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SEPARATION OF PROTEINS BY REVERSED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

I. OPTIMIZING THE COLUMN

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SUMMARY

In the process of developing a new analytical technology (the chromatophoresis process®) which couples reversed-phase high-performance liquid chromatography (HPLC) to sodium dodecyl sulfate polyacrylamide gel electrophoresis in a real-time automated system, it was apparent that improvements in resolving power for the first-dimension (HPLC) separation were necessary. The present paper describes the optimization of the column for our initial work on reversed-phase HPLC separations. Polymeric (polystyrene) packings having particle diameters of 5 μm and pore diameters of 300 Å were generally superior in terms of resolution, sample recovery and minimization of "ghosting". Optimum column dimensions were 50 × 1.0 mm I.D. for the flow-rates required in our system (10–100 μl/min).

INTRODUCTION

Among the many methods available for the high-resolution analysis of complex mixtures, it is clear that multi-dimensional procedures [*e.g.*, high-performance liquid chromatography (HPLC) with column-switching, liquid chromatography–mass spectrometry] deliver maximal resolving power. For the characterization of protein mixtures, two-dimensional (2-D) electrophoresis is by far unique in this respect[1–3]. This procedure makes use of two independent physico-chemical parameters (solute charge and molecular weight) to separate protein samples having more than 1000 components, including proteins having quite subtle differences in macromolecular composition. However the great power of 2-D electrophoresis carries a corresponding price in terms of procedural complexity and the need for skilled operators. As a result, this technique has so far not been adapted for fully automated operation in the routine laboratory.

We have chosen to explore a new strategy for the high-resolution analysis of complex protein samples. Laboratories currently responsible for the characterization

of protein mixtures (such as those that arise for recombinant products) today make heavy use of both reversed-phase HPLC and sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE). In this and following papers[4,5] we will describe a fully-automated procedure (the chromatophoresis process) for the two-dimensional separation of protein samples by means of reversed-phase HPLC (RP-HPLC) and SDS-PAGE.

In the chromatophoresis process illustrated in Fig. 1, proteins eluting from the HPLC column pass through a UV detector (UV) to a heated micromixing chamber (protein reaction system, PRS). Proteins in the eluate are denatured and complexed with SDS in the PRS when a denaturing solution (protein reaction cocktail, PRC) containing SDS, β-mercaptoethanol and buffer is mixed with the eluate. The SDS–protein complexes in the eluate stream exit the HPLC system through an outlet lying flush on the surface of a discontinuous polyacrylamide gradient gel. The outlet is moved across the surface of the gel by means of a computer-controlled tracking

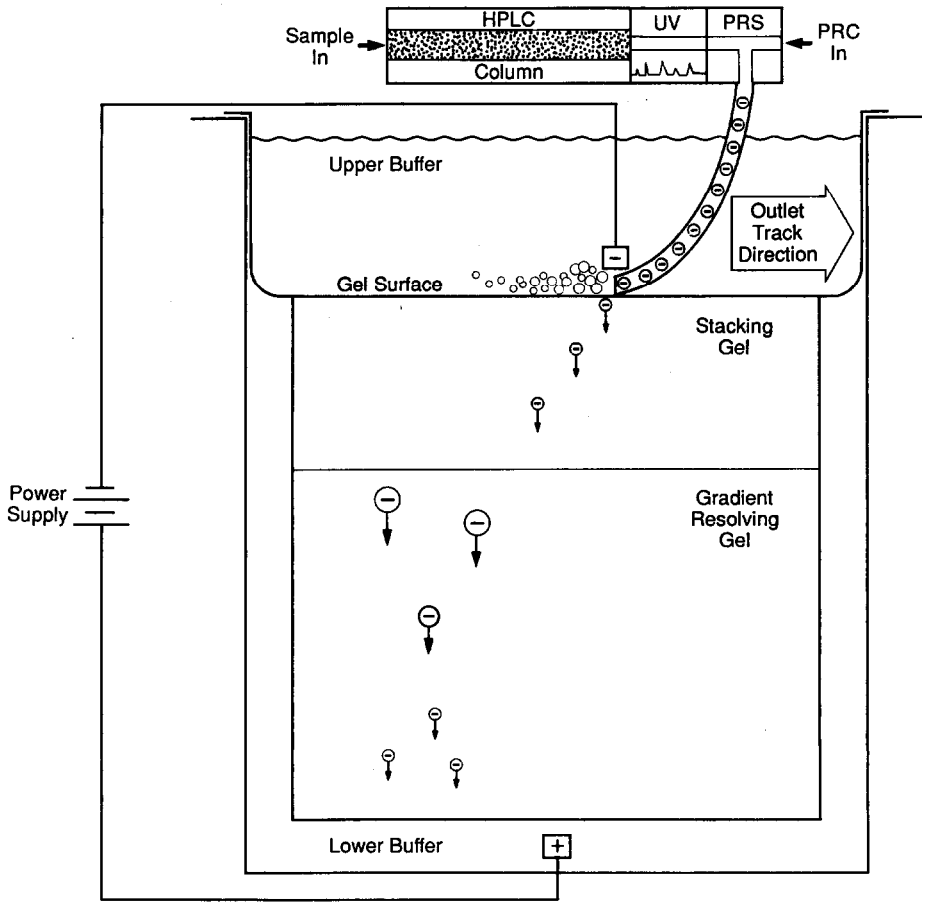

Fig. 1. Schematic illustration of the electrophoretic transfer of proteins in the chromatophoresis process. For details and explanation see text.

system. A d.c. electrical field is focused in the region of the HPLC outlet, which drives the negatively-charged SDS–protein complexes from the elution stream into the gel as a programmably-specified continuum, with no degradation of the resolution achieved in the first dimension HPLC separation.

The polyacrylamide gel uses a discontinuous stacking chemistry to stack proteins as the first dimension HPLC separation proceeds[4,5]. The mobility of the moving boundary of the discontinuous stacking chemistry is chemically regulated so that it will completely stack proteins loaded into the gel for up to 60 min. After stacking, separation in the gradient resolving gel requires 4 h, resulting in a total run time for the 2-D chromatophoresis process of no more than 5 h.

Unfortunately, the current performance of RP-HPLC falls far short of what is required, if the chromatophoresis process is to prove useful for a broad range of protein samples, particularly those having molecular weights $> 25\,000$. Our goals for the enhancement of the first-dimension RP-HPLC separation in the chromatophoresis process were as follows: (1) a peak capacity comparable to that of isoelectric focusing (about 70–100)[6], (2) minimum peak volumes for maximum detection sensitivity, (3) single, Gaussian peaks for most proteins, and (4) near-quantitative sample recovery with concommitant reductions in ghosting. This paper and the one that follows[4] describe how we have been able to achieve these objectives for RP-HPLC separation as part of the chromatophoresis system. Success in reaching these goals should also be of interest to workers interested in using RP-HPLC *per se* for the separation of protein mixtures.

