Journal of Chromatography, 445 (1988) 71–86
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROM. 20 506

RAPID ANALYSIS OF PROTEINS AND PEPTIDES BY REVERSED-PHASE CHROMATOGRAPHY WITH POLYMERIC MICROPELLICULAR SORBENTS

YIH-FEN MAA and CSABA HORVÁTH*
Department of Chemical Engineering, Yale University, New Haven, CT 06520 (U.S.A.)
(Received March 24th, 1988)

SUMMARY

Peptides and proteins were separated by reversed-phase chromatography on a 30 × 4.6 mm I.D. column packed with non-porous crosslinked polystyrene particles having a mean particle diameter of 3 μm and a rugulose surface. The polymeric support did swell slightly in organic solvents, but the estimated 5–8% change in particle diameter did not adversely affect the efficiency of the column which was used repeatedly with gradient elution from water to organic solvent under conditions typically employed in reversed-phase chromatography. In these experiments, the pH of the eluent was varied in a wide range in order to compare the effect of acidic and alkaline eluents on the separation of protein and complex peptide mixtures. The column showed no deterioration even after extensive exposure to alkaline mobile phases. The retention behavior of sixteen proteins having widely different p*I* values was studied as a function of the eluent pH. The chromatographic system exhibited large selectivity differences upon changing the pH of the eluent from 2 to 11. Analytical information about peptide and protein mixtures could therefore be enhanced by using eluents at the pH extremes. At the pH extremes of 2 and 11 peak sharpness and protein mass recovery were found to be superior to those obtained with neutral eluents. Usually the column temperature was held at 80°C and typical analysis times ranged from 30 s to 10 min as illustrated by chromatograms of protein mixtures and by peptide maps. With regular use under such conditions the column showed no deterioration after three months.

INTRODUCTION

Recently, micropellicular sorbents made of spherical particles of a few μm in diameter were introduced for rapid separation of proteins and peptides by reversed-phase[1], ion-exchange[2,3] and hydrophobic interaction[4] chromatography. A major advantage of the pellicular configuration is that significant intraparticular diffusion resistances are absent[5,6] and this is particularly useful for the rapid analysis of biopolymers with high efficiency and resolution. At the same time, the lack of internal pore structure offers certain other advantages such as good recovery of mass and

0021-9673/88/$03.50

biological activity in protein chromatography[7,8]. Theoretical and experimental studies[1,9] have shown that the high speed and efficiency of chromatographic analysis with micropellicular sorbents can be further enhanced by operating the column at elevated temperature. Due to their solid, fluid-impervious core, pellicular sorbents are generally more stable at high temperatures than the porous stationary phases traditionally used in high-performance liquid chromatography (HPLC). With increasing temperature, both diffusivity and sorption kinetics of the eluites are accelerated with a concomitant decrease in eluent viscosity. Hence, high flow velocities are possible and even columns packed with micropellicular sorbents can be operated below the upper pressure limit of the chromatograph. These benefits can be particularly significant in the rapid chromatography of proteins which have low diffusivities and slow sorption kinetics.

Reversed-phase chromatography has been widely employed for the separation of proteins and peptides with hydro-organic eluents having acidic or neutral pH values[10–12] but the instability of the commonly used non-polar siliceous bonded phases at high pH has precluded their employment with alkaline eluents[13,14]. Only recently have been introduced microparticulate, rigid, chemically stable stationary phases on polymeric supports that exhibit column efficiencies comparable to those of the corresponding siliceous sorbents[15,16]. Such polymeric stationary phases are stable in contact with alkaline eluents and allow separation of proteins and peptides at high pH, where their carboxylic and amino groups are ionized and neutral, respectively. Under such conditions, chromatographic selectivity is expected to be different from that obtained with commonly used acidic media[17]. Besides the possibility of expanding the operating pH range of the eluent in HPLC, stationary phases based on polymeric supports also allow convenient regeneration of the column by washing it with sodium hydroxide solution as often required in protein chromatography.

This report examines the potential of polymer-based micropellicular sorbents in the rapid analysis of proteins and peptides by reversed-phase chromatography at elevated temperature in a wide range of eluent pH.

EXPERIMENTAL

Instrument

The liquid chromatograph was assembled from Constametric I and IIIG metering pumps with a Gradient Master Controller and a SpectraMonitor D variable-wavelength detector (LDC/Milton Roy, Riviera Beach, FL, U.S.A.). A Rheodyne Model 7125 valve (Cotati, CA, U.S.A.) with Tefzel rotor seal and a 20-μl loop was used for sample injection,. The fluid lines from the pump, the sampling valve and the column were installed in a temperature-controlled column compartment (DuPont, Wilmington, DE, U.S.A.).

Columns

Highly crosslinked polystyrene microspheres having a rugulose exterior and a mean particle diameter of 3 μm were prepared by suspension polymerization. The particles were suspended in dioxane, the slurry was sonicated and packed into a 30 $\times$ 4.6 mm I.D. stainless-steel column with methanol as the driving solvent at 850 bar by using an air-driven fluid pump Model DSHT-300 from Haskel (Burbank, CA,

U.S.A.). Thereafter, water was pumped through the column at the same inlet pressure for 20 min.

Chemicals

HPLC-grade acetonitrile, methanol and dioxane as well as ethylenediamine, styrene and sodium acetate were purchased from Fisher (Springfield, NJ, U.S.A.) and divinylbenzene was obtained from Dow (Midland, MI, U.S.A.). Benzoic acid, benzene, trifluoroacetic acid (TFA), calcium chloride, sodium bicarbonate, tribasic and monobasic sodium phosphate were purchased from J. T. Baker (Phillipsburg, NJ, U.S.A.). Aniline, ammonium bicarbonate, sodium borate and sodium carbonate were purchased from Mallinckrodt (Paris, KY, U.S.A.) and tetramethylethylenediamine was obtained from Strem Chemicals (Newburyport, MA, U.S.A.). Water was purified by a Barnstead (Boston, MA, U.S.A.) NanoPure unit. Bovine serum albumin was purchased from J. T. Baker, L-asparaginase from Merck (West Point, PA, U.S.A.), and the other proteins, including TPCK-treated trypsin, were obtained from Sigma (St. Louis, MO, U.S.A.), with the exception of human growth hormone, which was a generous gift from Genentech (South San Francisco, CA, U.S.A.).

