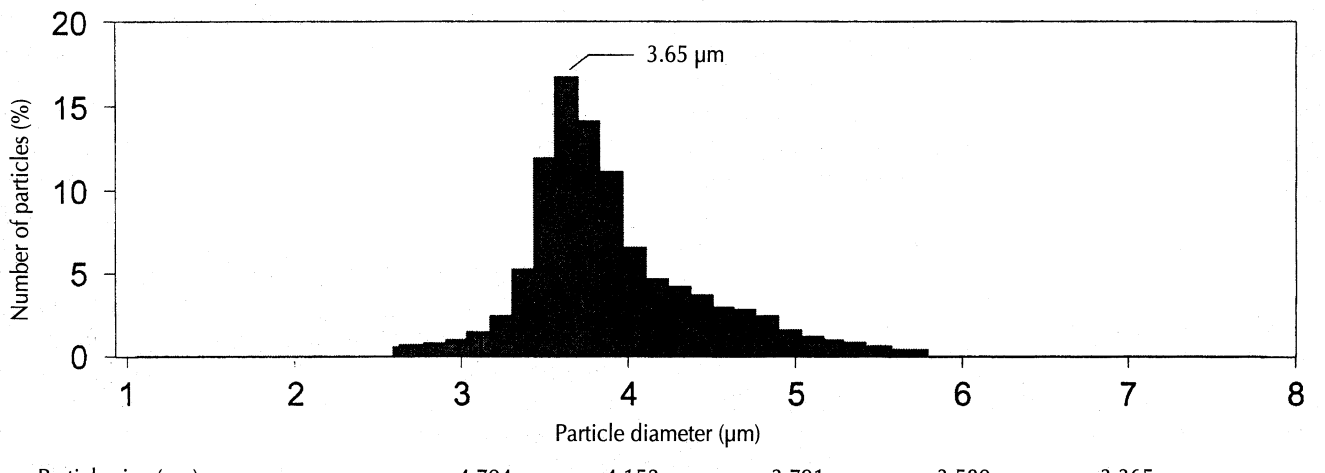
Table II presents the types of trade-offs involved when making the transition to a shorter length reversed-phase column, including the concomitant increases in productivity. The standard HPLC column is 25 × 0.46-cm i.d. with 5-µm particles, which has approximately 20,000 plates. To attain the same separating power (the same efficiency), one could use a shorter column of 3.5-µm particles, which will also accomplish the separation with the same resolution and with a 40% reduction in time. If, in addition, the separation only requires 10,000 plates or less, a shorter column of 7.5 cm would decrease the analysis time by another 50%. The 7.5 × 0.46-cm column configuration packed with 3.5-µm particles is compatible with standard instrumental band-broadening and is the best match with current HPLC equipment for achieving high-throughput analyses. In other words, the 3.5-µm particles are a compromise between attaining the highest performance and longest column lifetime using conventional instrumentation without modification. Table II is also a good way of evaluating the choices one has for increasing productivity by matching the particle size and column length to the required efficiency and to desired throughput.

Figure 2 provides validation of the capability implied in Table II. In this example, the separation of antibacterial compounds on the 25 × 0.46-cm column with 5-µm particles is compared with the separation obtained on the 3.5-µm particle, 15 × 0.46-cm i.d. configuration. As was predicted by the information in Table II, the resolution is

Table I. Calculated Effect of Particle Size on Chromatographic Parameters When Using Reversed-Phase Columns

	13,000 plates			*k* (last peak) = 5		
Particle diameter (µm)	Flow rate (mL/min)	Length (cm)	Volume (mL)	Pressure (psi)	Retention time (min)	Volume (*k* = 1) (µL)
21.0	6	0.6	2500	3.6	42	
31.0	10	1.0	1850	6.0	70	
51.0	15	1.5	996	9.0	106	
100.2	30	3.0	116	90	630	

Column i.d. = 4.6 mm Mobile phase = 100% H_2O



Particle size (µm)	4.704	4.152	3.791	3.580	3.365
Percentage of particles with greater size	10.00	25.00	50.00	75.00	90.00

Figure 1. Particle size distribution for Zorbax Rx-sil. Over 90% of the particles are greater than 3.365 µm, which is indicative of an extremely small amount of "fines" present.

comparable on both columns, and the separation was accomplished with a 40% reduction in analysis time.

Figure 3 is an example of an optimized separation that can be attained with a short column packed with 3.5-µm particles. This separation has about 6000 plates for each peak eluted during the 2.5-min analysis time. Each peak also has good symmetry. Additionally, experience has shown that the column lifetime of these small-particle, short-length configurations is comparable to the lifetime of conventional 15 and 25-cm columns containing 5-µm particles. The shorter analysis times achieved using short column configurations also mean that the amount of mobile phase per analysis is drastically reduced, as is shown in Table II. Certainly not every separation can be optimized to a 2–3 min analysis, but by using the guidance put forward in Table II, an appropriate choice of column length can be made for increasing the productivity for an applied separation.