While a substantial literature on RP-HPLC for protein separations already exists[7–12], it is widely appreciated that these separations commonly suffer from a number of practical problems[13–22]: broad bands which limit overall sample resolution, tailing or distorted bands which complicate quantitative analysis, incomplete recovery of individual proteins, carryover or "ghosting", with resulting contamination of the separation by components of a preceding sample, splitting of a single protein into two or more distinct bands, and fouling of the HPLC column so that only a small number of samples can be run.

In general, the likelihood of these separation problems in RP-HPLC increases with the mass and/or hydrophobicity of the protein molecule. Highly basic proteins can also exhibit poor performance in RP-HPLC. Because of the performance enhancements we were trying to achieve for the chromatophoresis process, we felt it was necessary to carry out a systematic investigation of all RP-HPLC parameters as a function of sample type and experimental conditions, in order to achieve adequate chromatographic behavior for proteins of any molecular weight, isoelectric point or hydrophobicity.

In this paper we will examine the effects on our performance goals of (a) column-packing parameters (silica *vs.* polymeric supports, different bonded phases, pore sizes and particle sizes), (b) column dimensions, and (c) extra-column effects. The following paper[4] discusses the effects of varying temperature, mobile phase composition, gradient parameters, sample pretreatment and sample size. We considered it unlikely that we could find a single set of optimized separation conditions for the entire spectrum of potential protein samples; therefore our initial goal was to develop general guidelines for the broadest possible range of proteins. This in turn requires compromises among different separation requirements; maximum resolution or peak

capacity, acceptable separation time, minimum peak width (for maximum sensitivity), minimum band tailing and adequate recovery.

The chromatophoresis process imposes further constraints on certain of the separation parameters, *e.g.*, minimum extra-column effects, as well as mobile phase compositions and flow-rates that are compatible with the transfer process of Fig. 1 and various electrochemical characteristics of the PAGE system. There is an additional requirement that final conditions must be suitable for an automated instrument to be used in routine, reproducible protein separations. Success in the attainment of these various goals should be of interest to many workers faced with the design of RP-HPLC separations of proteins (for other purposes).

BACKGROUND

A casual reading of the literature suggests that RP-HPLC protein separations are often complex and that every protein sample is unique. However, there now exists a well developed theory of chromatographic separation. This theory can be applied quantitatively to proteins, as discussed below, for (a) predicting and optimizing peak capacity and bandwidth[12,23–25], (b) controlling band-spacing and resolution[24,26] and (c) maximizing sample size for preparative separations[27,28]. In the present study we have used this theoretical background for designing experiments, assessing the effects of experimental variables and developing final optimized procedures and conditions for the RP-HPLC separation of proteins.

Ideal protein chromatography

Given a description of the experimental conditions and sample molecular weight, it is possible to predict average bandwidth and peak capacity for the various HPLC methods used to separate proteins[12,29]. Comparisons of such predictions with experimental data often show good agreement, *i.e.*, bandwidth or peak capacity values within ±20% of predicted values. In other cases it is found that experimental runs exhibit much poorer performance than predicted (wider and/or asymmetric bands). It is now believed that exceptions of the latter kind reflect factors that are not included or accounted for in simple models of the chromatographic process. We will refer to those factors responsible for poor chromatographic performance as "non-ideal" effects and discuss them further in the following paper[4].

The ability to predict "ideal" chromatographic separation as a function of experimental conditions and sample characteristics is useful in two ways. First, when bandwidths observed experimentally are wider than predicted by theory, it suggests that something is wrong with the chromatography. Once the existence of a problem has been confirmed, changes in conditions can be explored as a means of identifying and eliminating the deleterious effect and optimizing the separation. Comparisons of bandwidths (experimental *vs.* predicted) can then tell us when further improvements are unlikely, *i.e.*, when experimental bandwidths are within 20% of predicted values. A second use of theoretical predictions is for the design of optimum conditions and system configuration. Different possibilities can be explored via computer modeling before attempting more time-consuming experimental studies. A systematic optimization of all separation parameters can thus be achieved within a reasonable time.

Our model for the quantitative prediction of bandwidth and peak capacity in

TABLE I

SUMMARY OF HPLC SYSTEMS AND SEPARATION CONDITIONS USED IN INITIAL ("CONSENSUS") STUDIES COMPARED TO FINAL ("OPTIMIZED") CONDITIONS

Variable	*Consensus*	*Optimized*
Instrument		
Type	Hewlett-Packard 1090	Bionovus prototype
Precolumn volume (μl)	350	60
Postcolumn volume (μl)	30	0.3
Detector volume (μl)	4.6	1.2
Detection wavelength (nm)	214	214
Column		
Packing	5 μm C_4 silica	5 μm polymeric
Pore size (Å)	300	300
Dimensions (mm)	250 × 4.6	50 × 1.0
Stability	Limited	Good
Mobile phase		
Temperature (°C)	25	60
pH	2.1	2.1
Organic modifier	Acetonitrile	Acetonitrile
Additives	No	Yes
Gradient range (%)	5 to 80	10 to 60
Gradient time (min)	10 to 60	10 to 60
Flow-rate (μl/min)	600	30
Backpressure (p.s.i.)	1000	100
Peak volumes (μl)	300–2400	10–60
Sample pretreatment	No	Yes

RP-HPLC of proteins [12,29] can be simplified to the folllowing approximate relationships[23]

$$\text{peak capacity} \propto \frac{\sqrt{t_G}}{d_p} \quad \text{(independent of } L, d_c \text{ and } F\text{)} \tag{1}$$

$$\text{peak height} \propto \frac{1}{F d_p d_c^2 \sqrt{t_G}} \quad \text{(independent of } L\text{, for a fixed sample mass)} \tag{2}$$

Here t_G is the gradient time, d_p is the diameter of the column-packing particles, L is the column length, F is the flow-rate, and d_c is the column diameter. These equations suggest that (a) particles of smaller diameter improve resolution and favor smaller bandwidths, (b) an increase in gradient time helps resolution but has an adverse effect on detection sensitivity,(c) flow-rate and column diameter have no effect on resolution, but lower flow-rates and narrower columns give more concentrated, taller bands, and (d) column length has little effect on either resolution or detection sensitivity.

