Journal of Chromatography, 516 (1990) 99–114
Elsevier Science Publishers B.V., Amsterdam

CHROM. 22 650

Method optimization in capillary zone electrophoretic analysis of hGH tryptic digest fragments

R. G. NIELSEN*,[a] and E. C. RICKARD
Eli Lilly and Company, Lilly Research Laboratories, Lilly Corporate Center, Indianapolis, IN 46285 (U.S.A.)

ABSTRACT

The mobile phase composition was optimized for the separation of tryptic digest fragments of human growth hormone by capillary zone electrophoresis. The effect of pH (pH 2.4, 6.1, 8.1 and 10.4) was evaluated since pH determines the relative charge of species, the prime contributor to selectivity; pH 8.1 was selected for the optimization studies. Tricine (buffer), sodium chloride (ionic strength adjustor), and morpholine (mobile phase additive) concentrations were systematically varied at pH 8.1. All three exhibited major effects on the electroosmotic flow velocity and current, and minor effects on selectivity. Tricine was the most crucial for good resolution, although addition of morpholine helped to resolve closely eluting species. The optimum separation conditions were found to be pH 8.1 with 0.1 *M* tricine, 0.02 *M* morpholine and no salt.

INTRODUCTION

Capillary electrophoresis has been used in a variety of modes to separate and characterize diverse molecules, especially biomolecules[1–4]. The open tubular mode is referred to as capillary zone electrophoresis (CZE), high-performance capillary electrophoresis, or free solution capillary electrophoresis. CZE has been widely used for separations due to its simplicity, high separative power, ease of quantitation, and ability to perform rapid, automated analyses. It has been applied to numerous polypeptide and protein molecules[5–9]. One area of particular interest to us was the characterization of the identity and purity of proteins used as drugs. We reported separations of biosynthetic human insulin (BHI) and human growth hormone (hGH) from closely related species that could originate as impurities or as degradation products[10]. That paper also demonstrated that the results for the quantitation of the desamido-A21 BHI degradation product in BHI by CZE were equivalent to results obtained from reversed-phase high-performance liquid chromatography (RP-HPLC)

[a] Present address: Process Development, Hybritech, Inc., P.O. Box 269006, San Diego, CA 92126-9006. U.S.A.

and polyacrylamide gel electrophoresis. Other papers have reported the separation of peptide mixtures such as the 19 peptide fragments produced from an enzymatic (trypsin) digest of human growth hormone[11,12] and a series of synthetic peptides[13]. However, relatively few reports of a systematic approach to the selection of CZE operating conditions to achieve optimum separations have been given. This paper will describe the optimization of the separation of components in the complex tryptic digest of hGH.

The selectivity achieved in CZE separations is determined by differences in electrophoretic mobilities of the analytes. Mobility in the open tubular mode is predominantly related to charge, shape and size of the analyte as well as to the properties of the eluting solvent[2,13,14]. For impurities and degradation products, which usually have shapes and sizes similar to that of the main component, adjustment of pH to a value near the midpoint of the isoelectric point (p*I*) range of the analytes will tend to maximize their net charge differences[7]. However, digest fragments generally consist of a rather heterogeneous mixture of peptide fragments such that the pH value that will maximize selectivity may not be easily predictable.

In addition to differences in selectivity, efficiency also plays an important role in the separation process. The efficiency (or number of theoretical plates) can be quite large if processes that lead to band broadening can be minimized[5,15–18]. Some of the major processes that contribute to band broadening include interactions of the analyte with the capillary wall[6,8,15–19], temperature gradients (which affect viscosity and electrophoretic mobility)[5,17,18,20], sample loading (sample introduction technique, volume and amount)[17,18], sample solvent[5,15,17,20], and detector design[17]. The rate of electroosmotic flow also affects the efficiency since it decreases the amount of time that species are separated under the influence of the electric field but does not contribute to the separation[21].