Table II. Maintaining Resolution by Matching Required Plate Number to Column Length

	5 µm	3.5 µm	5 µm	3.5 µm
Dimension	250 × 4.6 mm	150 × 4.6 mm	150 × 4.6 mm	75 × 4.6 mm
Analysis time (min)	30 min —40% reduction→ 18 min		18 min —50% reduction→ 9 min	
Solvent waste (mL)	30 mL —40% reduction→ 18 mL		18 mL —50% reduction→ 9 mL	
Number of plates (N)	20,000	20,000	12,000	10,000
Resolution $\propto N^{1/2}$	unchanged		9% difference	

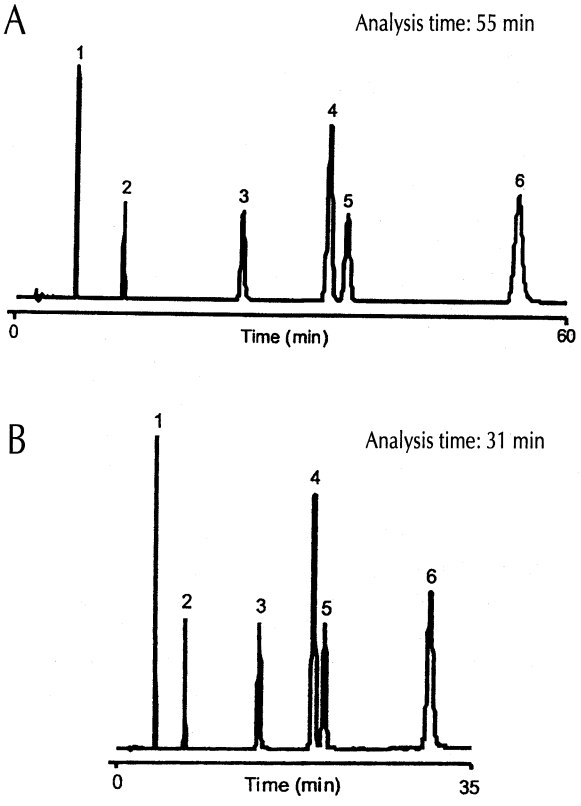


Figure 2. Maintaining resolution while increasing throughput by using short columns with 3.5-µm packing. Mobile phase: ACN–0.1M citric acid–0.2M Na_2HPO_4 (200:713:87, v/v/v); flow rate: 1 mL/min; column: (A) Zorbax SB-C_8, 5-µm particles, 4.6 × 150 mm; (B) Zorbax SB-C_8, 3.5-µm particles, 4.6 × 150 mm; temperature: ambient; sample: antibacterials. Peaks: 1, sulfamerazine; 2, furazolidone; 3, oxolinic acid; 4, sulfadimethoxine; 5, sulfaquinoxaline; 6, nalidixic acid.

The use of temperature has always been valuable for controlling the reproducibility of a separation, especially in laboratories without adequate air conditioning (4). However, currently there is a trend to use elevated temperatures to reduce the time of analysis in addition to controlling retention reproducibility. In reversed-phase chromatography, the retention time decreases as the temperature is increased, and if necessary, the exact relationship can be obtained by using a vanHoff plot. The impact on the modern laboratory is that sample throughput can be increased by using elevated temperatures. An example of the use of elevated temperature is shown in Figure 4. This routine separation of water-soluble vitamins is used by food and pharmaceutical producers for assays. It can be clearly seen that by using a temperature of 60°C, the throughput of this analysis may be doubled with the benefit of increased peak heights, which are useful for individuals concerned with low-level detection. Increasing the temperature above 60°C results in a threefold increase in throughput. Generally, temperature optimization can improve productivity for routine analyses. Because of this trend, instruments with ovens are now available as standard or as a commonly recommended option from most (if not all) manufacturers. Years earlier, ovens were not popular and sometimes even shunned by users. Today an HPLC without an oven would not effectively serve the applied user.