Non-ideal protein chromatography

The two most important causes of chromatographic non-ideality in RP-HPLC are (a) secondary retention, often due to silanol effects (in silica-based sorbents), and (b) slow changes in protein conformation during separation[12]. Secondary retention effects can be minimized by choosing appropriate experimental conditions, particularly the type of column packing. Protein conformation can be altered by sample pretreatment procedures, the choice of mobile phase and the column temperature.

TABLE II

CHARACTERISTICS AND CLASSIFICATION OF PROTEIN STANDARDS

Protein	*Abbreviation*	*Subunit mol.wt.*	*No of subunits*	*Hydrophobic***	*pI*	*Initial class****
Insulin*	INS	5700	1	95	5.3	G
Aprotinin*	APR	6500	1	66		B
Cytochrome *c*	CYC	12 500	1	92	9.2	G
Ribonuclease A*	RNS	13 500	1	75	8.8	G
α-Lactalbumin	ALA	14 200	1	114		G
Lysozyme*	LYS	14 300	1	100	11.0	G
Hemoglobin	HEM	17 100	4	123		B
Myoglobin	MYG	17 400	1	122	7.1	B
β-Lactoglobin	BLA	18 400	1	127	5.8	B
Trypsin inhibitor	TIN	20 500	1	119		G
Ferritin	FER	21 000	22	144	4.3	U§
Human growth hormone*	HGH	21 500	1	160	4.8	B
α-Chymotrypsin	CTN	21 600	1	120	8.4	B
Papain	PAP	22 000	1	117		B
α-Chymotrypsinogen	CTA	25 000	1	123	8.8	B
Carbonic anhydrase*	CAH	29 000	1	129		G
Lactic dehydrogenase	LDH	35 000	4	145	4.9	U
Alcohol dehydrogenase	ADH	37 000	4	131		B
Glycerol-3P-dehydrogenase	GPD	39 000	4	121	6.3	B
Ovalbumin*	OVA	45 000	1	150	4.7	B
β-Amylase	BAM	50 000	4	139		B
Human glycoprotein*	HGP	50 000	3	109	4.7	B
β-Glucosidase	BGU	65 000	2	130		U
Bovine serum albumin	BSA	68 000	1	136	5.0	U
Lactoperoxidase*	LPO	85 000	1	139	9.2	U
Jack bean urease	JBU	92 000	6	134		U§
Amyloglucosidase*	AGS	97 000	1	155		U
Phosphorylase B	PHB	97 000	1	146	6.3	U
β-Galactosidase	BGL	115 000	5	144	5.1	U
Collagen	COL	120 000	1	78		B
Immuno γ-globulins	IGG	160 000	1	116		U§
Thyroglobulin	THY	335 000	2	153		U§
Fibrinogen*	FIB	340 000	1	120		B

* Standard used in indicator test mixture.

** Scale of protein hydrophobicity is based on retention times relative to lysozyme (100) under the optimized conditions described in Table I.

*** G = "good", B = "bad" and U = "ugly", as defined in the text.

§ Protein which did not exhibit ideal behavior under final conditions (but now only B rather than U). Slight modification of these conditions make them behave in ideal fashion.

In this paper we will consider the effect of various column parameters on chromatographic non-ideality. Other contributions to chromatographic non-ideality are discussed in the following paper[4].

EXPERIMENTAL

Apparatus

We initially used a Hewlett-Packard (HP) Model 1090 liquid chromatograph equipped with an automatic sample injector, a photodiode array detector and a column oven; the system was controlled by the HP-Chemstation® chromatography software running on an HP 9000 computer. Chromatographic studies with columns having internal diameters of less than 2 mm were carried out on a prototype chromatophoresis system, consisting of a customized microbore liquid chromatograph with binary gradient pumping system, auto-sampler, column oven and UV detector (connected to the PAGE system as in Fig. 1). The entire chromatophoresis system was designed to minimize extracolumn bandspreading so as to ensure maximum performance and minimal loss in resolution in the first-dimension separation prior to electrophoretic transfer of proteins to the gel. Operating conditions and hardware configuration (for both HPLC systems) are summarized in Table I.

TABLE III

RESULTS OF COMPUTER SIMULATION (BIOG-RP3) FOR THE EFFECTS OF VARIOUS CONDITIONS ON SEPARATION

Conditions are "optimum" as defined in Table I, except for the variable being studied.

Condition varied	*Actual value of variable*	*Peak capacity*	*Peak volume (μl)*	*p.s.i.*	k
Column length (cm)	25	167	11	331	0.4
	10	205	9	132	0.8
	5*	215	8	66	1.6
	2	215	8	26	3.3
	1	205	9	13	6.6
	0.5	188	10	6	13
Column diameter (mm)	1*	215	8	66	1.6
	1.5	180	10	29	0.7
	2.0	145	12	16	0.4
Particle diameter (mm)	3	346	5	183	1.6
	5*	215	8	66	1.6
	10	113	16	16	1.6
Flow-rate (ml/min)	0.01	167	4	22	0.5
	0.03*	215	8	66	1.6
	0.05	227	13	110	2.7
Molecular weight (kD)	10	250	7	66	3.2
	50**	215	8	66	1.6
	200	100	18	66	0.9

* Preferred condition in Table I.

** Value assumed in computer simulations unless noted otherwise.

Columns

Column packings were obtained from the following suppliers: (1) PLRP-S (Polymer Labs., Church Stretton, U.K.), (2) Apex (Jones Chromatography, Denver, CO, U.S.A.,), (3) Synchropak (SynChrom, Lafayette, IN, U.S.A.) and (4) Vydac (The Separations Group, Hesperia, CA, U.S.A.). Hardware for all HPLC columns was obtained from Upchurch Scientific (Oak Harbor, WA, U.S.A.). All HPLC columns were packed in our laboratory.

Reagents

All proteins used in this study (Table II) were obtained from Sigma (St. Louis, MO, U.S.A.). Water and acetonitrile were "OmniSolve" grade supplied by EM Science (Cherry Hill, NJ, U.S.A.). Trifluoroacetic acid (Sequanal Grade) was obtained from Pierce (Rockford, IL, U.S.A.).

