High Performance Liquid Chromatography: Potential for Protein Separations

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During the past decade, technological innovations based on new knowledge of the principles underlying separations processes have transformed column liquid chromatography, the oldest chromatographic technique, into a sophisticated tool for rapidly achieving the most difficult of separations and analyses. Applications of this modern version called High Performance Liquid Chromatography (HPLC) are growing at a tremendous rate, as evidenced by an increase in instrument sales by more than 35% a year for the past several years. The HPLC chromatogram shown in Figure 1 depicts the separation of a homologous series of amphoteric surfactants. The direct analysis of such materials was not possible before we developed this HPLC method (Parris, 1977). Of particular importance to the food scientist is the fact that HPLC makes possible the analysis of chemical constituents which have insufficient volatility or are too labile for adequate separation by gas chromatography.

Published separations include fat and oil soluble vitamins (Yeransian, 1979), triglycerides (du Pont Technical Bulletin, 1979), sugars (Waters Bulletin, 1977; Doner, 1979; Parrish, 1980), food colors (Whatman Bulletin 123, 1977, aflatoxins (Stubblefield, 1977), and nitrosamines (Barford, 1974; Wolfram, 1977). Some of these are processed routinely by use of automated devices for sample workup and microcomputer-controlled chromatographs for analysis. Recently attention has turned to separations of peptides and proteins with some success. What is HPLC?

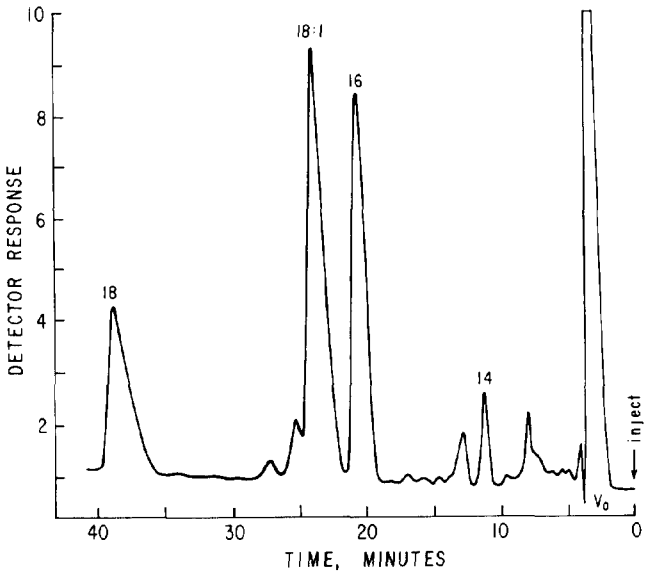

Figure 1. (a) Chromatogram of tallow-derived sulfobetaine. Tal-$CONHC_3H_6N^+(CH_3)_2CH(OH)CH\ SO_3^-$. Column: reversed phase. Mobile phase: methanol/water (80/20) RI detection (Parris, 1977).

Column Packings—The Critical Factor

Evaluation of the mathematical models that describe transport processes in chromatography reveals that faster and more efficient separations result as the diameter of the column packing is reduced. For this reason, special packings having narrow size distributions with average diameters of 5 or 10 microns are in common use. It was the developments in particle technology with methodology to pack stable beds of the particles (pressures of thousands of pounds/square inch are used) that led to rapid growth of the technique.

Usually the basic particles are silica. A comparison of (N_{eff}/t), the number of effective plates per second generated by a column, gives some idea of the advances that have occurred. The larger (N_{eff}/t), the faster an analysis can be performed. Traditional liquid chromatography was performed on open columns operated at atmospheric pressure with particles nearly 200 micrometers in diameter. An (N_{eff}/t) of 0.02 would be generated so that almost 7 hours would be required to obtain 500 plates, a reasonable number for general separation problems (Karger, 1971). Microparticulate columns generate 10-100 plates/sec (Parris, 1976), thereby reducing the time for the above separation to several minutes.