The most aggressively pursued detector now being used in the applied area is the mass spectrometer (MS). Several years ago, the HPLC–MS with an atmospheric pressure ionization source (API) was beginning to be used in the metabolism laboratories of pharmaceutical companies. These workhorse instruments were large and expensive, but their cost was justified by the productivity increase in the drug discovery and development process. Today the use of HPLC–MS as a tool for the metabolism scientist is quite universally accepted. Initially, some felt that the HPLC was not needed because samples could be introduced directly into the MS; however, most soon discovered that some HPLC retention was needed to separate the substances of interest from the other substances present. With the advent of benchtop HPLC–MS instruments, which have been introduced by most manufacturers, the HPLC–MS task is

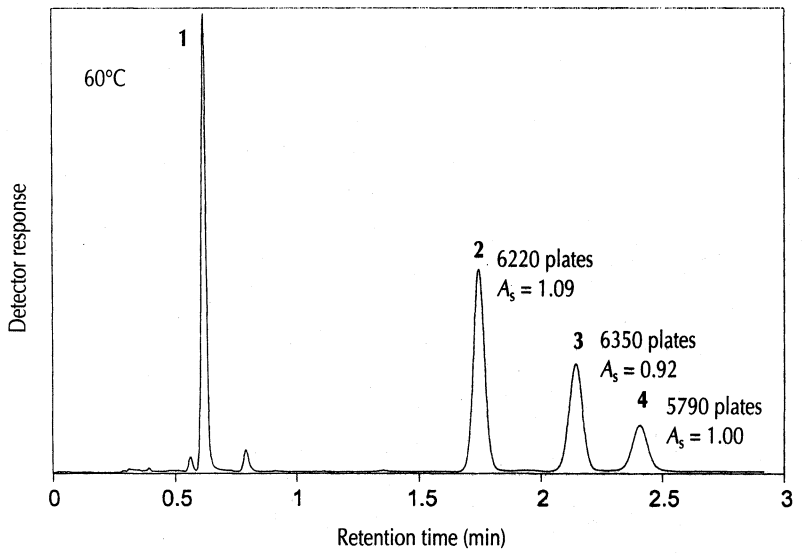


Figure 3. High-speed separation of antibiotics. Mobile phase: ACN–0.1% aqueous TFA (98:92); flow rate: 3.0 mL/min; column: Zorbax SB-C_8, 3.5-µm particles, 4.6 x 75 mm; temperature: 60°C; UV detector at 260 nm. Peaks: 1, ceftazidime (0.40 µg); 2, cefotaxime (0.36 µg); 3, ciprofloxacin (0.1 µg); 4, cefazolin (0.35 µg).

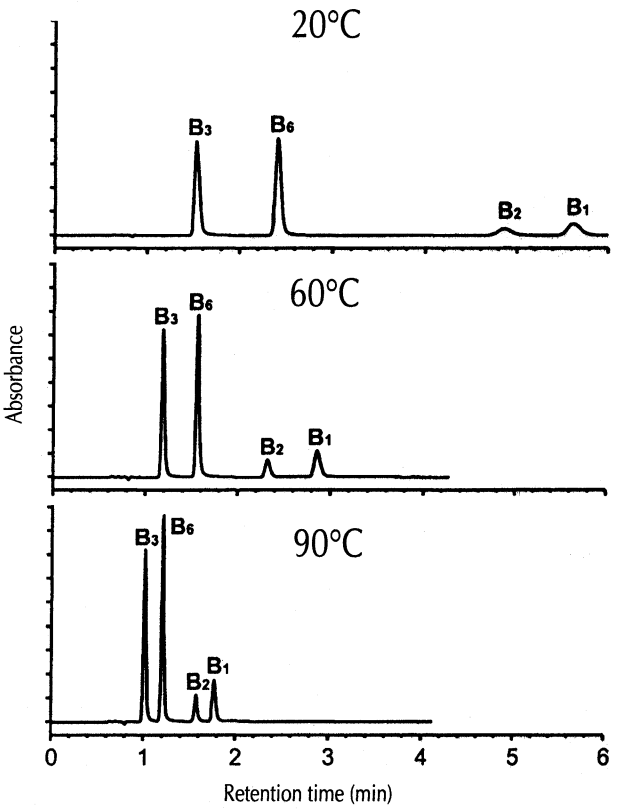


Figure 4. Effect of temperature on the separation of water-soluble vitamins. Mobile phase: MeOH–water containing 10mM hexane sulfonate with 0.1% phosphoric acid; column: Zorbax SB-C_8, 3.5-µm particles, 0.46 x 7.5 cm; flow rate: 1 mL/min; temperature: 20, 60, and 90°C; sample: vitamins B_1, B_2, B_3, and B_6.

easier and more convenient than with past instrumentation. This has resulted in a solidification of the trend toward using the HPLC–MS technique.