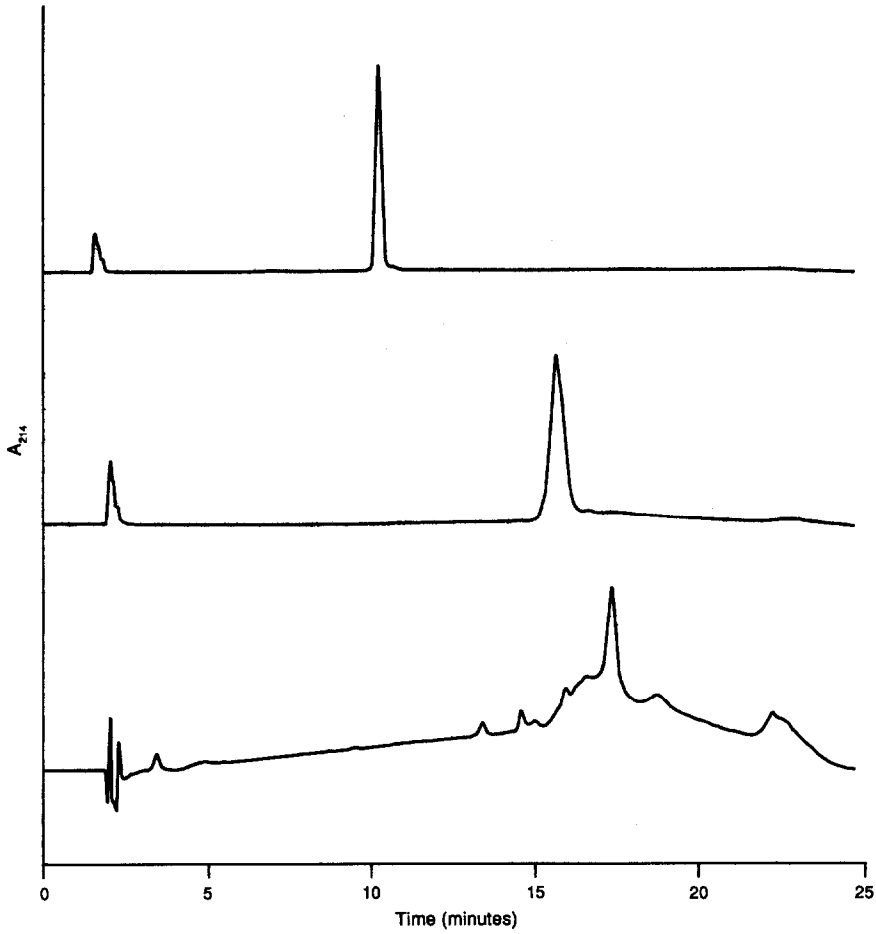

Fig. 2. Representative chromatograms showing the results obtained using consensus conditions for (from top to bottom) "good" (ribonuclease A), "bad" (alcohol dehydrogenase) and "ugly" (β-galactosidase) proteins.

Computer simulations

Predictions of chromatographic separation (Table III) were carried out with BIOG-RP3, a computer-modeling program from LC Resources (Lafayette, CA, U.S.A.). This program for the calculation of bandwidths in reversed-phase gradient-elution separations of proteins has been described previously[12]. Calculations were carried out on an IBM-XT personal computer. The previously described software was modified slightly for the more accurate prediction of results for very steep gradients (the factor J described in ref. 12 is set equal to 1.8 whenever the gradient-steepness parameter b exceeds 1.5).

RESULTS AND DISCUSSION

Most attempts to establish optimum conditions for the RP-HPLC separation of proteins fall into one of two categories: (a) "application-specific optimization", in which separation conditions are chosen to resolve and analyze a specific protein or set of proteins in a given sample; (b) "model-protein samples", in which one or more "standard" proteins (ribonuclease, lysozyme, etc.) are used to evaluate some aspect of RP-HPLC. Since our goal was to develop separation conditions that are generally applicable to a broad spectrum of proteins, we selected 33 proteins with molecular weights of 6000–670 000, including both very hydrophobic and very hydrophilic proteins, as well as acidic and basic proteins (Table II).

Initial separation conditions (Table I) were selected on the basis of recommendations and practice reported in the literature. With these "consensus" starting conditions, only 6 of the 33 proteins yielded acceptable results (labeled "good" in Table II). A representative chromatogram (ribonuclease) is shown in Fig. 2 (top). The six "good" proteins gave symmetrical bandshapes, reasonable recoveries (greater than 80%), and bandwidths that agreed (± a factor of 2) with our computer model (BIOG-RP3 predictions for "ideal" chromatographic behavior). Fifteen proteins (designated "bad" in Table II) gave broad bands and/or somewhat asymmetric peaks; Fig. 2 (middle) (alcohol dehydrogenase) illustrates such a chromatogram. The remaining eleven proteins gave broad, multiple and/or misshaped bands such as that illustrated in Fig. 2 (bottom) (β-galactosidase); these are designated as "ugly" in Table II. It is clear that these results (based on consensus conditions and entirely consistent with results reported in the literature[12–22]) were far from adequate for our intended purpose.

As summarized in this and the following paper[4], many variables were examined to assess their effect on the chromatographic behaviour of proteins under RP-HPLC conditions. However, experience shows that it is seldom meaningful to measure the effect on chromatographic performance of any given variable, if other conditions are far from optimal. Therefore, our initial approach to the study of a specific separation variable was to use sets of indicator proteins (mixtures containing one or more proteins from each of the three HPLC-performance classes of Table II) to assess that variable, with all other parameters defined by the "consensus" conditions of Table I. Later we repeated these studies for each variable, using "optimized" conditions (Table I) for all variables except the one being studied.

The computer-simulation program BIOG-RP3 proved useful in defining the first set of separation variables to be evaluated. As summarized in Table III, computer

modeling suggested optimum specifications for column length and diameter, particle size and flow-rate for use in the chromatophoresis system.

As preferred conditions for the column packing and other separation variables emerged, these conditions were then used as the baseline for another assessment in which progressively more complex mixtures of proteins were used. It is important to note that apparent improvements in chromatographic performance (bandwidths) were compared with BIOG-RP3 predictions at each stage. When optimum conditions