The silica surface consists of silanol groups that sorb many kinds of molecules. Differential desorption by the mobile phase, analogous to those obtained with thin layer chromatography, can bring about separations. However, most HPLC is carried out with packings to which various organic moieties (stationary phases) have been bound covalently so that different interaction mechanisms or modes of chromatography may be employed. Some examples are given in Table 1. In general, organochlorosilanes are used commercially for

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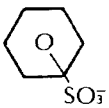
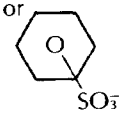
Reciprocal Meat Conference Proceedings, Volume 33, 1980

Table 1. Examples of HPLC Packings

Surface Group	*Type of Chromatography*	*Example Applications*
≡SiOH	Sorption	Aflatoxins (Pons, 1977)
≡Si(R₁)(R₁)OSi(R₁)(R₁)C₁₈H₃₇	Reversed phase	Fatty acids as phenacyl esters (Borch, 1975)
	Ion-pair	Bile acids (Parris, 1979)
≡Si(R₁)(R₁)-OSi(R₁)(R₁)(CH₂)₂ 	Ion exchange	Biogenic amines (Radhakrishnan, 1977)
or ≡Si-CH₂ 		Nitrosoproline isolation (Barford, 1974)
≡Si(R₁)(R₁)-OSi(R₁)(R₁)R₂CN	Normal phase partition	Spice essence components (du Pont Column Report, 1979)
≡Si(R₁)(R₁)-OSi(R₁)(R₁)CH₂CH₂OCH₂CHCH₂OH (OH)	Size exclusion	Water soluble polymers (Regnier, 1976)

attaching the organic groups, although the use of an organolithium pathway provided stationary phases with improved pH stability (Barford, 1974). A detailed review of the chemistry of silica, its surface modification, and its use in chromatography was published recently (Unger, 1979).

Role of Mobile Phase

In general, liquid (mobile phase) being pumped through the packing may be viewed as competing with the sample constituents for the interacting sites on the silica. It is these differential interactions between constituents, stationary phase, and mobile phase that cause the constituents to be resolved. While the nature of these complex interactions is not fully understood, models for some modes exist and aid selection of conditions.

Equation 1 is the basic expression that describes elution in chromatography where V_R is the retention volume or position of the

$$V_R = V_M + KV_S \qquad (1)$$

peak maximum, V_M and V_S the volumes of mobile and stationary phases respectively, and K the coefficient that describes the distribution of solute between mobile and stationary phase (conc in stationary/conc in mobile).

In reversed phase chromatography, decreasing the surface tension of the mobile phase, for example, by increasing the organic component in an acetonitrile- or methanol-water mobile phase, decreases K for most solutes. Comprehensive treatments of the thermodynamics of such systems have been published (Eon, 1973; Horvath, 1976). The use in mobile phase of organic ions opposite in charge to sample constituents has become more widespread. The added ion pairs with constituent ion to form a neutral ion-pair species which interacts with a reversed phase packing. By manipulating pH, mobile phase composition, or chain length of the added ion (Eksborg, 1973), complex mixtures may be resolved.

For cation exchange systems (Rothbart, 1973):

$$K = \frac{K_{E/}[E/]_S^Z}{[E/]_M^Z\left(1 + \frac{K_{eq}}{[H^+]_M^Z}\right)} = \frac{[C^Z]_S}{[C^Z]_M + [CA_Z]_M} \qquad (2)$$

where $K_{E/}$ is equilibrium constant for the ion exchange process, $K_{E/} = [E/]_M^Z\,[C^Z]_S \,/\, [E/]_S^Z\,[C^Z]_M$: $[E/]_M$ is the concentration of univalent counterion in the mobile (eluent) or stationary phase; K_{eq} is the dissociation constant of the solute; and Z is the absolute charge on the dissociated solute species. Under conditions of chromatography, virtually all sites on the support have counterions so that $[E/]_S$ is the capacity of the support. $K_{E/}$ and K_{eq} also may be considered constant under chromatographic conditions. The expressions show that in ion exchange, increasing concentration of counterion in the mobile phase decreases retention, and increasing pH diminishes retention of cationic solutes. For polyvalent solutes, the effects are more pronounced, since $[H^+]_M$ and $[E/]_M$ are raised to the absolute power of the charge on the solute. Analogous concepts may be derived for anion exchange.