In the column area of HPLC–MS, there is a general trend toward short columns including those less than 5 cm in length, even shorter than those shown in Table II, which are used only to give some separation prior to the effluent's introduction into the MS. Because of the need in the column area for the HPLC–MS application, manufacturers often call the very short columns "LC–MS columns" to denote the specific end use. As one would expect, these columns do not exhibit many plates; however, the efficiency performance is adequate because the MS accomplishes compound selectivity and identification. Additionally, in some situations there is concern to use a column that exhibits very low bleed. Under certain conditions, this concern is not easily met, and it requires the use of unique bonded phases that do not hydrolyze during use.

Today's general purpose HPLC instrumentation is designed to deliver greater performance and improved operator interaction (user friendliness) at a cost similar to or lower than the predecessor instruments. This is in keeping with the drive toward higher productivity. Many instruments can not only be controlled by the operator but may also be controlled by some sort of insertable PC card or disc so that a lesser skilled instrument user can automatically set up the desired method. The instruments are usually equipped with diagnostic software that helps the operator identify the causes of failure as well as alert the user when preventative maintenance is recommended. Many instruments can keep track of component usage and, using these logs, alert the user when preventative maintenance should occur (e.g., change the detector lamp or change or check the column). Troubleshooting and repair may be assisted using phone lines with the instrument available for on-line checkouts over a modem. Because of increased regulatory oversight, gathering of as much information as possible is encouraged. This desire to gather more information about the chromatographic peak has changed the detector part of the instrumentation. When appropriate, the need for additional information to confirm the compound's presence at the desired level has led to the incorporation of photodiode array detectors (PDA) into many instruments being used in the applied area. Use of PDA detectors has become fairly routine when the user incorporates the appropriate computer, memory, and documentation program.

Trends in Investigative HPLC

Because of the goals of the investigative work, trends in this area have tended toward increased capability and sophistication. These goals have resulted in an emphasis on generating more separating power through the use of higher resolution columns

with an increased number of theoretical plates, using conventional column configurations with more sophisticated detection for compound identification, or both. Because overall efficiency of the separation is dependent upon column and instrumentation contributions, the column trends in the investigative area require instrumentation improvements in order for the total benefit of the column to be appreciated.

Requirements in the investigative area have moved HPLC columns to narrow diameters (2.1 mm and lower) with lengths of 25 cm and longer, packed with small particles. Columns with small diameters (e.g., 0.1–0.2 cm) are preferred when high sensitivity is required or the amount of sample is limited. Columns of small diameters are often desired when analyzing complex biological samples. Using these narrow bore columns increases the absolute sensitivity for the analytes, as illustrated in Figure 5. In this figure, 50 pmol of a PTH-amino acid mixture is separated essentially to baseline resolution using gradient elution and a 0.21-cm-i.d. column. In this example, it should be noted that, compared to a 0.46-cm-i.d. column, the smaller 0.21-cm column was run at a low flow rate and required only one fourth of the mobile phase for the separation.

Columns such as the configuration used for the PTH-amino acid separation are often packed with 3.5-µm particles or smaller and are typically used to generate very high performance and high sensitivity. Nonporous (NP) particles with particle sizes of approximately 1–2 µm are being used to achieve high efficiencies. However, columns using NP material have the disadvantage of overloading with sample at smaller injected amounts (mass) than the totally porous particles because of the lower surface area of the NP particle. With proper operation, however, these columns can be used successfully. On the other hand, the high efficiencies that can be attained with the very small particles are of use in the investigative area. But these scientists must modify their instruments or purchase special instruments to attain the benefits of very small particles. Also, columns containing very small particles may not be as rugged as the 3.5-µm ones under usual applied laboratory operations. However, with careful attention to good chemical hygiene, attaining long life with high performance should be possible. As an example of the high performance, a chromatogram of a peptide map is shown in Figure 6. Peptide mapping benefits from high efficiencies, and often, because only a very small amount of material is available, it is appropriate to use a small-diameter column.

Because very high-efficiency columns spread the bands less, use of these high-performance columns do require instruments with very low dispersion. As a result, many instrument suppliers now offer low dispersion instrument capability. These recently introduced systems are designed with smaller diameter columns in mind. The main area of application is in biological research, in which small sample amounts are common and the sensitivity requirements highly favor the use of small-diameter columns. Traditional instrumentation requires modification to eliminate extra column volume and to operate with small-diameter columns. As mentioned, these columns are used when the mass of sample is limited; the largest application is in the area of biotechnology, in which small amounts of material are all that is initially available.