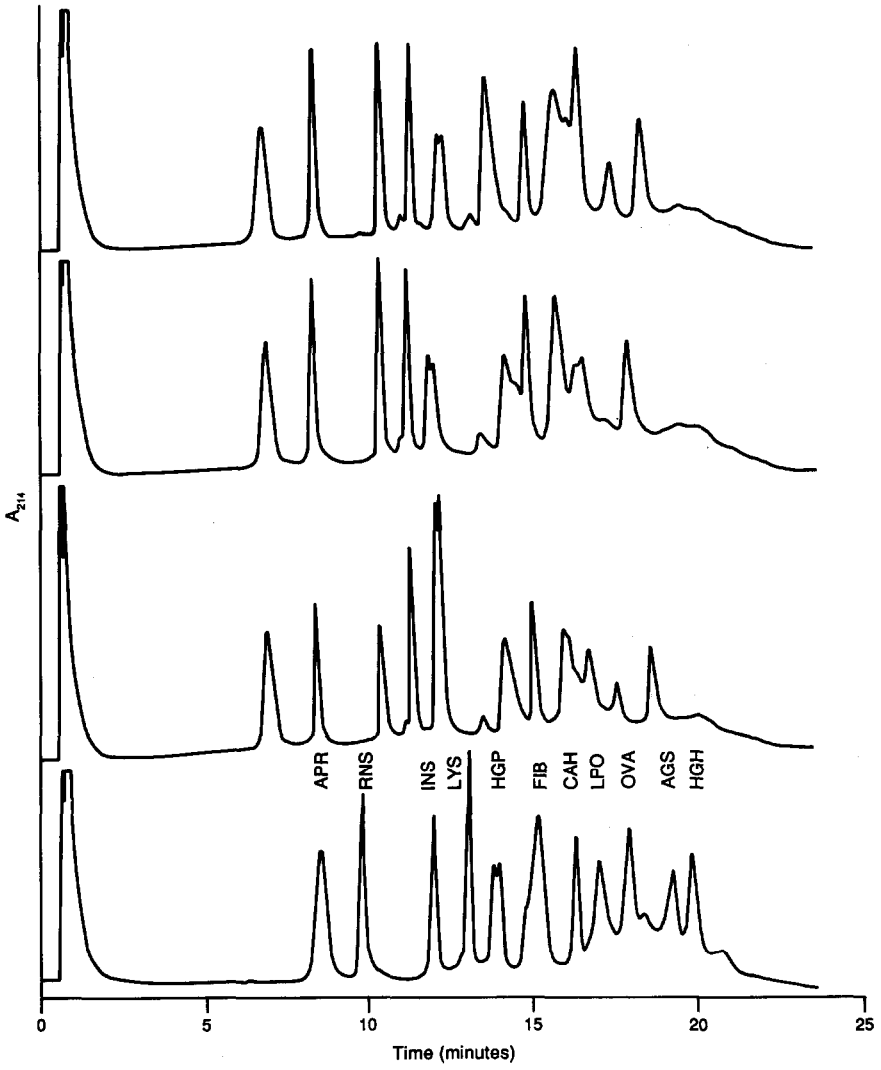

Fig. 3. Comparison of separations obtained for different C_4 column packings (one polymer-based and three silica-based) under optimum conditions (see Table I). The sample is a standard mixture of proteins ("indicator proteins"). From top to bottom: Apex (7 μm), Synchropak (5 μm), Vydac (5 μm), PLRP-S (5-μm). Pore size was 300 Å in all materials.

for each variable were established (summarized in Table I), confirmation of these conditions was established by re-chromatographing all 33 proteins.

Choice of column packing

Column-packing support. A wide variety of column-packing supports (silica- and polymer-based, various suppliers) were evaluated in this study (see Experimental). Chromatographic performance was evaluated using commercially available silica-based packings with a particle diameter of 5 μm, 300-Å pores, and a C_4 bonded phase (recommended by various manufacturers for separating proteins), or corresponding polymeric packings. Fig. 3 illustrates the chromatograms obtained for an

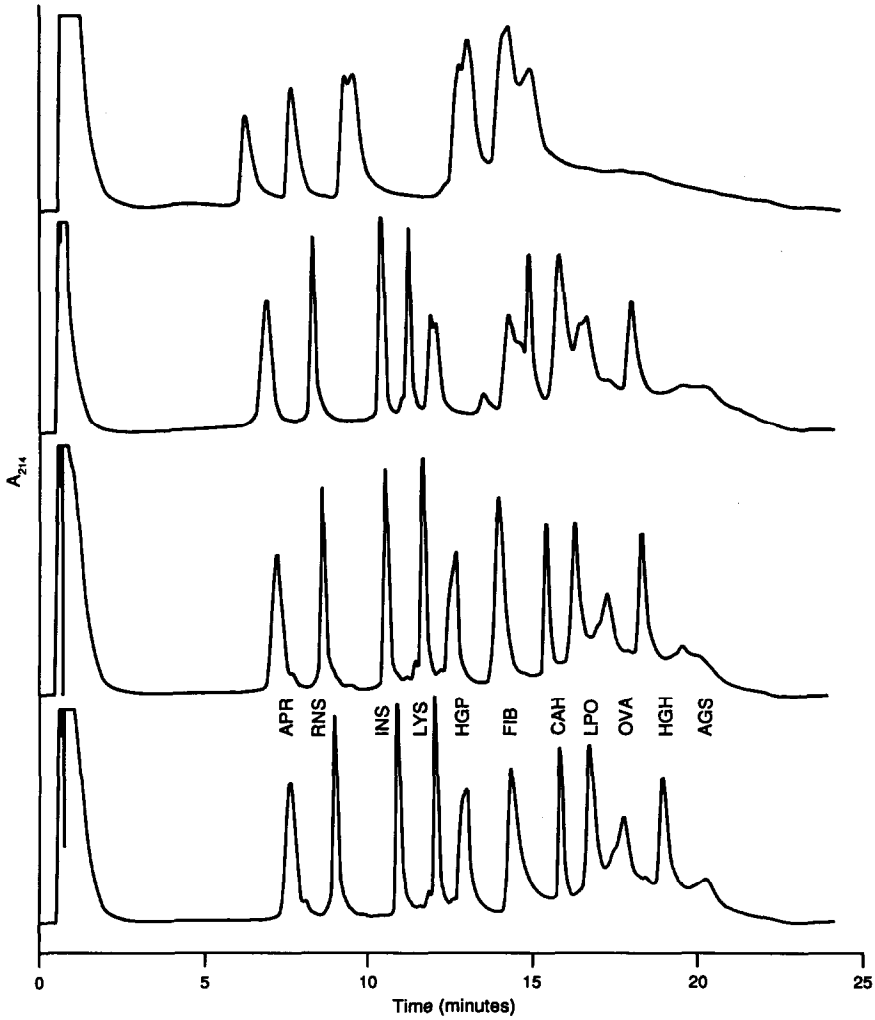

Fig. 4. Comparison of separations obtained on various Apex 7-μm 300-Å (Jones Chromatography) silica-based bonded phases. Conditions and sample are the same as in Fig. 3. From top to bottom: cyano, C_4, phenyl, C_{18} bonded phases.

eleven-component protein sample (Table II, proteins designated by an asterisk) on four column packings of this type. When run under otherwise optimal conditions (see Table I), the silica-based packings gave generally good performance for all proteins, including those initially classified as having non-ideal chromatographic behavior. However, as indicated in Table IV, the recovery from these columns was unacceptably low for hydrophobic proteins, and the elution profile of more basic proteins indicated significant tailing.