Tryptic digest

The human growth hormone (2 mg/ml) and TPCK-treated trypsin at a substrate to enzyme ratio of 100:1 were dissolved in a solution containing 0.1 m*M* calcium chloride, 10 m*M* Tris and 100 m*M* sodium acetate and then incubated at 37°C for 4 h. The reaction was quenched by heating in a boiling water bath for 10 min. The digest was stored at 0°C and used within 48 h.

RESULTS AND DISCUSSION

Stationary phase

Fig. 1 shows a scanning electron micrograph of the stationary phase particles made of a highly crosslinked styrene–divinylbenzene copolymer. The rugulose surface of the particle offers a contact area greater than that of a smooth spherical particle

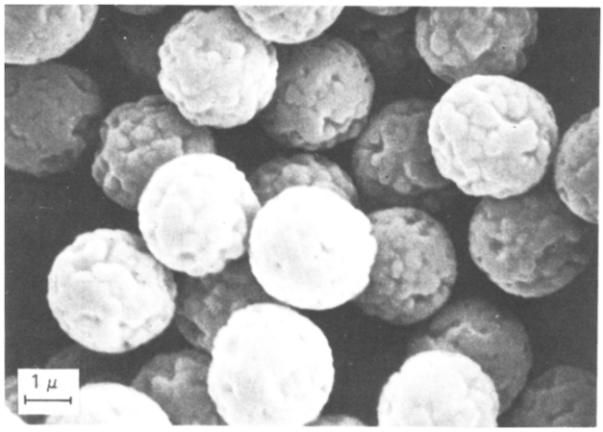

Fig. 1. Scanning electron micrograph of the crosslinked polystyrene particles used in this study.

having the same diameter. As polystyrene is hydrophobic, no chemical treatment of the particles was needed for use as the stationary phase in reversed-phase chromatography.

Porous polymeric stationary phases in contact with organic solvents often lack sufficient mechanical strength and the particles may be deformed under the pressure gradient normally encountered in HPLC columns. By contrast, non-porous column packing made of highly crosslinked polystyrene is expected to be stable under such conditions. In order to evaluate the mechanical stability of our column packing, the pressure drop across the column was measured upon perfusing it with various solvents in a wide range of flow-rate. The results presented in Fig. 2 show a linear dependence of the column inlet pressure on the flow-rate. Thus, with any given solvent the permeability of the 30 × 4.6 mm I.D. column was invariant up to at least 250 bar column inlet pressure. Nevertheless, the permeability of the column with water was greater than with any of the organic solvents, despite their lower viscosity. The results shown in Fig. 2 were reproducible when the different liquids were passed through the column in different order. Furthermore, continually changing the perfusing solvent from water to methanol and to water again over several days did not alter the permeability of the column.

It is believed that this solvent-dependent and reproducible change in column permeability is due to a slight and reversible swelling of the polymeric particles in contact with organic solvents and this effect has been examined in detail as follows.

The specific permeability B^0 of the column was calculated from Darcy's law

$$v = \frac{B^0}{\eta}\frac{\Delta P}{L} \tag{1}$$

where v is the superficial flow velocity, η is the viscosity, L is the column length and

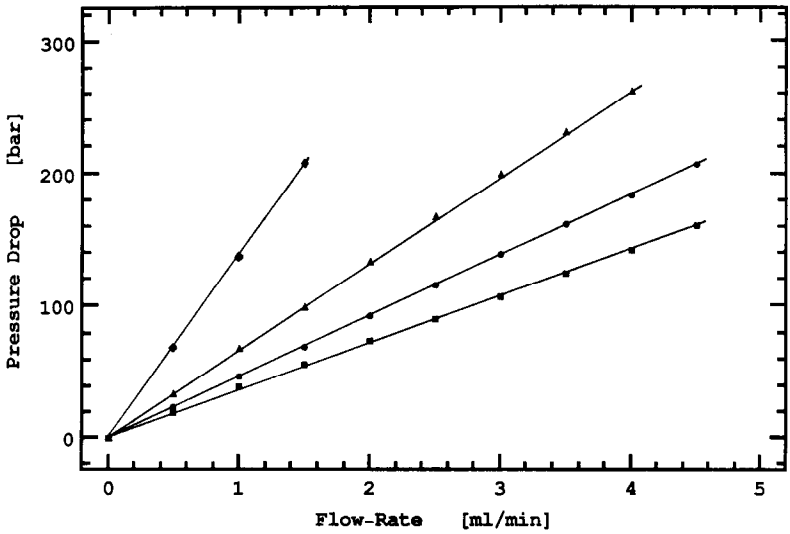

Fig. 2. Graph illustrating plots of the pressure drop *versus* flow velocity of different liquids. Column: 30 × 4.6 mm I.D. Mobile phases: (■) water, (●) acetonitrile, (▲) methanol, (◆) tetrahydrofuran. Temperature: 25°C.

ΔP is the pressure drop across the column. The specific permeability is related to the particle diameter and column porosity by the Kozeny–Carman equation

$$B^0 = \frac{d_p^2 \varepsilon^3}{180(1 - \varepsilon)^2} \tag{2}$$

where d_p is the particle diameter and ε is the interstitial porosity of the column. As described in the experimental section, columns were packed from a dioxane slurry first with methanol followed by water as the packing solvent. It is assumed that no swelling of the particles occurs in water and the particle diameter in contact with water is identical to that of the dry particles as determined by the microscope to be 3.2-μm. With this particle diameter and B^0 calculated by eqn. 1 from data measured with water, the interstitial porosity was calculated by eqn. 2 as 0.38, a value that is typical for random packing of spherical particles[18]. If swelling of the particles occured upon replacing water by an organic solvent in the column, the resulting increase in particle diameter and decrease in interstitial porosity can explain the observed changes in permeability. In order to calculate these changes, we assume that a cube of volume V is occupied by one polystyrene particle having diameter d_p. Then the relationship between V, d_p and fractional void volume of the cube, which is the porosity ε, is given by

$$V(1 - \varepsilon) = \frac{\pi d_p^3}{6} \tag{3}$$

When water is replaced by an organic solvent, the particle swells slightly and the porosity decreases to ε^*. We assume that the equivalent diameter of the slightly swollen particle, d_p^*, in the same cube can be expressed as

$$d_p^* = d_p(1 + \delta^*) \tag{4}$$

where δ^* is the fractional increase in the particle diameter. According to eqn. 3 we obtain that

$$V(1 - \varepsilon^*) = \frac{\pi[d_p(1 + \delta^*)]^3}{6} \tag{5}$$

and by dividing eqn. 5 by eqn. 3, we get the following relationship

$$\frac{(1 - \varepsilon^*)}{(1 - \varepsilon)} = (1 + \delta^*)^3 \tag{6}$$

The specific permeability of the column containing the slightly swollen particles, B^{0*}, in the presence of organic solvent can be expressed as

$$B^{0*} = \frac{[(1 + \delta^*)d_p]^2 \varepsilon^{*3}}{180(1 - \varepsilon^*)^2} \tag{7}$$