Although each of the above processes that lead to decreased efficiency can be addressed, they are generally not independent of the parameters that affect selectivity. For example, variation of pH to achieve optimum selectivity based on charge differences will also affect efficiency by changing the rate of electroosmotic flow in fused-silica capillaries. Furthermore, the net charge on the protein relative to that on the silica surface, which are both determined by pH, is one of the primary factors that determines the amount of analyte interaction with fused silica.

In addition to the pH effects described above, the buffer composition will affect the ionic strength and conductivity. Other additives may be included in the mobile phase. For example, the ionic strength may be adjusted with salts, and organic modifiers may be added to reduce wall interactions or to maintain analyte solubility. It is obvious that the effect on operating performance due to the interaction of the experimental parameters is complex. The effects of the most important parameters are reported herein: pH, tricine (buffer) concentration, sodium chloride (ionic strength adjustor) concentration, and morpholine (mobile phase modifier) concentration.

EXPERIMENTAL

Reagents and materials

Biosynthetic hGH was obtained from Eli Lilly and Co. (Lilly Research Labs., Indianapolis, IN, U.S.A.). Morpholine and glycine were purchased from Fisher Sci-

entific (Pittsburgh, PA, U.S.A.); tricine, 2-[N-morpholino]ethanesulfonic acid (MES), and 3-[cyclohexylamino]-1-propane-sulfonic acid (CAPS) were purchased from Sigma (St. Louis, MO, U.S.A.). Tris(hydroxymethyl)aminomethane (Tris) was purchased from Bio-Rad Labs. (Richmond, CA, U.S.A.). Trypsin (TPCK, 267 units/mg protein, 98% protein) was purchased from Cooper Biomedical (Malvern, PA, U.S.A.). Reagent-grade water obtained from a Milli-Q purification system from Millipore (Bedford, MA, U.S.A.) was used to prepare all solutions. All other reagents were analytical grade and were used without further purification. Polyimide-coated, fused-silica capillaries, 50 μm internal diameter and 360 μm outside diameter, were purchased from Polymicro Technol., (Phoenix, AZ, U.S.A.).

Tris–acetate buffer was prepared by adjusting the pH of a 0.05 *M* Tris solution to pH 7.5 with acetic acid. The pH 2.4 buffer was prepared from a 0.1 *M* glycine solution. The pH 6.1 buffer contained 0.1 *M* MES and 0.0363 *M* sodium chloride. The pH 8.1 buffer contained 0.1 *M* tricine and 0.0343 *M* sodium chloride. The pH 10.4 buffer contained 0.1 *M* CAPS and 0.0381 *M* sodium chloride. Minor adjustments of the pH of the latter four buffers were made with 0.1 *M* hydrochloric acid or 0.1 *M* sodium hydroxide as necessary.

Methods

The trypsin digestion was carried out according to reported methods using non-reducing conditions so that both the correct amino acid sequence and the presence of the correct disulfide linkages could be confirmed[22]. Aliquots of the digest mixture were frozen (−20°C) for use at a later time. The thawed digest mixture was injected directly. The concentration of the analyte in all studies was about 2 mg/ml total protein or 90 μM for each digest fragment except for fragments 17–19 which will be present at lower concentrations since fragments 17 and 19 derive from cleavage of fragment 18.

The mobile phase used in the initial CZE separations was 0.01 *M* tricine, 0.02 *M* sodium chloride, and 0.045 *M* morpholine adjusted to pH 8.0. The four buffers described above were used as mobile phases in the pH optimization study; the mobile phases used in the pH 8.1 optimization experiments are described below. The column was rinsed with mobile phase between injections or successively with 0.1 *M* sodium hydroxide and mobile phase when the mobile phase composition was changed.

Preliminary data were obtained using the CZE instrumentation previously described[10] except that both CZE instruments now include vacuum injection devices and a constant temperature environment. The data presented in the figures were obtained on an Applied Biosystems (Santa Clara, CA, U.S.A.) Model 270A instrument. Sample (approximately 10 nl as estimated from the Poiseuille equation for a 3-s injection) was introduced by applying vacuum (127 mmHg) to a capillary that was approximately 100 cm in length with 80 cm to the detector. Separation conditions were: 30 kV applied voltage and 30°C. The components were detected by UV absorbance at 200 nm. Analog data were collected directly from the absorbance detector on an in-house centralized chromatography computer system based on the Hewlett-Packard Model 1000 minicomputer that has storage, manipulation, and graphics capabilities.