Tables of solvent strengths of common eluents in chromatography with sorbents such as silica, alumina, and carbon, have been published (Snyder, 1979), as has detailed discussion of sorption mechanisms (Snyder, 1968). Increasing solvent strength decreases K.

The role of the mobile phase in size exclusion chromatography is primarily as a solvent, since molecules are separated on the basis of their relative accessibility to the pores of the support. The largest molecules are excluded, while the smallest have access to the pores. The distribution coefficient in

this case is defined as the concentration of solute in the pores/concentration of solute in the interstitial volume between the particles and varies between 0 and 1.

Instrumentation

A high performance liquid chromatograph is shown schematically in Figure 2. The pump must be capable of delivering a constant, reproducible flow of mobile phase at pressures up to several thousand pounds per square inch. The pressure drops across columns packed with the microparticles are typically in this range. Capability for changing solvent composition in a programmed fashion (gradient) is also required for many separations. The separation in Figure 1 could have been completed in less time if a gradient program had been used to elute the C_{18} component nearer to the 18:1 component. Commonly, samples are loaded into a loop at atmospheric pressure and the loop is switched instantaneously into the flowing mobile phase stream.

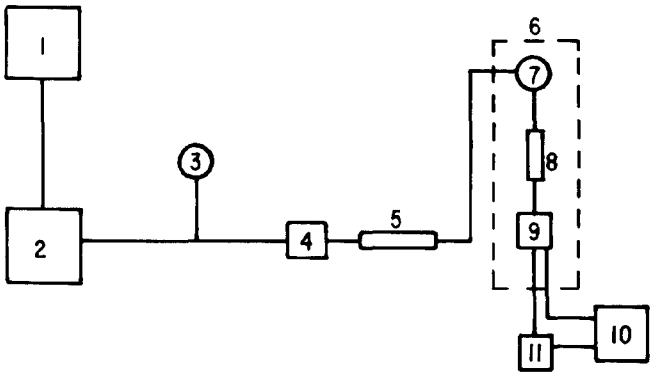


Figure 2. Schematic of simple LC apparatus. (1) mobile phase reservoir, (2) pump, (3) pressure gauge, (4) line filter, (5) pulse damper, (6) thermostated oven, (7) sample injector, (8) chromatographic column, (9) detector, (10) recorder, (11) data handling equipment.

Efficiency of the separation and the resolution are diminished as the volume of sample solution injected is increased. However, the amount of sample chromatographed is also increased, and this may be important when quantities of separated components are needed for confirmation of identity or for further study. Equations that quantitatively evaluate the effective sample volume have been derived (Barford, 1978).

No universally applicable device exists for detecting the separated constituents as they elute from the column. The ultraviolet detector is the most widely used. Because of its exceptionally high signal-to-noise ratio, it can detect several nanograms of solutes having only moderate absorptivities. Refractive index, fluorescence, electrochemical, infrared and colorimetric detectors are used also, as are specific detectors such as the thermal energy analyzer for nitroso compounds (Lafleur, 1980).

Since volumes of HPLC columns are only a few milliliters and detector cell volumes a few microliters, the dead volume of the total system must be kept as small as possible. Therefore, short pieces of capillary tubing are used to interconnect components. Because variation in temperature may influence resolution and cause variation in analysis, thermostated columns or ovens are often employed in HPLC. State-of-the-art liquid chromatographs utilize microprocessors to control the instrument operation, make calculations, and perform automated analysis. For a comprehensive review of HPLC instrumentation, see Huber, 1978.