Experimental B^{0*} values as well as eqns. 6 and 7 were used to calculate d_p^* and ε^* for the different cases and the results are given in Table I. According to these calculations the porosity of the column decreased from 0.38 to 0.28 upon changing from water

TABLE I

SOLVENT EFFECTS ON THE SWELLING OF MICROPELLICULAR POLYMERIC SORBENT AND ON THE SPECIFIC PERMEABILITY AND INTERSTITIAL POROSITY OF THE COLUMN PACKED WITH SUCH PARTICLES

Mobile phase	*Specific permeability (Darcy) × 10^4*	*Porosity*	*Percent increase in particle diameter**
Water	81	0.38	0
Methanol	24	0.28	5.1
Acetonitrile	24	0.28	5.1
Tetrahydrofuran	13	0.24	7.8

* With the particle diameter in contact with water, d_p = 3.2 μm, as the reference.

to methanol. With the 3.2-μm particles, this would correspond to a 5.1% increase in particle diameter whereas with acetonitrile and tetrahydrofuran, the respective increases in particle diameter were calculated as 5.1 and 7.8%. Evidently, swelling of such magnitude did not have an untoward effect on the mechanical stability of the packing that manifested itself in constant permeability at least up to 250 bar column inlet pressure. It is believed that the use of water as the final packing solvent and the high (850 bar) packing pressure resulted in a compact packing structure that retained its integrity and exhibited only reversible changes in the porosity upon contact with different solvents.

Retention behavior of small molecules

In order to gain some insight into the characteristics of the polymeric micropellicular stationary phase, the retention behavior of small molecules under conditions employed in reversed-phase chromatography was investigated. Benzene, aniline and benzoic acid were chromatographed isocratically with aqueous acetonitrile as the eluent and the retention factors were plotted as a function of the organic modifier concentration and the pH of the eluent, as shown in Fig. 3. The dependence of log k' on the organic modifier concentration was very close to linear for all three eluites under conditions of the experiments, as expected for a regular reversed phase system[19–21]. The slopes of the lines for neutral species decrease in the order of protonated benzoic acid, unprotonated aniline and benzene, that corresponds to the decreasing molecular size of the eluites. The close similarity of the slopes for dissociated benzoic acid, protonated aniline and benzene is likely the result of a balance of the electrostatic and hydrophobic effects involved in the retention free energy[21]. These results imply that the underivatized resin-based packing material has retentive properties similar to *n*-alkylsilica and can be used as an effective alternative stationary phase in reversed-phase chromatography. The small swelling of the stationary phase particles upon changing the composition of the hydro-organic eluent, as described in the previous section, did not affect the retention behavior of the stationary phase so that even retention of small molecules followed the pattern customarily observed in reversed-phase chromatography. However, column efficiency in terms of reduced plate height was inferior to conventional packings, therefore further advances are needed to exploit the potential of this type of columns for the HPLC of small molecules.

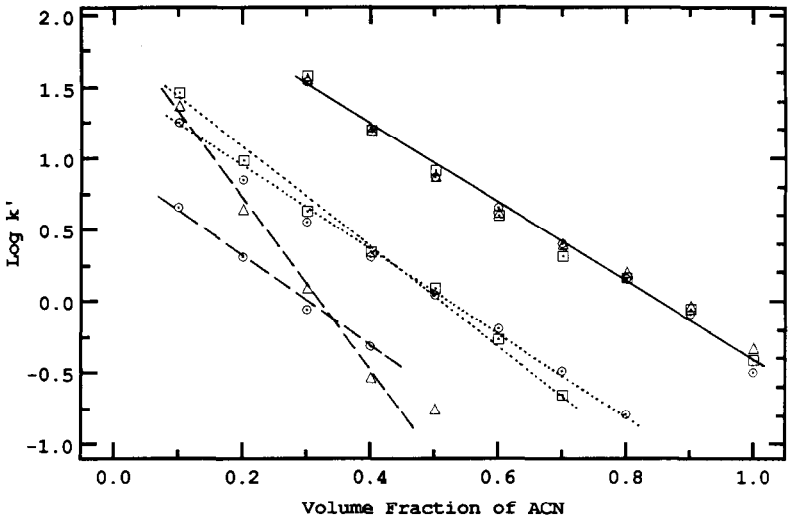

Fig. 3. Plots of the logarithmic retention factor of aromatics against the volume fraction of acetonitrile (ACN) in the aqueous eluent. Column: 30 × 4.6 mm I.D. Eluent: (△) 0.1% TFA, pH 2.2; (○) pH 6.5; (□) 0.1% ethylenediamine, pH 10.8. Flow-rate: 2 ml/min. Temperature: 25°C. Samples: benzoic acid (---), benzene (——) and aniline (· · · · ·).