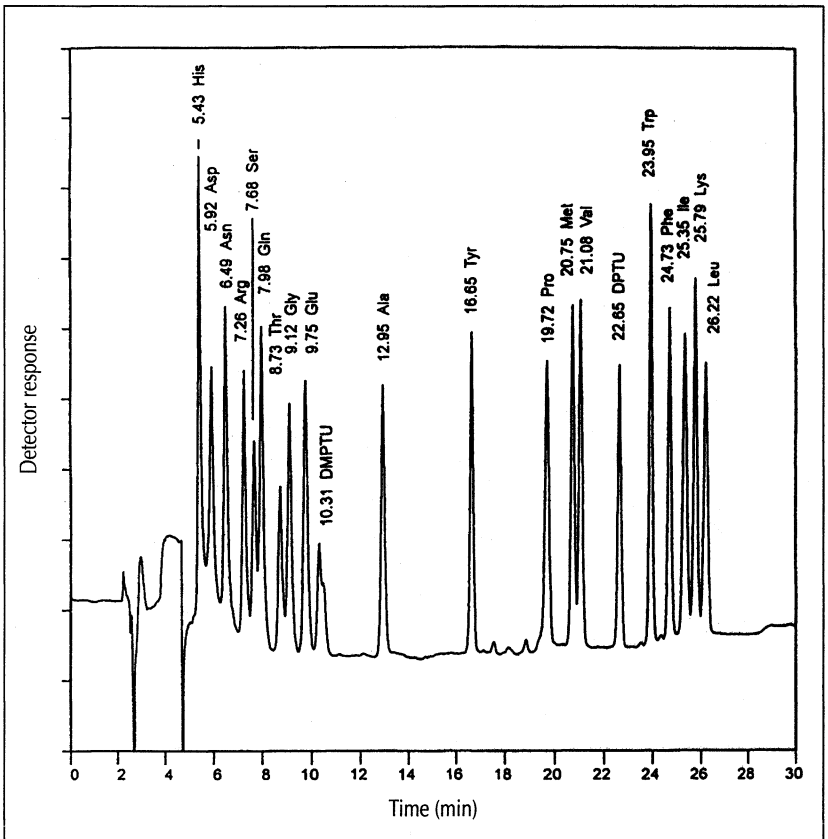


Figure 5. Narrow bore column separation of PTH-amino acids. Mobile phase: 0.04M acetic acid with 5% tetrahydrofuran–triethylamine to pH 4.1, solvent B, acetonitrile; gradient: 12–38% B in 18 min; column: Zorbax SB-C_8, 5-µm particles, 2.1 × 250 mm; flow rate: 0.21 mL/min; temperature: 55°C; sample: 5 pmol of each amino acid.

Because of the need to solve complex problems in the investigative area, there has been emphasis on a multidimensional HPLC approach or, as it is sometimes called, hyphenated HPLC, in which two analytical techniques are coupled. The combination of two analytical methods using a standard physical property or bulk property detector with a specific identification-type detector produces an ideal investigative research tool for identification and quantitation. Obviously, the analytical power made possible through this approach is much greater than that achieved through nonhyphenated techniques. The difficulties in combining HPLC with other techniques and/or detectors is usually in the interface between the techniques; this has limited the application in the investigative area.

Active hyphenated techniques are infrared (HPLC–IR) (5), Fourier transform infrared (HPLC–FTIR) (6), and gas chromatography (HPLC–GC) (7), but the biggest trend in the investigative area has been in the coupling of HPLC with MS (HPLC–MS). For example, within the pharmaceutical industry there is a movement to