Proteins and Peptides

Traditionally, many separations of proteins have been accomplished by size exclusion chromatography (SEC) on soft gels. Unfortunately, these separations take many hours or even days to complete, although with some automated systems a number of samples a day could be run (Fishman, 1976). Rigid glass or silica particles having pores of a specified size and narrow size distribution have been used for faster separation of organic-solvent soluble polymers. However, proteins were sorbed to these materials under most conditions, so that diffusion in and out of the pores was not the controlling mechanism and thus elution volume was not related to molecular size. Chemical modification of the surface (Table I, bottom) was proposed to reduce sorption and shown to be somewhat effective. We have evaluated commercial versions of this modified controlled pore glass and found that nonideal retention of some proteins still occurred (Barford, 1979a). In fact, at high salt concentrations elution order (ribonuclease (13.7 K Daltons) < chymotrypsinogen (25 K) < ovalbumin (45 K)) was the inverse of that expected for SEC. When sodium dodecylsulfate was added to the buffer mobile phase, normal elution order was observed. While this latter approach had some shortcomings, it has been used in our Center for comparing leaf-protein isolates (Figure 3) from different genotypes and for monitoring the biodegradation of keratin-protein isolates (Barford, 1977). Chromatograms were obtained in less than 30 minutes instead of many hours previously required. Recently, new size exclusion materials, whose chemical nature is proprietary, have been introduced commercially. Some results with one of these is shown in Figure 4. The elution volumes for these proteins fall on a straight line when plotted as a logarithmic function of molecular weight for the lower Na_2SO_4 concentrations shown. Thus, molecular weight information can be obtained on protein mixtures rapidly and the use of detergent or denaturing buffers is avoided. At 0.33 M Na_2SO_4 the larger proteins were retained longer than at the lower salt concentrations, while the retention of the lower molecular weight proteins was essentially unchanged. These retentions could be related to the effect of salt on the shape of these proteins.

While useful information may be obtained by SEC, it is a relatively low resolution chromatographic technique. In our search to develop improved methods for separating complex protein mixtures from agricultural sources, we view SEC as a fractionating tool which would be coupled with other approaches such as ion exchange or reversed phase chromatography to achieve the best results. Several researchers have reported protein separations for which silica-based ion exchangers were used (Alpert, 1979). Of significance was the separation of the clinically important LDH-isoenzyme system

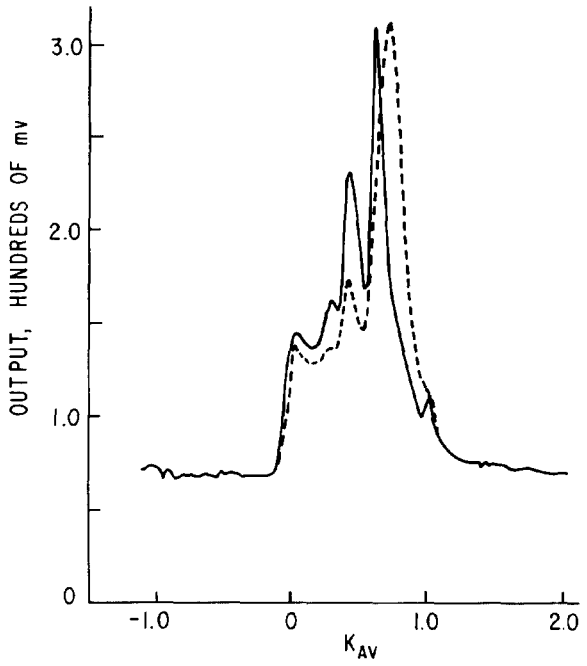


Figure 3. Rapid SEC of fractions from alfalfa protein isolates. Column: SEC-500 (DuPont, Inc.). Mobile phase: phosphate buffer (0.005 mol dm^{-3}, pH = 7.5) containing 0.02% NaN_3 and 1% sodium dodecylsulfate.