The electrophoretic separations were evaluated for selectivity, efficiency and resolution. Selectivity was determined by identification of peaks under selected condi-

tions. Efficiency was qualitatively judged by the peak sharpness. Overall resolution was qualitatively judged by the number of peaks which could be observed and the spacing between peaks.

The buffer capacity[23] and ionic strength of the buffers were calculated from the following values: glycine, pK_{a1} = 2.35 (carboxylate), pK_{a2} = 9.78 (amine); MES, pK_a = 6.10; tricine, pK_{a1} = 2.33 (carboxylate), pK_{a2} = 8.15 (amine); CAPS, pK_a = 10.40; morpholine, pK_a = 8.40. The buffer capacity is defined as the amount of acid or base required to change the pH by one unit. The calculated ionic strength includes contributions from all species with a net charge; concentrations of buffer components were determined using the acid dissociation constants given above without consideration of activity effects.

RESULTS AND DISCUSSION

The large number of hGH cleavage fragments and their diverse structure contribute to the complexity of designing an acceptable separation. The structure of the individual fragments and their RP-HPLC behavior were described by Becker *et al.*[22]. The properties of the fragments that are relevant to CZE and RP-HPLC separations are listed in Table I. Note that the fragments range from highly basic (*e.g.*, fragments

TABLE I

FRAGMENTS FROM ENZYMATIC DIGEST OF HUMAN GROWTH HORMONE

Fragment number	*Isoelectric point*[a]	*Hydrophobicity*[b]	*Molecular weight*	*Amino acid residues*	*Amino acid sequence*[c]
1	10.1	18.1	930	8	FPTIPLSR
2	5.8	17.5	978	8	LFDNAMLR
3	10.4	−12.3	382	3	AHR
4	4.2	45.5	2343	19	LHQLAFDTYQEFEEAYIPK
5	6.4	−15.7	404	3	EQK
6–16	7.3	74.8	3763	32	6: YSFLQNPQTSLCFSESIPTPSNR 16: NYGLLYCFR
7	4.5	−19.0	762	6	EETQQK
8	5.9	9.4	844	7	SNLELLR
9	6.4	72.1	2056	17	ISLLLIQSWLEPVQFLR
10	3.5	33.3	2263	21	SVFANSLVYGASDSNVYDLLK
11	4.0	13.0	1362	12	DLEEGIQTLMGR
12	4.0	−3.4	773	7	LEDGSPR
13	9.2	0.4	693	6	TGQIFK
14	9.0	−14.2	626	5	QTYSK
15	3.8	0.9	1490	13	FDTNSHNDDALLK
17	9.0	−13.9	146	1	K
18	6.1	10.5	1382	11	KDMDKVETFLR
19	4.0	12.1	1253	10	DMDKVETFLR
20–21	5.9	19.9	1401	13	20: IVQCR 21: SVEGSCGF

[a] Calculated from a computer program based on Skoog and Wichman[27].
[b] Calculated according to Meek and Rossetti[28].
[c] Single-letter code for amino acids used.

1 and 3) that will be positively charged at neutral pH values to highly acidic (*e.g.*, fragments 10 and 15) that will be negatively charged at neutral pH values. They also differ significantly in size, from fragment 17 that is a single amino acid residue (lysine) to fragment 6–16 that contains 32 amino acid residues in two chains linked by a disulfide bond. (Fragment 20–21 also consists of two chains connected by a disulfide bond.) Finally, the fragments range from hydrophobic (*e.g.*, fragments 6–16, 9, 4 and 10) to hydrophilic (*e.g.*, fragments 7, 5, 14, 17 and 3).