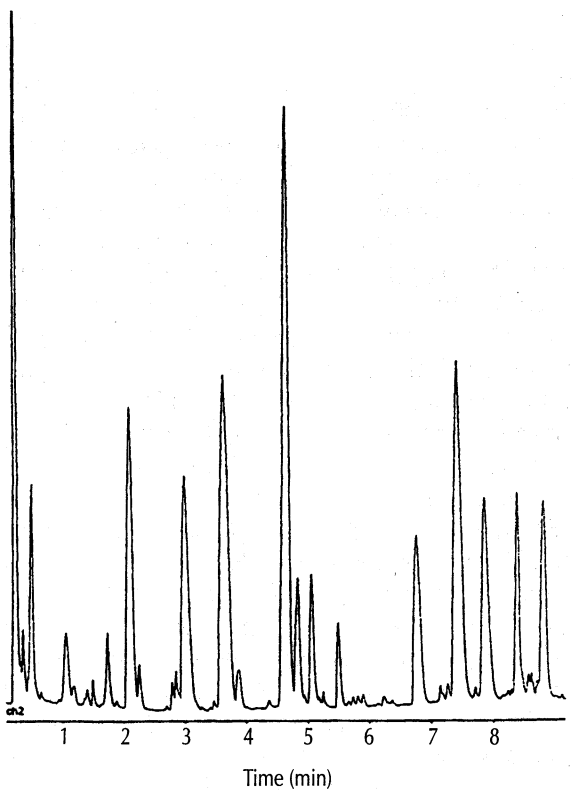


Figure 6. Separation of a tryptic digest of cytochrom C on an NP reversed-phase column. Mobile phase: solvent A, 0.1% trifluoracetic (TFA) acid in water; solvent B, 0.1% TFA in ACN; gradient, 0–50% B in 20 min, 50–70% B in 3.5 min, return to initial conditions in 5.5 min; flow rate: 1 mL/min; detector: UV at 215 nm; temperature: 50°C.
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accelerate the drug discovery process through the use of combinatorial chemistry. Until recently, the weak link in drug discovery was chemical synthesis. Today, however, it is possible with combinatorial chemistry to make a group or "library" of approximately 50,000 chemically similar compounds in a few hours or days. Then it is necessary to determine if the produced compounds are effective and, if so, it is necessary to elucidate the chemical nature of the active compounds. This puts a very large demand on the separation of the compounds made by the combinatorial chemist. Effective utilization of HPLC analyses is paramount to achieving the discovery of new drugs.

The use of HPLC columns interfaced to MS units has quickly become a pervasive trend. In combinatorial work, the rapid analysis of compounds in the reaction mixture is often equally as important as the determination of the major compound present. As mentioned earlier, combinatorial applications require the rapid and accurate analysis of tens of thousands of compounds. In these laboratories, the UV–vis detectors, once the preferred choice, are no longer as productive as the MS. The MS delivers a higher degree of confidence for the chemist who needs to know the structure of the "right" compound. The trend in HPLC columns is often toward short columns (e.g., 2.1 × 15 mm and 2.1 × 30 mm, rather than 2 × 15 mm and 2.1 × 250 mm columns, which have commonly been used by individuals doing HPLC–MS).

The use of elevated temperature is also becoming a trend in the investigative area of HPLC for a different reason than in the applied area. In the investigative area, the motivation is not to use temperature to decrease retention time, but to accomplish previously unattainable separations. The use of temperature as a variable should be investigated when it is necessary to optimize a separation or to increase recovery. For example, membrane proteins have typically exhibited low recovery on reversed-phase HPLC packings. The use of elevated temperature appears to solve this problem. An example of the utility of high-temperature HPLC can be found in the field of neurology, in which researchers are studying the brains of individuals who have died with Alzheimer's disease. At the present time, the cause of this disease is unknown. The hallmark pathological finding is the presence of significant quantities of the amyloid peptide in the insoluble amyloid plaques. These peptides readily form aggregates in vitro that mimic the fibrils found in diseased brains. Most researchers believe these plaques develop on the nerve cells and cause the death of the brain. Thus, there is interest in studying these proteins. Figure 7 shows the separation of two beta-amyloid peptides (BAP) under the same mobile phase conditions but different temperatures. In this example, the increased peak area is accompanied by an increase in the recovery of the BAP, 1–43 peptides. Initial recovery was on the order of 10%, and the recovery at 80°C was greater than 92% (8). The separation shown in Figure 7 was obtained using gradient elution and, in keeping with the discussion of isocratic chromatographic behavior in the applied area (Figure 4), the compounds also eluted at an earlier retention time as the temperature was raised. The elevated temperature in this example appears to be an advantage due to increased solubility of the analyte in the mobile phase, which results in higher recovery at the elevated temperature.

Increasing the temperature to improve solubility can also be used to enhance chromatographic separations of crude mixtures that might not be obtained at room temperature. An example of this is shown in Figure 8. The chromatogram represents an injection of a crude extract of brains from individuals with Alzheimer's disease directly onto the reversed-phase column at an elevated temperature. In this case, the entire sample was injected. In addition to the BAP region, a new region in the chromatogram was found that showed BAP immunoreactivity (9). This new region may represent a new group of compounds not previously identified.

Finding this new information was possible because of the use of elevated temperature. This new region is believed to have been removed from the sample in room-temperature HPLC, due to the sample preparation technique developed to remove material that could not be injected at room temperature.

Elevated-temperature HPLC at low pH brings an increased capability to the investigative area. However, the ability of the column to withstand higher temperatures needs to be verified by the manufacturer because not all bonded-phase columns can operate successfully at the higher temperatures with concomitant long lifespans. It should be noted that elevated temperatures above pH 7 using phosphate buffers will tend to be harmful to all

silica-based columns due to dissolution of the silica.

