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New developments in bioanalytical chromatography*

MILOS V. NOVOTNY

Department of Chemistry, Indiana University, Bloomington, IN 47405, USA

Abstract: Recent trends in the chromatography of biological molecules are reviewed, emphasizing advances in liquid chromatography column technology, miniaturized separation systems, ultrasensitive detection, and supercritical fluid chromatography.

Keywords: *Biological macromolecules; column technology; microcolumn liquid chromatography; laser-induced fluorescence detection; capillary zone electrophoresis; supercritical fluid chromatography.*

Introduction

The demands of modern biochemistry and medical science provide continuous incentive for the further development of chromatographic and electromigration methods. Since the biological mixtures contain molecular species with a respectable range of molecular weights and functionalities, even the most effective separation principles and information-rich detection techniques are challenged considerably by various "real-world" samples. The great advances in molecular biology and biotechnology which have been made during the last decade turn the attention of numerous separation scientists toward the unique analytical problems of biological macromolecules. While basic investigations in the chromatography of smaller molecules appear to have reached a plateau, biomacromolecular separations are likely to receive attention for many years to come.

A number of directions are clearly evident in the current development of bioanalytical separations. A comprehensive investigation of these trends would be difficult to carry out in one article, by one author. The present article, therefore, reviews several selected topics in this field:

(a) advances in liquid chromatography (LC) column design (by no means a comprehensive treatment);
(b) miniaturized instrumentation;
(c) new detection opportunities;
(d) recent advances in supercritical fluid chromatography (SFC).

Biomacromolecular separations and analyses will be emphasized.

Advances in LC column design

Certain distinct advantages of organic gels, such as their easily adjustable separation selectivity and inertness toward polar biological molecules, have been responsible for

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much progress in biochemical separations. Their main disadvantage, i.e. the lack of a rigid structure, has accelerated investigations into the substitutes based on the silica framework, and ultimately, toward faster and more efficient separations by high-performance liquid chromatography (HPLC). While the problems of siliceous surface chemistry have certainly not been overcome at present, effective approaches to surface modification in relation to biological molecules (small and large) have been extensively studied. Simultaneously, developments in the design of more rigid organic materials have also continued. Certain novel materials actually have the combined characteristics of polymers and silica.

The deleterious effects of the surface silanol groups are particularly evident in reversed-phase chromatography, an otherwise extremely popular separation mode. A controlled deposition of polysiloxanes onto the silica gel materials has recently shown considerable promise [1–4]. An example of this is shown in Fig. 1 [4], emphasizing the difference of this surface treatment technology from the conventional approach. The resulting beads show good stability at high pH-values and dramatically decreased irreversible adsorption phenomena, together with the usual pressure resistance of silica-based packings. Since the "unmasked" surface silanol groups present reproducibility problems in the routine analyses of peptide [5] and nucleic acid fragments, these column technology developments are likely to reduce the problem.

With the notable exception of chiral separations, the design of selective stationary phases for small biological molecules appears less important than for biomacromolecules. With the increasing complexity of biopolymeric mixtures, the entire "arsenal" of chromatographic methods (reversed-phase, size exclusion, ion-exchange, hydrophobic interaction, etc.) becomes important. While the bioaffinity separations represent

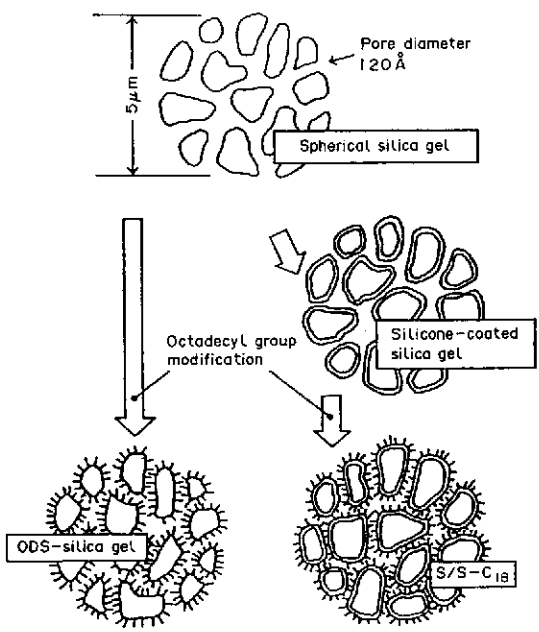

Figure 1
Illustration of the capsule-type packings (right) as compared with conventional reversed-phase packing (left). Reproduced from ref. 4 with permission of Friedr. Vieweg and Sohn, Pergamon Press.