In previously reported data, the peaks in the electropherogram of the hGH digest were identified using the pH 8.0 CZE mobile phase described for the initial CZE separations[11]. Identification was accomplished by spiking individual fragments isolated by RP-HPLC or, in cases where RP-HPLC did not produce a complete separation, material that was further fractionated by anion-exchange chromatography. The integrity of the isolated material was verified by reinjection on RP-HPLC; the identity of the material isolated by anion exchange was confirmed by amino acid analysis.

Note that fragment 14 gives more than one peak following isolation by RP-HPLC and solvent evaporation. Authentic fragment 14 elutes early in the electropherograms at pH 8.1 as labeled in Figs. 2 and 8. A degradation product of this fragment, labeled 14*, elutes much later. Therefore, fragment 14* must be more negatively charged than fragment 14; it probably arises from a spontaneous cyclization of the N-terminal glutamine (sequence, QTYSK) to give pyrrolidone carboxylic acid at the N-terminal. However, this postulated structure has not been confirmed. The subtle rearrangement of fragment 14* was missed in the preliminary peak assignments for the digest[9]. Fragment 11 also yields fragment 11* (of unknown composition), which is resolved only under the optimum separation conditions at pH 8.15 (Fig. 8).

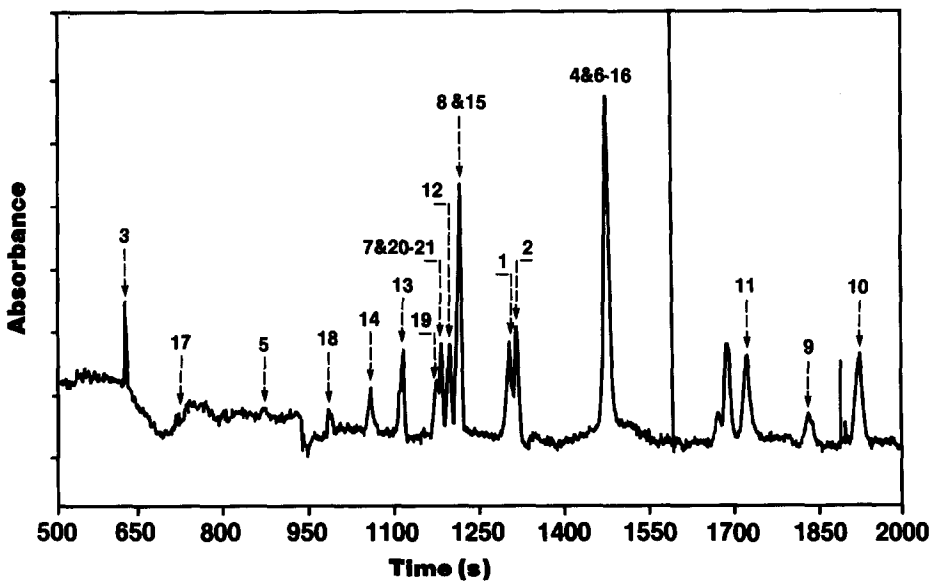

Fig. 1. Electropherogram of hGH digest in 0.1 *M* glycine buffer, pH 2.4.