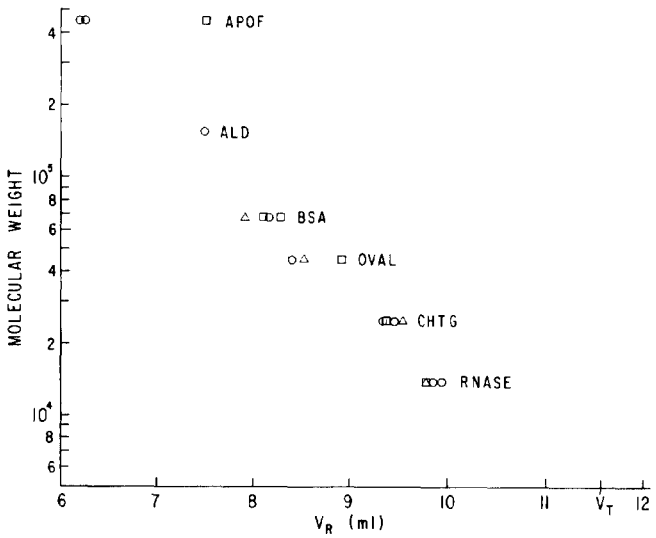


Figure 4. Protein elution from SEC column. Column: TSK-3000SW (Varian Assoc.). Mobile Phase: 0.067M phosphate (pH 6.8) + Na_2SO_4 [○] 0.033M, [△] 0.067M, [□] 0.33M. APOF: apoferritin (450 KD); ALD: aldolase (160 KD); BSA: bovine serum albumin (68 KD); OVAL: ovalbumin (45 KD); CHTG: chymotrypsinogen (25 KD); RNASE: ribonuclease (13.7 KD).

(Schlabach, 1978). These studies were implemented by use of a post-column reactor which enhanced the selectivity of the analysis. We have studied the interactions of proteins with several commercial bonded phase ion exchangers (Barford, 1979b). The elution of proteins from a cation exchanger showed that interactions other than ionic ones occurred. For example, bovine serum albumin and ovalbumin were retained at a pH above their isoionic points, and their retention times increased rather than decreased with increasing eluent molarity as ion exchange theory would predict (Figure 5). The mixed mechanisms and the lower diffusivities of protein led to higher heights equivalent to a theoretical plate. Nevertheless, mixtures of reference proteins and protein isolates were resolved in less than 30 minutes (Figure 6).

Peptides and small proteins have been separated by use of reversed phase columns and mobile phases consisting of hydrophylic or hydrophobic ion-pairing reagents contained in aqueous buffer/alcohol or acetonitrile mixtures (Rivier, 1978; Hearn, 1979). We are investigating the effect of parameters such as pH, temperature, and nature of organic mobile phase components on retention of larger proteins such as bovine serum albumin (68 K Daltons). Mobile phase composition was observed to be critical with changes of less than a percent determining whether protein was eluted at the void volume or retained for exceedingly long times (Breyer, 1979). In these and the ion exchange studies, tests to confirm the presence of protein in eluted peaks were employed. This was found necessary to avoid false identification of peaks which would lead to faulty interpretations of the data.