the ultimate in selectivity, the column design can be a very tedious task. Intermediate on the scale of selectivity are selective metal complexation phenomena, which can often be responsible for remarkable separations of protein mixtures [6].

A gradual reduction of particle size has been at the centre of the HPLC approach. The column technologies featuring 5-μm particles have now been extensively developed, while the use of smaller particles (1–3 μm) is aimed primarily toward faster separations with the use of very short columns. Packing such small particles remains technologically demanding. There is now general agreement that large-pore materials are more suitable for biomacromolecular separations than the packings with small pore sizes.

The unique separation problems of biological macromolecules and biotechnologically important problems, such as resolution of structurally similar proteins or purification of DNA restriction fragments, will continue to stimulate the search for new sorption materials.

The columns with drastically reduced inner diameters have been the subject of intensive research for over a decade. These so-called microcolumns possess certain distinct advantages over the conventional LC columns. These advantages are of both a kinetic and an instrumental nature [7–9]. The LC microcolumns can be roughly divided into three types: (a) open tubular columns; (b) semipermeable packed capillaries; and (c) slurry-packed capillaries. Although open tubular columns for LC are still viewed as the least "practical" column type because of their stringent technological and instrumental requirements, considerable progress has been made in the area during the last 2 years. At least three research groups [10–12] reported successful experiments with the open tubular microcolumns with inner diameters of <10 μm. Whilst the column technology aspects of semipermeable capillaries continues to receive attention, the slurry-packed capillary columns have been the most successful column variety.

Originated in the early 1980s [13–16], slurry-packed capillaries have now been developed into an effective tool for biochemical research. Reproducible column technologies have been developed for the reversed-phase mode [16, 17] and normal-phase separations [18]. More recently, Karlsson and Novotny [19] have demonstrated that fused silica capillaries can be efficiently packed using inner diameters of <50 μm; with one such column, 226,000 theoretical plates in 33 min were achieved.

An extremely important aspect of working with microcolumns is the drastically reduced amount of the sorptive material that a sensitive biomolecule, such as a protein, encounters during its passage through the system. Recent investigations by our laboratory [20, 21] emphasize the superior inertness of slurry-packed fused silica microcolumns toward proteins and other biomolecules. It appears that isolations can be carried out at subpicomolar levels with such systems, while the previous reports with conventional columns indicate difficulties for peptides [22] and proteins [23, 24] at levels below 1–10 μg. An example is shown in Fig. 2, where chromatography of several standard proteins is shown at the level of 200 pg each [20].

Miniaturized separation systems

Microcolumn LC, capillary SFC, and capillary zone electrophoresis (CZE) share many common instrumental characteristics which make them very attractive for work with small biological samples. In somewhat different fashions, they share kinetic advantages of small dimensions that lead to exceptionally high resolution in multicomponent separation. The extremely low flow-rates associated with microcolumns often make it easier to interface them with unconventional types of detectors and ancillary techniques.