Polymeric supports such as the 5-μm (diameter), 300-Å pore PLRP-S packing [Fig. 3 (bottom)] gave bandwidths and resolution comparable to the C_4 silica-based packings; they also offered clear advantages in terms of reduced tailing and improved recoveries (across-the-board), as well as significantly increased column-stability over a much wider range of operating conditions[4].

Both polymeric and silica-based column packings required some initial conditioning in order to stabilize recoveries and retention times. However, the chromatographic performance of polymeric packings stabilized after fewer sample injections (3–5) than for the silica-based packings (> 10). The polymeric packings also had a column life up to 10 times longer than the silica-based packings under our final optimized conditions (Table I). Since current polymeric supports do not offer a wide choice of stationary-phase compositions, we also examined a variety of silica-based packings having different bonded phases.

Silica-based packings with different bonded phases. To evaluate the effect of bonded-phase type on chromatographic performance, we obtained from a single supplier a wide range of bonded phases, all coupled to the same silica particle [7-μm diameter, 300-Å pore (see Experimental)]. Fig. 4 presents elution profiles obtained when our eleven-component indicator sample was chromatographed on each of the various bonded phases. While only minor differences can be seen in the elution profile of samples chromatographed on the non-polar phases (C_{18}, phenyl, C_4), the more polar CN phase gave poorer peak shapes and a more limited elution range (bunching of peaks). It was also found (Table IV) that this column gave significantly lower recoveries. A highly polar Diol-phase column was found to retain none of the proteins of the indicator sample under these conditions.

Pore diameter. Theory suggests that the pore diameter of the column packing will become more important for larger protein molecules; larger pores should favor better separation[12]. However, most workers currently use packings with 300-Å pores. Fig. 5 compares the results obtained with PLRP-S columns packed with particles with pore diameters of 300, 1000 and 4000 Å. It can be seen for proteins of very high molecular weight (*i.e.*, fibrinogen, mol. wt. 340 000, marked by an asterisk in Fig. 5) that peak shape improves with increasing pore diameter. However, for most proteins the 300-Å pore packing offers the best overall performance in terms of bandwidth, tailing, recovery and ghosting (Table IV). This is illustrated in Fig. 5 for the doublet band of human glycoprotein (mol. wt. 150 000, glycosylated isomers; identified by two asterisks).

Particle diameter. As shown in Table III, computer modeling predicts that resolution (as measured by peak capacity) and peak height should each increase markedly as particle diameter is decreased. Other workers have confirmed this experimentally and have found dramatic improvements in resolution and/or separation time with smaller particles for protein separations[30,31]. We have observed a similar

performance trend for particles as small as 5 μm in diameter (Table IV). In our hands, 3-μm particles (experimental materials, not commercially available) gave broader bands and increased tailing compared to similar 5-μm particles. However, computer simulations (BIOG-RP3) suggested that the wider bands found for the 3-μm packing were due to factors other than particle size.

Column dimensions

Column length. Eqns. 1 and 2 (above) suggest that resolution and peak height should be relatively insensitive to changes in column length. This has been confirmed by several laboratories[12,23]. While some workers[32] have observed comparable resolution for columns 2–50 mm in length, other studies have shown that resolution generally decreases for very short columns[33]. However, simulations using our computer model[12,33] show that maximum peak capacity and resolution occur for longer columns when gradient time is allowed to increase.

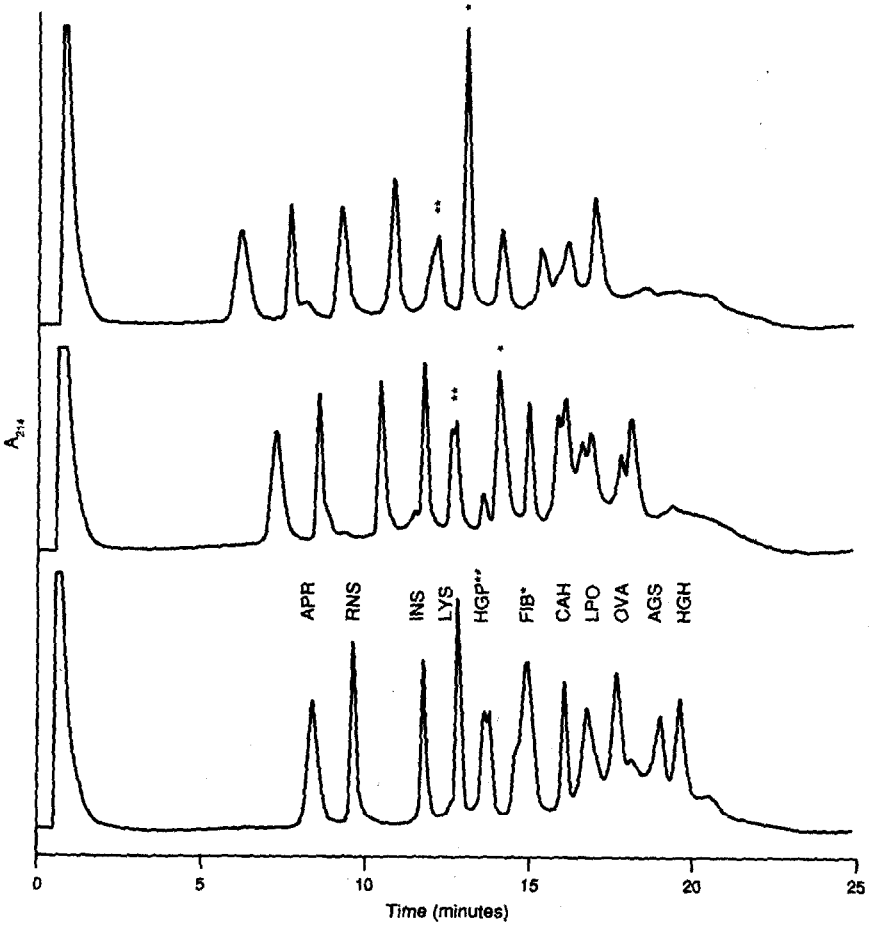

Fig. 5. Comparison of separations obtained on various PLRP-S columns with different pore diameters. Conditions and sample are the same as in Fig. 3. From top to bottom: 4000-Å pores (8 μm); 1000-Å pores (8 μm); 300-Å pores (5 μm).

TABLE IV

SUMMARY OF THE COLUMN PARAMETERS INVESTIGATED TO OPTIMIZE RP-HPLC OF PROTEINS AND THE RESULTS OBTAINED

(O) No effect compared to consensus conditions; (−) negative effect compared to consensus conditions; (+) positive effect compared to consensus conditions.