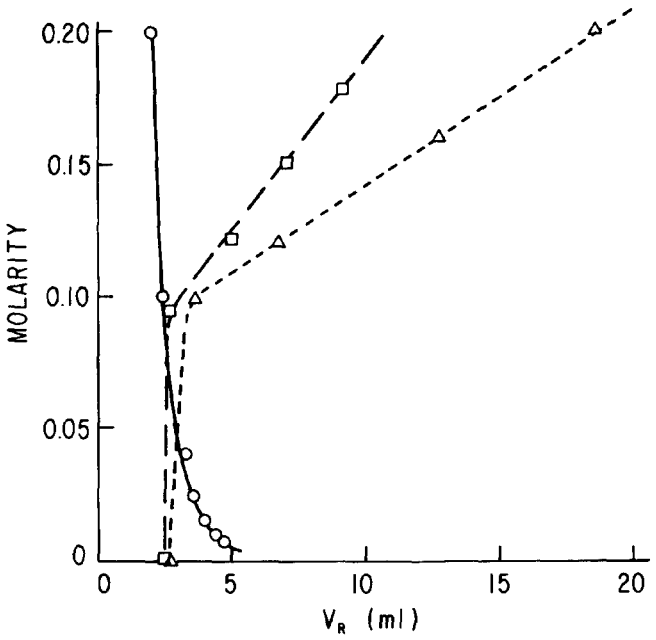


Figure 5. Elution of p-aminobenzoic acid (pK_{a_1} 2.5) (○), ovalbumin (□) and bovine serum albumin (△) from pellicular cation exchanger. Mobile phases: 0, 0.01 M citrate buffer (0.02% NaN_3) + $NaNO_3$ (pH 2.5) 20 μg injected in 10 μl mobile phase, flow 1 ml min^{-1}; for proteins, 0.01 M citrate (0.02% NaN_3) + $(NH_4)_2SO_4$ (pH 6), 400 μg injected in 200 μl mobile phase. Flow-rate 0.5 ml min^{-1}.

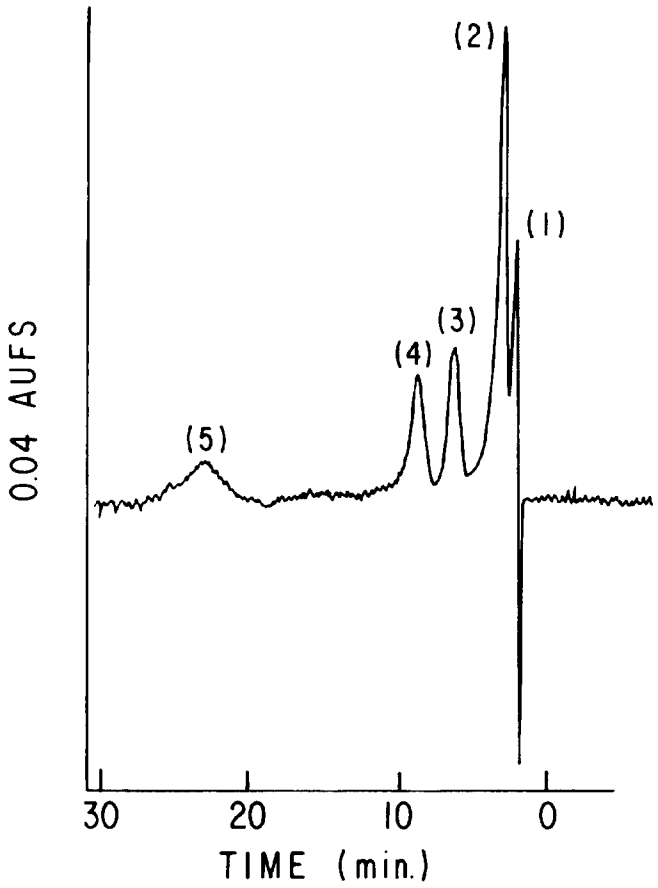


Figure 6. Separation of an alfalfa leaf protein isolate on pellicular cation exchanger. Mobile phase: 0.01 M citrate buffer + 0.05 M $NaNO_3$ (pH 2.9). Peaks 2, 4, 5 gave definite positive protein test; peak 3 slightly positive; peak 1 negative. 200 μg injected in 200 μl mobile phase. Flow-rate: 1 ml min^{-1}.

In conclusion, separations of proteins and peptides in about 30 minutes or less is now possible with modern liquid chromatographic approaches. However, research such as that being performed at our Center and in other laboratories to understand the underlying retention mechanisms is needed before off-the-shelf, routine methods for analysis of proteins from complex matrices such as meat can be developed.

Acknowledgment

The author acknowledges the contributions of H. L. Rothbart, Bernadene J. Sliwinski, and Arthur C. Breyer to the ERRC research reported herein.

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