Fast analysis of proteins

The major objective of this study was to demonstrate the use of non-polar micropellicular polymer-based stationary phases in high-speed analysis of proteins and peptides by using gradient elution. The potential efficiency of the column packed with this type of sorbent is illustrated in Fig. 4A by the chromatogram of a mixture

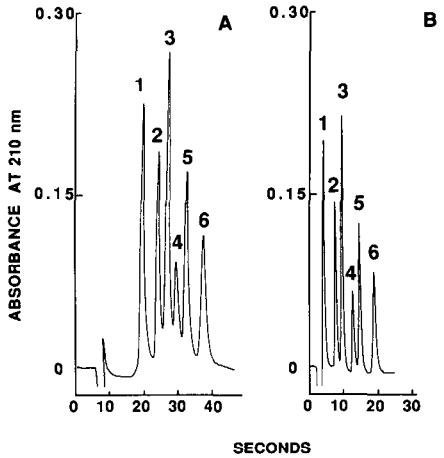

Fig. 4. Chromatograms of a protein mixture obtained on a 30 × 4.6 mm I.D. column under two different conditions. Flow-rate and temperature: (A) 3 ml/min, 25°C; (B) 7 ml/min, 80°C. Linear gradient in 1 min from 10 to 100% acetonitrile in water containing 0.2% TFA. Samples: 1, 100 ng of ribonuclease A; 2, 50 ng of cytochrome *c*; 3, 10 ng of lysozyme; 4, 100 ng of L-asparaginase; 5, 100 ng of β-lactoglobulin A; 6, 100 ng of ovalbumin.

of six proteins obtained at room temperature in about 40 s at an eluent flow-rate of 3 ml/min. As discussed in the literature[9], the speed and efficiency of separation can be further increased by operating at elevated temperatures. The effect of increasing column temperature is shown in Fig. 4B by a chromatogram of the same mixture at 80°C and 7 ml/min. Comparison of the two chromatograms obtained at commensurable column inlet pressures shows that the analysis time could be almost halved by increasing the column temperature from 25 to 80°C. Further increase in operating temperature was constrained by the difficulties encountered with sample introduction at higher temperatures.

The results support the proposition[9] that short columns packed with micropellicular sorbents, particularly at elevated temperatures, do offer a means to reduce the analysis time customary at present in HPLC from hours and minutes to minutes and seconds, respectively. The chromatograms shown in Fig. 4 indicate that with gradient elution the separation efficacy of the polymer-based micropellicular stationary phases is comparable to that of similar silica-based packings described in the literature[1].

The column was extensively used with gradient elution at 80°C for three months without changes in the retention behavior or permeability. So far no untoward effect of high temperature on the column stability was observed.

Effect of eluent pH

Reversed-phase chromatography of proteins has been almost exclusively carried out with acidic eluents due to the instability of the commonly used siliceous bonded stationary phases in contact with alkaline mobile phase.

In reversed-phase chromatography, the retention factors of ionogenic eluites are known to change upon appropriate change in the eluent pH[21]. This effect was extensively investigated with small molecules, such as organic acids[22], bases[23] and amino acids[24] by using polymeric stationary phases, mainly polystyrene based materials, that are stable in both acidic and alkaline solutions. Nevertheless, studies on the retention behavior of proteins with alkaline eluents were hampered by the lack of macroporous polymeric sorbents. The columns packed with the polystyrene based micropellicular stationary phase discussed here offered an intriguing opportunity to investigate protein chromatography in a wide range of eluent pH. Since the influence of eluent pH on the chromatographic behavior of such complex molecules is expected to be complicated, the effect of eluent pH was examined not only on the retention but also on peak sharpness and peak area that give information on the separation efficiency and mass recovery of the protein, respectively.

The retention volumes, peak widths and relative peak areas of sixteen proteins listed in Table II, were evaluated from data obtained with eluents having different pH by using the same column and gradient conditions. The dependence of the retention volumes on the pH of the eluent is illustrated in Fig. 5 that allows us to draw the following conclusions. First, the retention of most proteins is the strongest at pH 2.2 with 0.1% TFA in the hydro-organic eluent, *i.e.* under conditions most commonly employed in the reversed-phase chromatography of peptides and proteins[12]. Second, the separation of the proteins as measured by the spacing between their peaks is the greatest when the pH of the eluent is between pH 7 and 9. Third, for some proteins, such as α-chymotrypsinogen A, the retention can vary dramatically with the eluent

TABLE II

SYMBOLS AND PROPERTIES OF THE PROTEINS USED IN THIS STUDY

Symbol	*Protein*	*pI*	*Molecular weight*	*Source*
A	Pepsin	3.0	34 000	Porcine stomach mucosa
B	Fetuin	3.2–3.8	48 300	Fetal calf serum
C	Concanavalin A	4.5–5.5	96 000	Jack bean
D	Ovalbumin	4.7	44 000	Chicken egg
E	Bovine serum albumin	4.9	67 000	Bovine serum
F	β-Lactoglobulin A	5.1	35 000	Bovine milk
G	L-Asparaginase	5.2	106 000	*Escherichia coli*
H	Insulin	5.7	5700	Bovine pancreas
I	Conalbumin	6.8	77 000	Chicken egg white
J	Hemoglobin	6.8	68 000	Bovine blood
K	Myoglobin	6.8–7.3	16 900	Equine skeletal muscle
L	Ribonuclease A	9.4	13 700	Bovine pancreas
M	α-Chymotrypsinogen A	9.5	25 500	Bovine pancreas
N	Trypsin	10.1	23 000	Bovine pancreas
O	Cytochrome *c*	10.6	12 200	Horse heart
P	Lysozyme	11.0	14 000	Chicken egg white

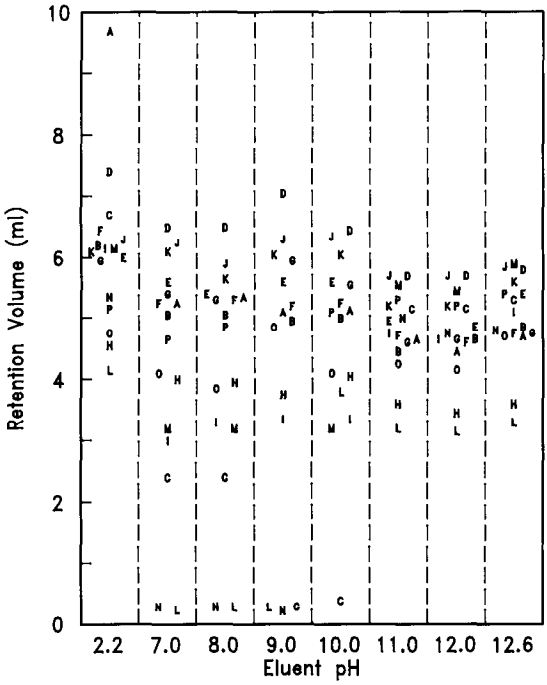

Fig. 5. Retention map of proteins. Column: 30 × 4.6 mm I.D. Flow-rate: 2.5 ml/min. Aqueous starting eluents: (1) 0.1% TFA, pH 2.2; (2) 0.1 *M* $NaHCO_3$, pH 7.0; (3) 0.1 *M* $NaHCO_3$, pH 8.0; (4) 5 m*M* $Na_2B_4O_7$, pH 9.0; (5) 0.2% ethylenediamine, pH 10.0; (6) 5 m*M* Na_2CO_3, pH 11.0; (7) 0.2% tetramethylethylenediamine, pH 12.0; (8) 1.5 m*M* Na_3PO_4, pH 12.6. Gradient former: 75% acetonitrile in the starting eluent. Linear gradient from 10 to 100% of the gradient former in 4 min. Temperature: 25°C. Sample proteins are listed in Table II.

pH whereas some other proteins, such as lysozyme, exhibit only slight changes in the retention with changing eluent pH. Finally, no correlation was found between the pH dependence of the retention and the isoelectric point of the proteins.