Variable	*Parameter*	*Result*					
		Band width	*Tailing*	*Recovery*	*Ghost-ing*	*High mol.wt.*	*Hydro-phobic*
Support	Silica	O	O	O	O	O	O
	Polymeric	O	+	+	O	O	+
Bonded phase	C_{18}	O	−	−	−	−	−
	Phenyl	O	O	−	O	−	−
	C_4	O	O	O	O	O	O
	Cyano	−	−	−	−	−	−
	Diol	−	−	−	−	−	−
Pore size	Solid	−	−	−	−	−	−
	300 Å	O	O	O	O	−	O
	1000 Å	−	O	O	−	O	−
	4000 Å	O	O	−	O	+	−
Particle size	3	−	−	O	O	O	O
(μm)	5	O	O	O	O	O	O
	10	−	O	O	O	O	O
Column I.D.	0.5	−	−	−	−	−	−
(mm)	1.0	+	O	+	O	O	O
	2.0	O	O	O	O	O	O
	4.6	−	O	−	O	O	−
Column length	2	−	O	+	O	O	+
(cm)	5	O	O	O	O	O	O
	10	O	O	−	−	−	−
	25	+	O	−	−	−	−
Materials	316 Stainless steel	O	O	O	O	O	O
	Titanium	O	O	O	O	O	O
Excess volume	Precolumn	O	O	−	O	O	−
	Postcolumn	−	−	O	O	O	O

These observations suggest that there is an optimum (intermediate) column length from the standpoint of maximum resolution for some preferred run-time (20–60 min in the present study). For the present system (data of Table III), computer simulations predict that column lengths of 2–5 cm should provide slightly greater resolution (peak capacity) than shorter or longer columns, as well as exhibit quite low pressures. The results of our experiments (Table IV) confirm these computer predictions and further indicate that a column length of 5 cm is a good compromise between maximum resolution and maximum recovery for protein samples.

Column diameter. For the present application, system constraints require a minimum column diameter, in order to keep the flow of mobile phase to the electrophoresis system within the required limits (10–100 μl/min) and maximize detection

sensitivity (via narrower bandwidths, eqn. 2). The lower limits on column diameter are determined by several other considerations: (a) extra-column effects, which become progressively more serious as the column diameter is decreased; (b) column capacity, which decreases with column diameter and eventually limits sample detection in both the RP-HPLC and electrophoresis separations by chromatophoresis; (c) increasing difficulty in packing efficient columns as the column diameter is reduced below 1 mm.

Computer simulations (Table III) predict that peak capacity increases significantly as the column diameter is decreased; this is the result of the requirement (for the chromatophoresis process) that flow-rates be 10–100 μl/min. We found that columns of 1 mm I.D. yielded optimal peak capacity and detection sensitivity, without overloading the column. Larger (2 or 4.6 mm) or smaller (0.5 mm) column diameters gave poorer results (see Table IV).

Column material. In light of the concern expressed in the literature[34,35] regarding the effect of 316 stainless steel on protein recovery, we conducted a quantitative assessment of non-specific protein binding on the column frit. Using "consen-

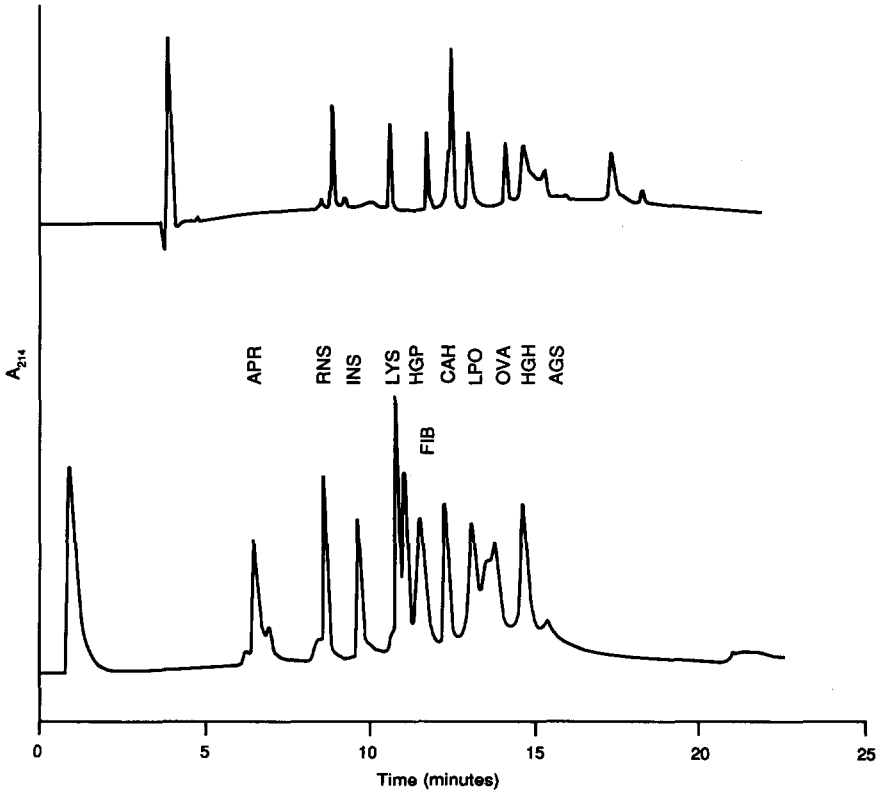

Fig. 6. Example of the improvements obtained in this study. Upper trace shows the elution profile for 20 μl of standard indicator mixture (see Fig. 3) run using "consensus" conditions from Table I. Lower trace shows the elution profile for 1 μl of standard indicator mixture run using the optimized instrument and column conditions and the "consensus" mobile phase and pretreatment. Note that ratio of sample amount to column volume is equal.

sus" conditions, we were able to confirm literature reports[3,5] of the absorptive loss of certain proteins (at very low concentrations) on stainless-steel frits, but not on titanium frits (data not shown). However, we have since determined that appropriate pretreatment of the sample[4] completely eliminates this loss and allows the use of stainless-steel frits in the RP-HPLC column of the chromatophoresis system.

Extra-column effects. Because of the flow-rate constraints of our system, extra-column effects have a pronounced impact on overall system performance (regardless of the optimization of other separation conditions). For example, a five-fold decrease in the length of the 0.007 in. tubing (from 25 to 5 cm) between the column and the detector reduced peak width by 50%. Although not as important as postcolumn volume, excessive precolumn volume also degraded separation by increasing gradient delay time and lowering recoveries for more hydrophobic proteins. We believe that this dwell-time-dependent recovery is a reflection of progressive, irreversible binding of hydrophobic proteins to the column during the time they spend sorbed at the column inlet.