As technical papers appear, it is tempting to predict the future directions of a technology. This is dangerous because not all of the strengths and weaknesses of the evolving technique are evenly discussed. One such new technique, capillary electrochromatography (CEC), is not yet a trend but deserves mention in this paper because it is directed at combining two popular investigative tools. First reported by Pretorius (10) for use in thin-layer chromatography (TLC) applications, it appears today as a combination of capillary electrophoresis (CE) and capillary HPLC. The techniques of CEC incorporate capillary tubing; the column container is packed with a standard HPLC packing and uses a CE instrument or a modification of one. Electroosmotic flow "pumps" the mobile phase and sample through the column. In CEC, the separation results from partitioning of the sample between the stationary phase and the mobile phase in addition to differences in electrophoretic mobility—a combination of the reversed-phase retention mechanism and electophoretic behavior. High efficiencies can be attained because of the plug-flow profile and the ability to use particles (1–2 µm) that are typically smaller than those normally used in HPLC. There are, however, practical hurdles to overcome, such as bubble formation due to use of very high voltages and poor reproducibility for electroosmotic flow and retention times. Recent research papers have addressed these issues (11–13).

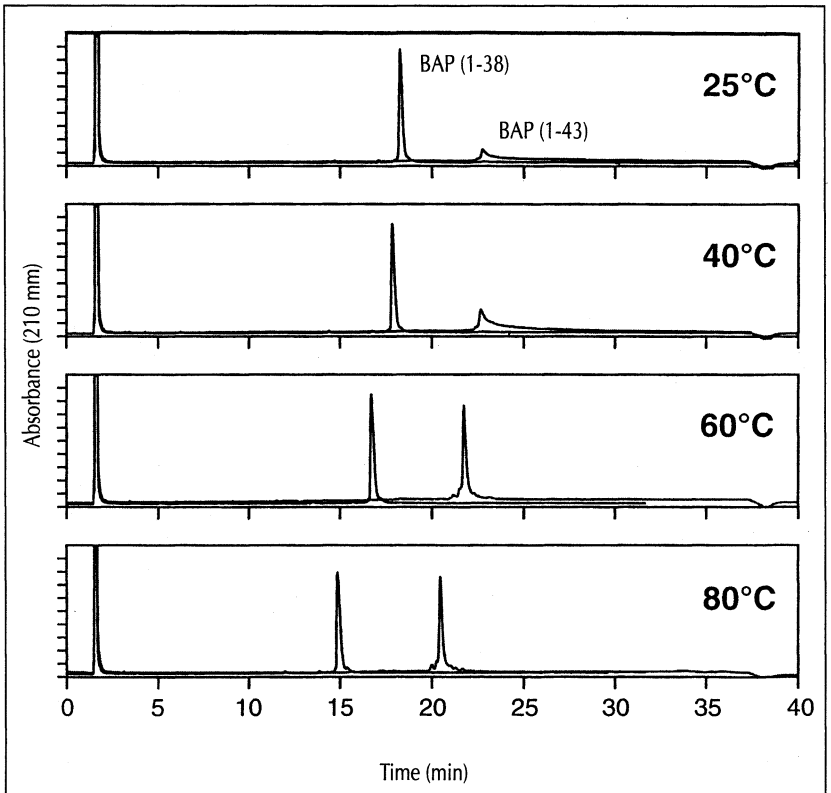

Figure 7. Effect of temperature on BAP peptide separation. Mobile phase: solvent A, 0.1% TFA acid in water; solvent B, 0.09% TFA in CAN; gradient: 20–45% in 35 min; column: Zorbax 300 SB-C_8, 5-µm particles, 4.6 x 150 mm; sample: 10-µL injection of 5 µg peptide in 6M urea with 5% acetic acid.

An example of attained separations in CEC is displayed in Figure 9, which shows the reversed-phase separation of a mixture of some polynuclear hydrocarbons (14). As seen in this figure, the separation of these nonpolar molecules is highly dependent upon the type (and manufacturer) of the packing. Each electropherogram was obtained using a different C_{18} column. Note that the different columns give strikingly different retention times for these neutral compounds. This type of difference is much greater than would be typical in the HPLC of neutral