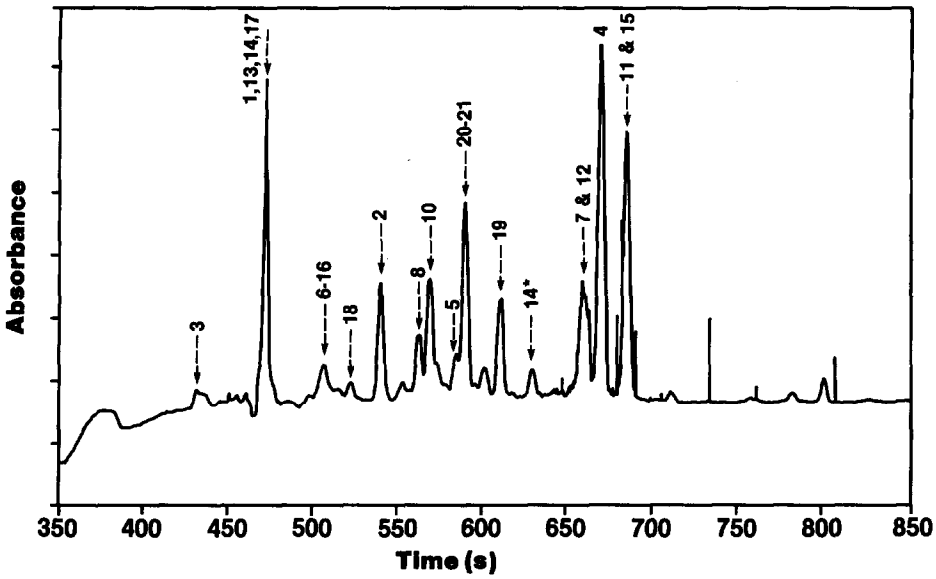

Fig. 2. Electropherogram of hGH digest in 0.1 *M* tricine buffer containing 0.0343 *M* sodium chloride, pH 8.1.

Fragment 9 also exhibits anomalous behavior. At low pH, it is soluble and migrates as labeled in Fig. 1. At high pH, more than one peak is associated with this fragment. The very limited solubility of fragment 9 at high pH produces insoluble particles that elute near fragment 4, usually as sharp spikes with variable migration times that are not representative of its true mobility. The soluble portion of fragment 9 is present at such low concentrations that it cannot be reliably observed, although it should elute near the position of fragment 6–16. Therefore, the identity of fragment 9 is not indicated on Figs. 2 and 8.

pH optimization

Various strategies have been used for selection of the pH of the mobile phase to minimize the interaction of proteins with the silica surface. These strategies are based on the reduced electrostatic interactions between the protein and the silica when they have charges of the same sign (compared to pH regions where they are oppositely charged). When the pH is greater than the p*I* of the protein, the protein will carry a net negative charge and, thus, be repelled from the negatively charged silica wall[6,16]. Others have used low pH values to minimize wall interactions[7,8,13] since the proteins are positively charged while the silica is relatively uncharged at pH values below about 2 (refs. 6, 8). Low pH also produces a drastic reduction in the electroosmotic flow velocity that will give greater efficiency (when other factors are maintained constant) at the cost of longer elution times. However, both of these strategies dictate that the pH value be chosen by factors other than optimization of selectivity. It is obvious that it would be better to minimize the interaction of the analyte with the silica by other means (buffer strength, mobile phase additives, ionic strength, coating

of capillaries, etc.) so that the pH of the mobile phase may be chosen to give optimum separations.

Four pH values over the range of 2.4 to 10.4 were selected for this study. These values encompass the extremes in the above two approaches and cover the p*I* range of the digest fragments (see Table I). A high buffer strength was used to maintain a constant pH within the analyte zone; the buffer capacity for all four buffers was about 52 m*M* or approximately 600 times the analyte concentration. The conductivity of the three higher pH buffers was empirically adjusted to be approximately equal to that of the pH 2.4 buffer by the addition of sodium chloride. This procedure resulted in approximately equal ionic strengths except for the pH 2.4 buffer where the hydrogen ion contributes disproportionately to the conductivity (calculated ionic strengths about 28, 61, 59 and 63 m*M* for pH 2.4, 6.1, 8.1 and 10.4 buffers, respectively). The high ionic strengths should minimize analyte interactions with the silica and maintain constant conductivity within the analyte zone.

The identities of the individual peaks in the electropherograms were assigned for pH 2.4 and 8.1 (Figs. 1 and 2; sequences given in Table I). The peak identification strategy was similar to that used in the earlier work and described briefly above. For pH 6.1 and 10.4, the efficiency and resolution were poorer than those at other pH values (data not shown). Since these conditions are not commonly used, no additional work was performed at these pH values.

The separation at pH 2.4 required a somewhat longer time (about 30 min) than the separations at higher pH values (about 18 min). There are two effects that contribute to the separation time. First, the electroosmotic flow is drastically reduced at low pH so that elution times are lengthened. Second, the electrophoretic velocity (which is proportional to the charge) is higher at low pH since the species carry a high net positive charge; this shortens the elution time and partially offsets the reduction in electroosmotic flow velocity.