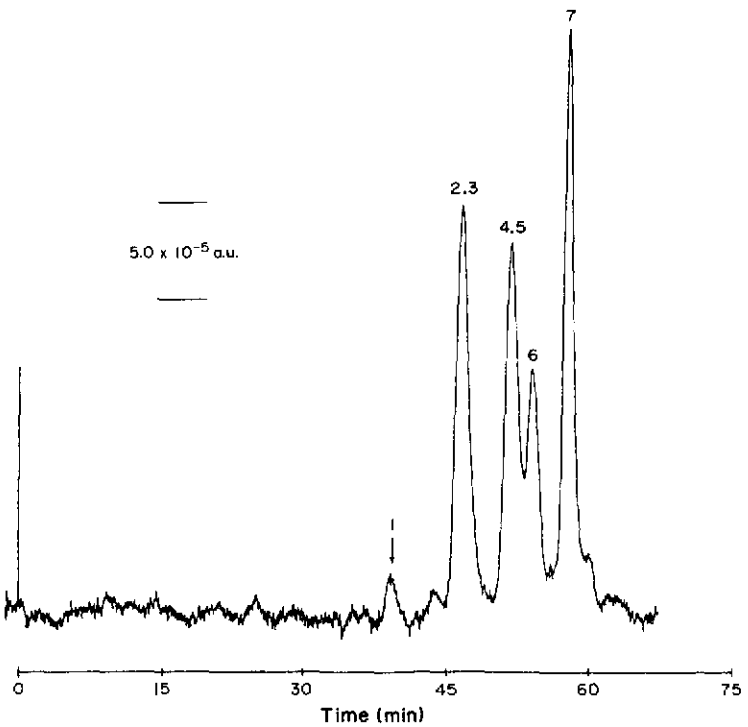

Figure 2
Chromatogram of standard proteins on a 48 cm × 250 μm, i.d., GPC 300 column. Proteins in order of elution: (1) 204 pg thyroglobulin; (2) 231 pg transferrin; (3) 202 pg bovine albumin; (4) 195 pg β-lactoglobulin A; (5) 216 pg carbonic anhydrase; (6) 210 pg ribonuclease A; and (7) 405 pg tetraglycine. Mobile phase: 10% methanol in 0.1 M sodium phosphate buffer, pH 7, at 0.30 μl min^{-1}. Reproduced from ref. 20 with permission of the American Chemical Society.

Another distinct advantage of miniaturized separation systems pertains to an increase in mass detection sensitivity with concentration-sensitive detectors.

While the principles and advantages of these miniaturized techniques have been known for several years, the development of the instrumentation necessary for commercialization still lags behind. Commercialization of capillary SFC has taken place during the last 2 years, and, with some reluctance, instrument companies have now been pursuing the microcolumns with inner diameters of <1 mm. However, CZE appears to be on the verge of commercialization, at present. While these techniques borrow heavily from each other in certain instrumental aspects, they are very complementary in their potential of meeting different bioanalytical tasks of the future.

The microcolumn LC developments of recent years have produced the slurry-packed fused silica capillaries as a remarkably effective tool for a variety of biochemical separation problems. Interfacing such columns with miniaturized UV absorbance, fluorimetric and electrochemical detectors has now been widely demonstrated, while their utilization in the LC–mass spectrometry combination is also highly promising [25]. Although sample introduction techniques for microcolumn LC have steadily progressed toward reproducible injection of nanolitre samples, improvements are still needed. Likewise, gradient elution in the miniaturized LC systems remains non-trivial.

Instrumentation for CZE is relatively straightforward. A high-voltage power supply provides voltage for migrating various charged species through a short piece of capillary (<100 μm, i.d.). Small diameters are crucial, since the heat generated at up to 30,000 V gradients must be dissipated through the capillary wall. A variety of detectors can be used for detection, although cross-flow UV absorbance and fluorimetric measurements are most common. Recent important innovations in detection for CZE include electrochemical [26] and conductivity [27] cells as well as interfacing to a mass spectrometer via an electrospray device [28, 29]. It appears that automated sampling and fraction collection are becoming feasible [12] to develop CZE into a fully instrumental separation method.

The recent interest in CZE is due to its superior potential for extremely efficient separations of peptides and proteins [30–32] and nucleotides [33], although a variety of other charged species can be adequately separated and detected. Certain separations can be further enhanced by the use of gel-filled capillaries [34] and micellar systems [35].

The virtues and limitations of capillary SFC will be discussed below as a separate subject.