The results shown in Fig. 5 also indicate that the selectivity of the separation can be adjusted by manipulating the eluent pH. This is illustrated in Fig. 6 by chromatograms of five proteins obtained with the same column with eluents having low and high pH. It is seen that fetuin, β-lactoglobulin A and α-chymotrypsinogen A were not separated at pH 2.2 with 0.1% TFA in the aqueous acetonitrile as the eluent. In contrast, all proteins were almost completely resolved at pH 11.0 with sodium phosphate in the otherwise identical hydro-organic eluent.

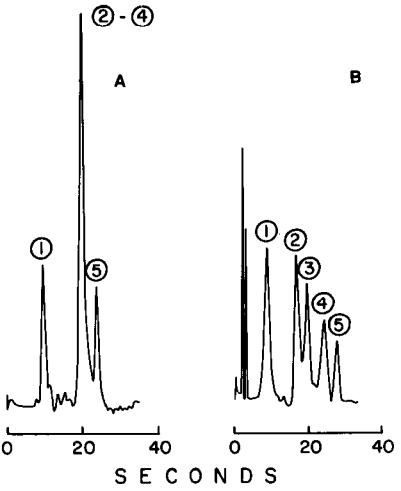

Fig. 6. Effect of eluent pH on the separation of proteins. Column: 30 × 4.6 mm I.D. Flow-rate: 5 ml/min. Aqueous starting eluent: (A) 0.1% TFA, pH 2.2; (B) 1.5 m*M* Na_3PO_4, pH 11.0. Linear gradient in 1.5 min from 5 to 70% acetonitrile in the starting eluent. Temperature: 80°C. Samples: 1, ribonuclease A; 2, fetuin; 3, β-lactoglobulin A; 4, α-chymotrypsinogen A; 5, ovalbumin.

Various other protein mixtures were also chromatographed on the same column with acidic, neutral and alkaline eluents. The number of peaks and the relative retention times were often greatly affected by the pH of the eluent. Chromatograms of commercial transferrin and wheat germ agglutinin are shown in Figs. 7 and 8. It is seen that the number of peaks was the greatest when alkaline eluent was used. The number of peaks on the respective chromatograms are believed to be various forms of transferrin containing different amounts of iron[25], and to be isolectins of wheat germ agglutinin[26].

The peak widths and areas of the sixteen proteins were also evaluated and the values obtained at a given eluent pH were averaged. Furthermore, the peak areas thus obtained were normalized to those obtained under conditions most commonly used in reversed-phase chromatography, *i.e.* with eluent containing 0.1% TFA. The results are shown in Table III. For most of the proteins, the peak area was the largest at pH 2.2 with 0.1% TFA in the eluent. On the other hand, the average peak width

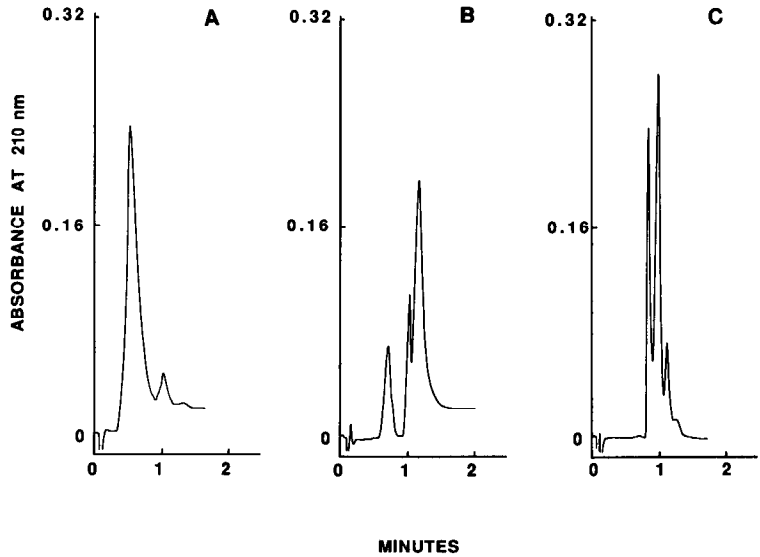

Fig. 7. Chromatograms of transferrin obtained with three eluents of different pH. Column: 30 × 4.6 mm I.D. Flow-rate, 3 ml/min. Linear gradient in 1.5 min (A) from 30 to 40% acetonitrile in water containing 0.1% TFA, pH 2.2; (B) from 5 to 50% acetonitrile containing 50 m*M* NH_4HCO_3, pH 7.8; (C) from 10 to 30% acetonitrile in water containing 3 m*M* Na_3PO_4, pH 11. Temperature: 25°C. Sample size: 10 μg.