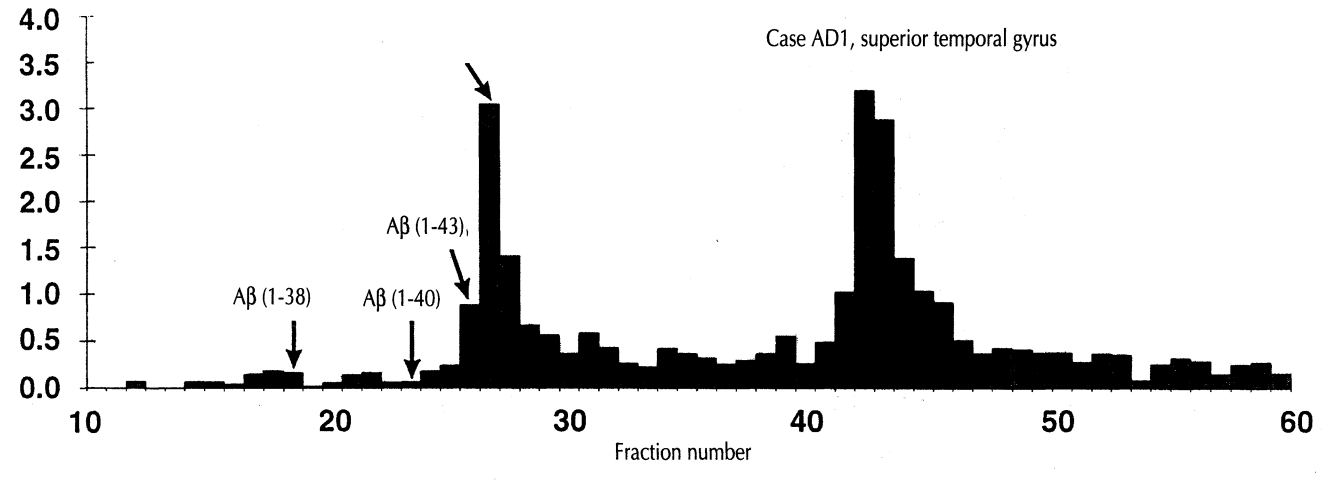

Figure 8. Analysis of HPLC fractions of brain tissue for antibody immunoreactivity. Mobile phase: solvent A, 0.1% trifluoracetic acid in water; solvent B, 0.09% TFA in CAN; gradient: 15–28% in 30 min, 34–55% in 25 min; sample: 100 µL of 70% formic acid extract injected (approximately 0.75–1 mg of protein); column: Zorbax 300 SB-C_8, 5-µm particles, 4.6 x 250 mm; flow rate: 1 mL/min. Results expressed as AB immunoreactivity per mass of protein injected.

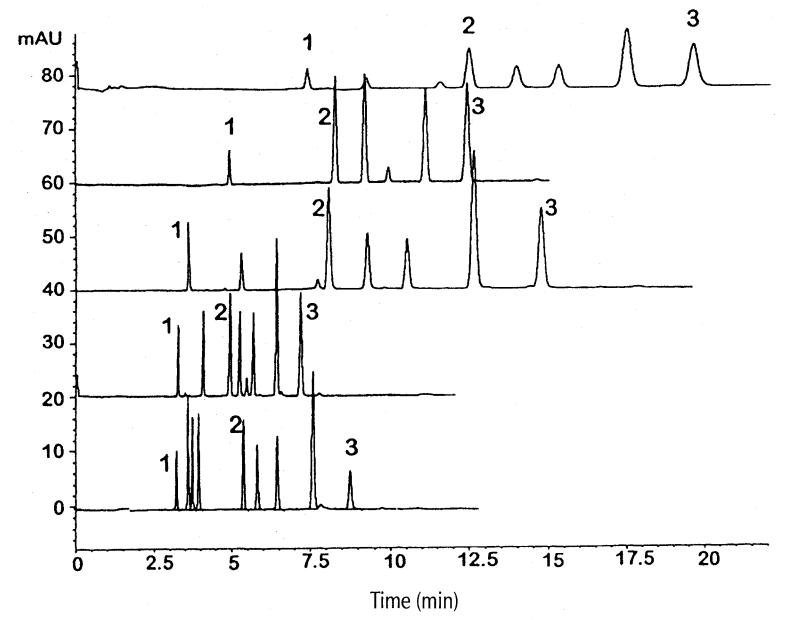

Figure 9. Separation of polynuclear hydrocarbons on different C_{18} stationary phases. Mobile phase: ACN–*tris*-HCl, pH 8 (80:20); voltage: 20 kV using 10 bar pressure on both sides of the capillary; temperature: 20°C; peaks: 1, thiourea; 2, naphthalene; 3, fluoranthene.
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compounds. In CEC, this behavior is probably due to the fact that the electroosmotic flow is dependent upon the silanol content of the packing as well as the silanol content of the capillary. Thus, there may be more problems attaining reproducible separations on columns from different batches of reversed-phase packings; slight silanol variations in packings that would go unnoticed in HPLC could be significant in CEC because the silanol population is very important for the reproducibility of the technique. The difficulty in having reproducible packings was a problem in the early years of HPLC but has been significantly reduced in modern HPLC, especially for neutral compounds.

Reports on the CEC separation mode are limited because this technique is just emerging, but it is one of the most promising investigative developments in recent years. CEC should be watched for its utilization in solving application problems. Only time will tell if CEC will have the capability to compete in the applied area, in which consistency of the method is paramount to the potential of the separation technique.

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Manuscript accepted June 17, 1997.