Sample-specific conditions. While the "optimized" conditions of Table I favor the separation of most proteins, larger and/or more hydrophobic (more strongly retained) proteins sometimes benefit from somewhat different conditions. These are summarized in the last two columns of Table IV, where a "+" indicates improved separation (bandwidths, recoveries, ghosting, etc.) relative to "optimized" conditions, and a "−" indicates poorer separation. Thus, polymeric packings gave generally higher recoveries of more hydrophobic proteins. Likewise, packings with larger-pore (4000 Å) were better for higher molecular mass proteins (*cf.*, fibrinogen, Fig. 5). Columns of smaller volume (either shorter or narrower) also gave generally higher recoveries of more hydrophobic proteins.

Finally, we noted above that more hydrophobic proteins exhibit decreased recoveries and increased ghosting when the time the sample spends on the column is increased (by longer gradients, increased dwell volume, etc.).

Optimized conditions. Fig. 6 shows the result of optimizing column and hardware conditions. The upper trace is a chromatogram of our indicator-protein mixture obtained when the "consensus" conditions of Table I were used. The lower trace was obtained when the same sample was separated with column conditions optimized as described in this paper. Although the lower separation is better (higher recoveries, less ghosting), further improvements were necessary for RP-HPLC to be usable in chromatophoresis. These improvements are described in the following paper[4].

CONCLUSIONS

In the process of developing a new analytical procedure for the automated, two-dimensional analysis of protein samples, we conducted a series of experiments to optimize and extend the utility of RP-HPLC. In this paper we describe the optimization of the column parameters for the reversed-phase separation of proteins. We found that a 50 × 1.0 mm I.D. column packed with 5-μm, 300-Å polymeric material gave the best performance for the majority of the proteins tested (when used with the optimized sample-pretreatment and mobile phase conditions described in the following paper[4]). Although our primary purpose was the adaptation of RP-HPLC for use in the chromatophoresis system, our findings should be relevant to other researchers trying to use RP-HPLC separation for complex protein samples.

REFERENCES

1 M. J. Dunn and A. H. M. Burghes, *Electrophoresis*, 4 (1983) 97.
2 R. P. Tracy, R. M. Currie and D. S. Young, *Clin. Chem.*, 28 (1982) 908.
3 P. H. O'Farrell, *J. Biol. Chem.*, 250 (1975) 4007.
4 K. D. Nugent, W. G. Burton, T. K. Slatterly, B. F. Johnson and L. R. Snyder, *J. Chromatogr.*, 443 (1988) 381.
5 W. G. Burton, B. F. Johnson, K. D. Nugent and T. K. Slatterly, *Biotechnology*, submitted for publication.
6 B. S. Welinder, H. H. Sørenson and B. Hansen, *J. Chromatogr.*, 398 (1987) 309.
7 F. E. Regnier and K. M. Gooding, *Anal. Biochem.*, 103 (1980) 1.
8 F. E. Regnier, *Science*, 222 (1983) 245.
9 M. T. W. Hearn, in Cs. Horvath (Editor), *High-performance Liquid Chromatography — Advances and Perspectives*, Vol. 3, Academic Press, New York, 1984, p. 87.
10 M. T. W. Hearn, *J. Chromatogr.*, 418 (1987) 3.
11 F. E. Regnier, *J. Chromatogr.*, 418 (1987) 115.
12 L. R. Snyder and M. A. Stadalius, in Cs. Horvath (Editor), *High-performance Liquid Chromatography — Advances and Perspectives*, Vol. 3, Academic Press, New York, 1986, p. 195.
13 E. C. Nice, M. W. Capp, N. Cooke and M. J. O'Hare, *J. Chromatogr.*, 218 (1981) 569.
14 J. D. Pearson, N. T. Lin and F. E. Regnier, in M. T. W. Hearn, C. T. Wehr and F. E. Regnier (Editors), *High-performance Liquid Chromatography of Proteins and Peptides*, Academic Press, New York, 1982, p. 81.
15 W. Kopaciewicz and F. E. Regnier, *Anal. Biochem.*, 133 (1983) 2517.
16 M. T. W. Hearn and B. Grego, *J. Chromatogr.*, 296 (1984) 61.
17 A. Köck and T. A. Luger, *J. Chromatogr.*, 296 (1984) 293.
18 M. T. W. Hearn, *Methods Enzymol.*, 104 (1984) 190.
19 M. T. W. Hearn, A. N. Nodder and M.-I. Aguilar, *J. Chromatogr.*, 327 (1985) 47.
20 K. Benedek, S. Dong and B. L. Karger, *J. Chromatogr.*, 317 (1984) 227.
21 C. T. Wehr, *J. Chromatogr.*, 418 (1987) 27.
22 A. J. Alpert, *J. Chromatogr.*, 359 (1986) 85.
23 L. R. Snyder, M. A. Stadalius and M. A. Quarry, *Anal. Chem.*, 55 (1983) 1412A.
24 M.-I. Aguilar, A. N. Hodder and M. T. W. Hearn, *J. Chromatogr.*, 327 (1985) 115.
25 M. T. W. Hearn and M.-I. Aguilar, *J. Chromatogr.*, 359 (1986) 31.
26 M. Kunitani, D. Johnson and L. R. Snyder, *J. Chromatogr.*, 371 (1986) 313.
27 L. R. Snyder, G. B. Cox and P. E. Antle, *J. Chromatogr.*, 444 (1988) 303.
28 G. B. Cox, P. E. Antle and L. R. Snyder, *J. Chromatogr.*, 444 (1988) 325.
29 M. A. Stadalius, B. F. D. Ghrist and L. R. Snyder, *J. Chromatogr.*, 387 (1987) 21.
30 J. L. Meek and Z. L. Rossetti, *J. Chromatogr.*, 211 (1981) 15.
31 N. D. Danielson and J. J. Kirkland, *Anal. Chem.*, 59 (1987) 2501.
32 R. M. Moore and R. R. Walters, *J. Chromatogr.*, 317 (1984) 119.
33 M. A. Stadalius, M. A. Quarry and L. R. Snyder, *J. Chromatogr.*, 327 (1985) 93.
34 J. D. Pearson and F. E. Regnier, *J. Liq. Chromatogr.*, 6 (1983) 497.
35 P. C. Sadek P. W. Carr, L. D. Bowers and L. C. Hadded, *Anal. Biochem.*, 144 (1985) 128.