Ultrasensitive detection — new opportunities for biochemical investigations

It is often said that there are no limits to the biochemist's desire to have detection methods of higher and higher sensitivity. The microcolumn techniques lend themselves to ultrasensitive detection due to the previously mentioned enhanced mass sensitivity of concentration-sensitive detection. This is perhaps most evident in research with electrochemical detectors [10, 36], but other cases involve fluorescence measurements, and even miniaturized UV detectors are considerably more sensitive than their conventional counterparts. In cases where column capacity limitations exist and dilute samples are necessary for trace analysis, microcolumns are not preferable unless they are coupled with a suitable sample enrichment technique [37].

The advantages of miniaturized systems for detection were shown in the applications to polar lipids [38] and environmental samples [37]. Under some circumstances, the capability of sampling of small volumes and subsequent microcolumn LC can lead to ultrasensitive detection in small biological objects, as evidenced recently by the analysis of neurochemically important substances from a single neuron cell (approx. 1-nanolitre vol) of *Helix aspersa* [12], as demonstrated in Fig. 3. The potential of these capabilities in the fields of modern biology and medical science is quite obvious.

The utilization of laser-based detectors in ultrasensitive measurements is becoming extremely beneficial. Laser technologies naturally combine with microcolumns because of the highly collimated nature of laser beams, as nanolitre to picolitre volumes are easily probed at the column outlet. In this area of research, our laboratory has concentrated on high-sensitivity fluorescence measurements utilizing the helium–cadmium laser at 325 and 442 nm for excitation [39–41]. Since there are only a few cases where the available light frequency coincides with the maximum excitation of the biological molecules of interest, the initially non-fluorescent molecules must be derivatized to ensure high sensitivity. This strategy of matching the laser wavelength and excitation maxima of derivatized compounds has been successfully explored for detection of steroids and prostaglandins [39, 42] bile acids [43], and, more recently, primary amines [40, 41].

Laser-induced fluorescence detection for microcolumn LC is likely to become a very powerful way of measuring biological compounds at extremely high sensitivity following the development of suitable fluorogenic reagents for a variety of substances. While the

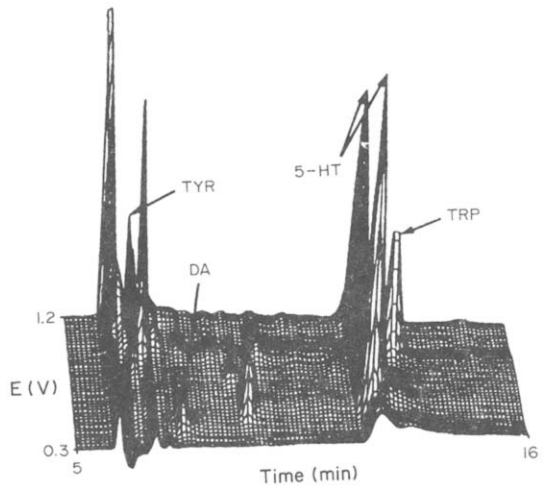

Figure 3
Capillary LC analysis of a single neuron for neurochemically interesting solutes. Reprinted from *Am. Lab.* **20**, 32 (1988). © 1988 International Scientific Communications, Inc.

helium–cadmium laser and the argon ion laser appear the most "practical" as excitation sources, additional laser technologies and detection principles are likely to develop in time. Whenever the appropriate fluorescent derivatives can be "engineered" to possess charged moieties, capillary electrophoresis coupled to laser-based detectors becomes a very attractive option.

The recent advances in ultrasensitive measurements by laser-induced fluorescence are viewed as highly complementary to our efforts in using microcolumn LC for preparative purposes in the areas of protein [20, 21] and nucleic acids research. Our ability to separate and recover subpicomolar quantities of biopolymers projects the necessity of quantifying their degradation products at femtogram levels and below. In Fig. 4, a chromatogram from 430 fmol of hydrolysed bovine insulin is demonstrated [20].