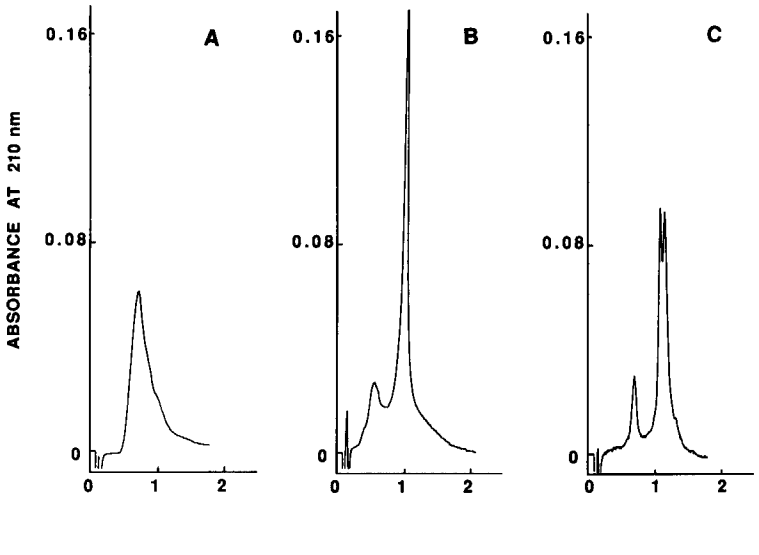

Fig. 8. Chromatograms of wheat germ agglutinin obtained with three eluents of different pH. Column: 30 × 4.6 mm I.D. Flow-rate: 3 ml/min. Linear gradient in 1.5 min (A) from 15 to 20% acetonitrile in water containing 0.1% TFA; (B) from 8 to 25% acetonitrile in water containing 50 m*M* NH_4HCO_3, pH 7.8; (C) from 10 to 15% acetonitrile in water containing 3 m*M* Na_3PO_4, pH 11. Temperature: 25°C. Sample size, 3 μg.

TABLE III

AVERAGE PEAK WIDTHS AND RELATIVE PEAK AREAS MEASURED AT DIFFERENT ELUENT CONDITIONS WITH THE PROTEINS LISTED IN TABLE II

	*Eluent pH**				
	2.2	*7.0*	*8.0*	*9.0*	*11.0*
$W_{0.5}$ (ml)**	0.22	0.34	0.26	0.29	0.17
Area***	1.0	0.5	0.6	0.8	0.9

* Operating conditions and eluent compositions as in Fig. 5.

** Average value of the peak width at half height for all sixteen proteins.

*** Average peak area of all sixteen proteins relative to that measured at pH 2.2 with 0.1% TFA in the eluent.

at half peak height, $W_{0.5}$, was the smallest at pH 11.0 with 5 m*M* sodium carbonate in the eluent and under these conditions the peak areas of most proteins were almost as high as those measured at pH 2.2 with 0.1% TFA in the eluent. In the pH range between 7 and 9, the average peak widths and the average peak areas were generally large and small, respectively, with eluents employed in this study.

The results discussed above likely represent the combined effect of pH, the particular buffer salt and additives, *e.g.*, TFA, a known ion pairing agent. Generally, it is hard to investigate the effect of each of these factors: yet we attempted to explore the effect of TFA concentrations in the eluent as well as that of the eluent pH in two sets of experiments.

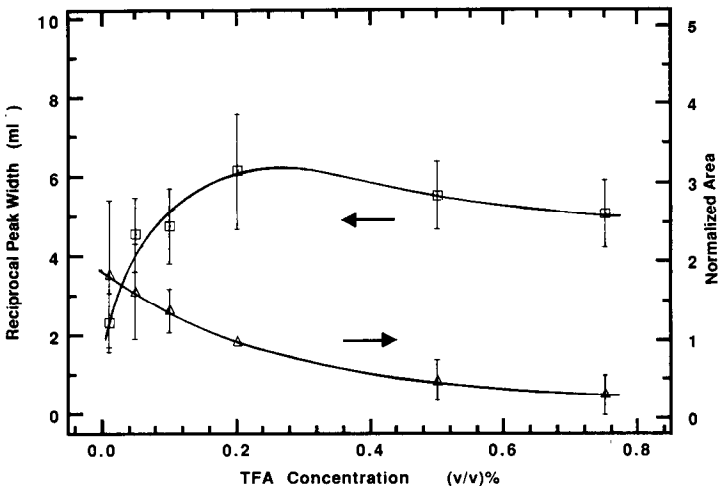

Fig. 9. Effect of TFA concentration on averaged widths and normalized areas of protein peaks. Column: 30 × 4.6 mm I.D. Flow-rate: 2 ml/min. Linear gradient in 3 min from 10 to 60% acetonitrile in water containing TFA. Temperature: 25°C. Samples: 2 μg of ribonuclease A, 1 μg of cytochrome *c*, 1 μg of lysozyme, 1 μg of α-chymotrypsinogen A and 2 μg of ovalbumin. Peak areas were normalized to that of the same protein measured with the eluent containing 0.2% TFA, pH 2.0.

In agreement with reports on the mass recovery and peak shape of proteins in reversed-phase chromatography with siliceous[27] and polymeric[28] stationary phases, we also have found that these properties of the chromatographic system are influenced by the concentration of the phosphate buffer or TFA. As shown in Fig. 9 both the area and width of the protein peak decrease with increasing concentration of TFA. The peak sharpness was expressed as the reciprocal of the peak width at half peak height. These results suggest that efficiency and protein recovery change in opposite ways with respect to the concentration of TFA in the hydro-organic eluent for the five proteins tested, and a good compromise range seems to be a TFA concentration between 0.05 and 0.2% (v/v).

Other experiments were carried out with phosphate buffers of widely different pH in order to study the effect of eluent pH alone on the chromatographic behavior of bovine serum albumin, myoglobin and ovalbumin. These three proteins were chromatographed by using gradient elution with 5 m*M* sodium phosphate of pH 2.6, 7.2 and 11.5, respectively, as the starting eluent and 50% acetonitrile in the aqueous buffer as the gradient former. The peak area and the peak sharpness, of the three proteins were drastically affected by the pH as shown in Fig. 10. It is seen that a gain in peak area is accompanied by a loss in peak sharpness when the pH of the phosphate buffer is changed from 11.5 to 2.6.

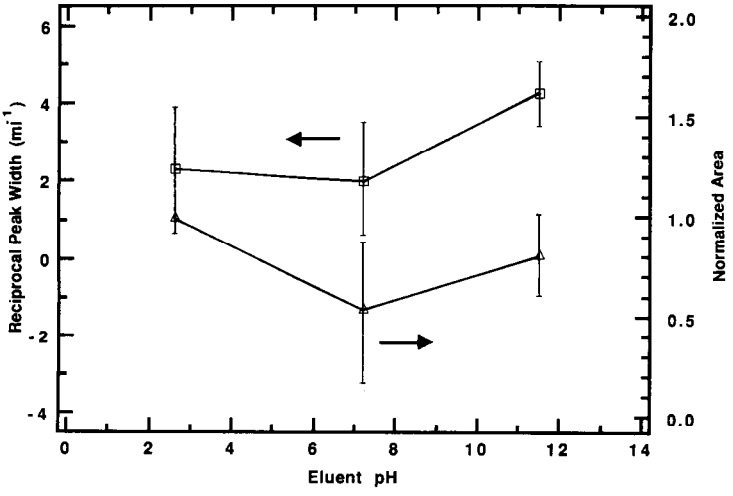

Fig. 10. Dependence of averaged peak widths and normalized peak areas on the eluent pH. Column: 30 × 4.6 mm I.D. Flow-rate: 3 ml/min. Linear gradient in 1 min from 5 to 50% acetonitrile in water containing 5 m*M* sodium phosphate and followed by isocratic elution with 50% acetonitrile for 1 min. Temperature: 25°C. Sample: 1 μg of bovine serum albumin, 1 μg of myoglobin and 1 μg of ovalbumin. Peak areas were normalized to that of the same protein measured with the eluent of pH 2.6.

The data show that the selectivity of the chromatographic system for proteins is quite different in reversed-phase chromatography with acidic and alkaline eluents. Such selectivity differences can be very useful in practical analytical work with proteins and can be exploited because under proper conditions, the area and sharpness of the peaks obtained with alkaline and the commonly used acidic eluents are commensurate.

Analysis of protein impurities

Rapid assay of protein purity can facilitate on-line and at-line monitoring of downstream processing and quality control. The use of columns packed with micropellicular sorbent is illustrated by the chromatogram in Fig. 11, which shows the composition of commercial bovine trypsin. The time scale of analysis is much shorter than that usual in reversed-phase HPLC[29].

Commercial ovalbumin is known to contain at least eight forms[30,31] and they were separated by chromatography on agarose bound lectins such as concanavalin A and wheat germ agglutinin[32,33]. Fig. 12 shows that at least seven of the components can be resolved with our chromatographic system by gradient elution in 2 min.

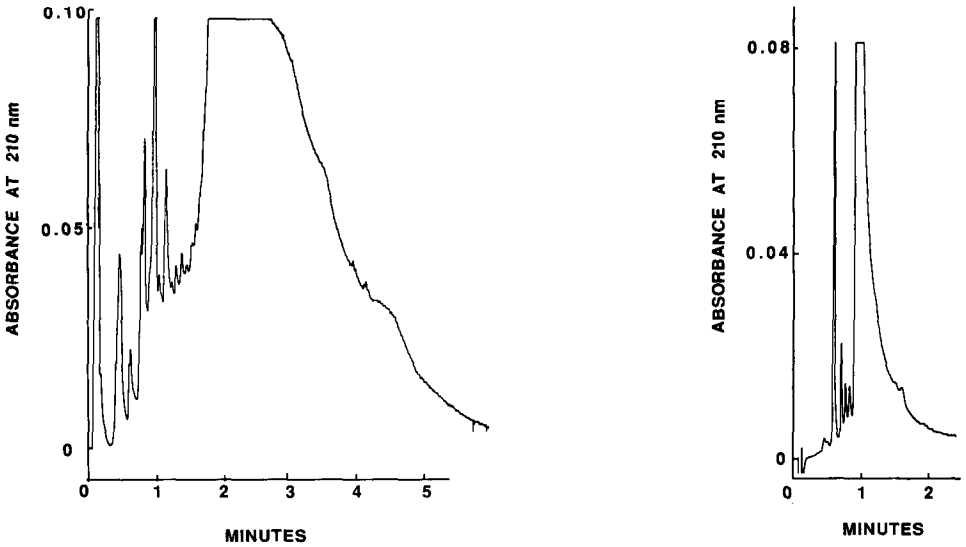

Fig. 11. Rapid analysis of commercial trypsin by reversed-phase chromatography with alkaline eluents. Column: 30 × 4.6 mm I.D. Flow-rate: 5 ml/min. Linear gradient in 3 min from 5 to 70% of acetonitrile in water containing 1.5 m*M* $Na_2B_4O_7$, pH 10. Temperature: 80°C. Sample size: 40 μg.

Fig. 12. Chromatogram of chicken ovalbumin at alkaline pH. Column, 30 × 4.6 mm I.D. Flow-rate: 4 ml/min. Linear gradient in 1 min from 5 to 50% acetonitrile in water containing 3 m*M* Na_2CO_3, pH 10.8; and followed by isocratic elution with 50% acetonitrile for 1 min. Temperature: 80°C. Sample size: 40 μg.

Peptide mapping

Preliminary investigations were made to examine the potential of the polymeric micropellicular sorbent in the separation of enzymic digests. The interest in reducing the time of such separations stems from the recent popularity of peptide mapping by reversed-phase chromatography not only in research but also in industrial quality control. The use of siliceous micropellicular sorbents has been shown[34] to facilitate rapid peptide mapping, but such columns are not stable in contact with alkaline eluents. Therefore, our interest was focused on peptide mapping not only under the

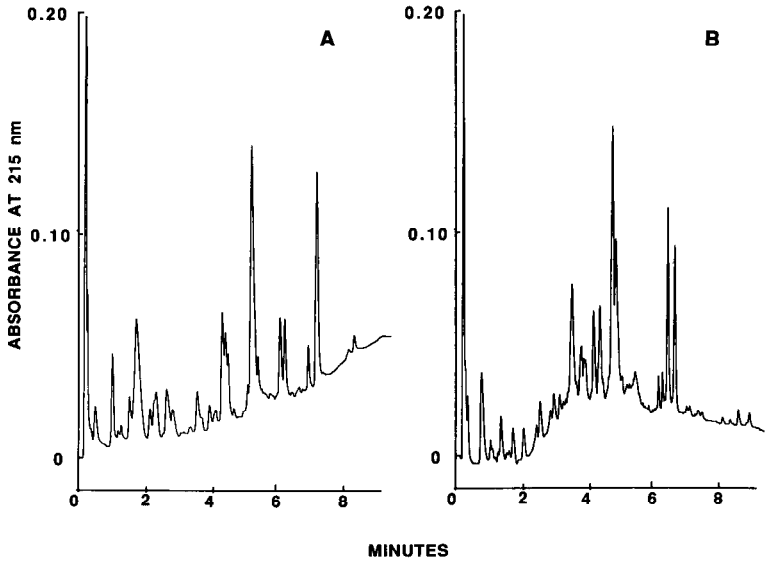

Fig. 13. Tryptic maps of human growth hormone obtained with acidic and alkaline eluents. Column: 30 × 4.6 mm I.D. Flow-rate, 3 ml/min. Gradient in 9 min from 0 to 50% acetonitrile. Aqueous starting eluent either with (A) 0.2% TFA, pH 2.0 or with (B) 3 m*M* Na_3PO_4 and 0.5 m*M* decyltrimethylammonium bromide, pH 11. Temperature: 80°C. Sample size: 20 μg.