SFC — a bridge between gas chromatography (GC) and LC

Although the initial studies in SFC actually preceded the development of HPLC by several years, only during recent years has SFC been considered a viable analytical alternative in some areas of GC and LC. Following the introduction of open tubular columns to SFC [44], commercial instrumentation has recently been developed. Whereas most samples successfully dealt with by SFC are of a hydrophobic nature, there are expectations of analysing more polar molecules at present.

For certain applications, the unique properties of supercritical fluids make SFC an ideal analytical approach. The molecules of intermediate size and polarities appear readily solvated by "comfortable" supercritical phases such as carbon dioxide and nitrous oxide, yet the kinetic attributes of SFC favour more efficient and faster analyses. In addition, the flame-based detectors are easily utilized, since, unlike with organic solvents used in LC, the flames tolerate many dense gases. Consequently, the flame ionization detector allows universal detection, while thermionic and plasma-based detectors show considerable promise toward element-specific detection. SFC–mass spectrometry and SFC–Fourier transform IR spectroscopy have equally intriguing analytical attributes.

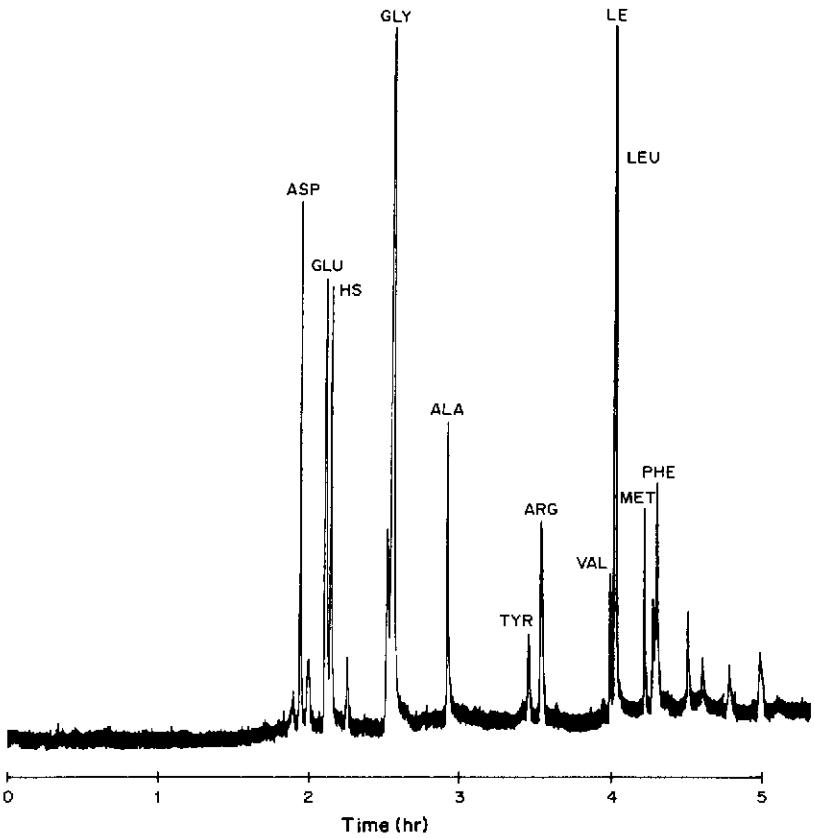

Figure 4
Chromatogram of amino acids from 430 fmol of hydrolysed bovine insulin obtained with a laser-based detector. Reproduced from ref. 20 with permission of the American Chemical Society.

The growing number of SFC applications [45] attests to the increasing popularity of this method.

In order to expand SFC into the area of biochemical applications, three basic approaches appear worth further exploration: (1) adaptation of SFC systems to more polar mobile phases; (2) use of polar retention modifiers, including the recently explored reverse micellar systems and other organized molecular assemblies [46–48]; and (3) improving the mobile-phase solubilities of polar molecules by their derivatization [49–51]. There is considerable room for innovation in either of these directions. While it is currently hard to imagine easy solvation of biopolymers in supercritical media, the extraction and analysis of medium-sized molecules has become a very distinct possibility. A "bridge" between GC and LC has thus been created.

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