conventional acidic but also under alkaline eluent conditions. The use of such widely different eluents is believed to enhance the chromatographic information ensconced in the peptide map about the structure and/or purity of the protein under investigation[35]. Two different peptide maps of the same tryptic digest of human growth hormone are shown in Fig. 13. The chromatograms were obtained at pH 2.0 and 11.0 and reveal even greater selectivity differences for the peptides than those found for the proteins at the two pH extremes. The results suggest that rapid peptide mapping by reversed-phase chromatography on micropellicular polymeric stationary phases at different eluent pH values could gain importance in protein chemistry and technology.

ACKNOWLEDGEMENTS

The authors are indebted to John Novak for providing the electron micrograph. This work was supported by Grants No. CA 21948 and GM 20993 from the National Institutes of Health, U.S. Department of Health and Human Services.

REFERENCES

1 K. Kalghatgi and Cs. Horváth, *J. Chromatogr.*, 398 (1987) 335.
2 D. J. Burke, J. K. Duncan, L. C. Dunn, L. Cummings, C. J. Siebert and G. S. Ott, *J. Chromatogr.*, 353 (1986) 425.
3 Y. Kato, T. Kitamura, A. Mitsui and T. Hashimoto, *J. Chromatogr.*, 398 (1987) 327.
4 R. Janzen, K. K. Unger, H. Giesche, J. N. Kinkel and M. T. W. Hearn, *J. Chromatogr.*, 397 (1987) 91.

5 Cs. Horváth, B. A. Preiss and S. R. Lipsky, *Anal. Chem.*, 39 (1967) 1422.
6 Cs. Horváth and S. R. Lipsky, *J. Chromatogr. Sci.*, 7 (1969) 109.
7 D. J. Burke, J. K. Duncan, C. Siebert and G. S. Ott, *J. Chromatogr.*, 359 (1986) 533.
8 Y. Kato, K. Nakamura and T. Hashimoto, *J. High Resolut. Chromatogr. Chromatogr. Commun.*, 8 (1985) 154.
9 F. D. Antia and Cs. Horváth, *J. Chromatogr.*, 435 (1988) 1.
10 C. E. Dunlap III, S. Gentleman and L. I. Lowney, *J. Chromatogr.*, 160 (1978) 191.
11 F. E. Regnier, *Methods Enzymol.*, 91 (1983) 180.
12 W. S. Hancock and D. R. K. Harding, in W. S. Hancock (Editor), *CRC Handbook of HPLC for the Separation of Amino Acids, Peptides, and Proteins*, CRC Press, Boca Raton, FL, 1984, pp. 303–312.
13 W. R. Melander and Cs. Horváth, in Cs. Horváth (Editor), *High-Performance Liquid Chromatography, Advances and Perspectives*, Vol. 2, Academic Press, New York, 1980, pp. 113–319.
14 A. M. Krstulović and P. R.Brown, *Reversed-Phase High-Performance Liquid Chromatography*, Wiley, New York, 1982.
15 Y.-B. Yang and M. Verzele, *J. Chromatogr.*, 387 (1987) 197.
16 T. Isobe, Y. Kurosu, Y. Fang, N. Ishioka, H. Kawasaki, N. Takai and T. Okuyama, *J. Liq. Chromatogr.*, 7 (1984) 1101.
17 W. S. Hancock and J. T. Sparrow, in Cs. Horváth (Editor), *High-Performance Liquid Chromatography, Advances and Perspectives*, Vol. 3, Academic Press, New York, 1983, pp. 49–85.
18 B. L. Karger, L. R. Snyder and Cs. Horváth, *An Introduction to Separation Science*, Wiley, New York, 1973.
19 W. R. Melander, B.-K. Chen and Cs. Horváth, *J. Chromatogr.*, 185 (1979) 99.
20 W. R. Melander, A. Nahum and Cs. Horváth, *J. Chromatogr.*, 185 (1979) 129.
21 Cs. Horváth and W. Melander, *J. Chromatogr. Sci.*, 15 (1977) 393.
22 M. D. Grieser and D. J. Pietrzyk, *Anal. Chem.*, 45 (1973) 1348.
23 C. H. Chu and D. J. Pietrzyk, *Anal. Chem.*, 46 (1974) 330.
24 E. P. Kroeff and D. J. Pietrzyk, *Anal. Chem.*, 50 (1978) 502.
25 H. Stibler, S. Borg and C. Allgulander, *Acta Med. Scand.*, 206 (1979) 275.
26 I. Matsumoto, T. Koyama, H. Kitagaki-Ogawa and N. Seno, *J. Chromatogr.*, 400 (1987) 77.
27 K. A. Cohen, K. Schellenberg, K. Benedek, B. L. Karger, B. Grego and M. T. W. Hearn, *Anal. Biochem.*, 140 (1984) 223.
28 Y. Kato, T. Kitamura and T. Hashimoto, *J. Chromatogr.*, 333 (1985) 93.
29 M. P. Strickler, M. J. Gemski and B. P. Doctor, *J. Liq. Chromatogr.*, 4 (1981) 1765.
30 G. D. Longmore and H. Schachter, *Carbohydr. Res.*, 100 (1982) 365.
31 C. Ceccarini, P. Lorenzoni and P. H. Atkinson, *Biochim. Biophys. Acta*, 759 (1983) 214.
32 Y. Kato, H. Iwase and K. Hotta, *J. Biochem.*, 95 (1984) 455.
33 Y. Kato, H. Iwase and K. Hotta, *Anal. Biochem.*, 138 (1984) 437.
34 K. Kalghati and Cs. Horváth, *J. Chromatogr.*, 443 (1988) 343.
35 T. Sasagawa, L. H. Ericsson, D. C. Teller, K. Titani and K. A. Walsh, *J. Chromatogr.*, 307 (1984) 29.