Overall, the selectivity observed at pH 2.4 was different, but not necessarily better, than that at pH 8.1. For example, fragments 7 and 20–21, fragments 8 and 15, and fragments 4 and 6–16 overlap at pH 2.4; several other pairs migrate at very similar velocities. Note that fragments 5 and 17 show very low absorbance in the pH 2.4 glycine buffer since they have very similar absorptivities to the mobile phase. The separation in the glycine buffer is very similar to that obtained with another commonly used low pH buffer, pH 2.5 citrate (data not shown; peaks 5 and 17 have greater relative peak height in the citrate buffer).

At pH 8.1, fragments 7 and 12, fragments 11 and 15, and fragments 1, 13, 14 and 17 co-migrate. However, the separation previously reported for pH 8.0 (refs. 9, 11) is superior to either Fig. 1 or 2. The addition of morpholine in the previous report is important to achieve the best resolution because of combined changes in selectivity and efficiency. The changes in selectivity between the previous work at pH 8.0 and pH 8.1 in this work seem to be minor since the peak order is preserved, although fewer peaks overlap for the pH 8.0 conditions. The changes in efficiency are apparently the result of a reduction of the electroosmotic velocity due to morpholine (which coats the capillary walls) and the higher buffer strength; both contribute to a greater effective separation time. Additives such as morpholine may also interact with other species in ways that are only poorly understood as discussed below.

Although pH 8.1 is not the midpoint of the p*I* range of the fragments (average

p*I*, 6.3; median p*I*, 5.9), previous empirical data suggested that it would give a good separation. Thus, the remainder of the optimization was performed at pH 8.1.

Tricine (buffer) optimization

It is well known that the buffer concentration must be sufficiently high to maintain a constant conductivity and pH within the analyte zone to prevent band broadening[5,6,15,24]. Buffer concentrations at least 100 times that of the sample may be necessary to meet this requirement. However, high concentrations of ionic buffers produce high conductivity, and therefore high currents and high heat loads. Use of a zwitterionic buffer allows one to adjust pH with only a minimal effect on conductivity[6,25]. For the optimization at pH 8.1, tricine was chosen after examination of a number of possible buffer systems since it met all of the above criteria and gave reproducible electropherograms.

The initial concentration of tricine was 0.01 *M*, which gives a buffer capacity of about 5 m*M* at pH 8.1. Thus, the buffer capacity was about 50 times the concentration of the individual fragments. The upper tricine concentration value of 0.1 *M* gave a ten-fold increase in buffer capacity. Morpholine also contributed to the buffer capacity when it was present, *e.g.*, 0.01 *M* morpholine has a buffer capacity of about 5 m*M* at pH 8.1. The total buffer capacity (range of 5 to 78 m*M*) and approximate ionic strengths are given in Table II.

TABLE II

OPTIMIZATION OF CZE MOBILE PHASE COMPOSITION AT pH 8.1

Concentrations, buffer capacity and ionic strength are given as mmol/l.

Condition	*Mobile phase concentration*			*Buffer capacity*	*Ionic strength*
	Tricine	*Sodium chloride*	*Morpholine*		
A	10	0	0	5	2
B	50	0	0	26	12
C	100	0	0	52	25
D	10	20	0	5	22
E	10	40	0	5	42
F	10	0	5	8	4
G	10	0	15	13	7
H	10	0	45	27	17
I	10	20	45	27	37
J	10	40	45	27	57
K	50	0	45	48	27
L	50	20	30	41	42
M	100	40	0	52	65
N	100	0	20	62	31
P	100	0	30	67	35
Q	100	0	45	74	39
R	100	40	45	74	79
S	100	20	0	52	45
T	100	0	10	57	28
U	150	0	0	78	38

An increase in the tricine concentration from 0.01 to 0.1 *M* in the absence of morpholine and sodium chloride produced increased current (approximately 3 μA to 28 μA, conditions A and E, respectively) and a decreased electroosmotic flow (approximately 2.4 mm/s to 1.6 mm/s under the same conditions). However, the greatest

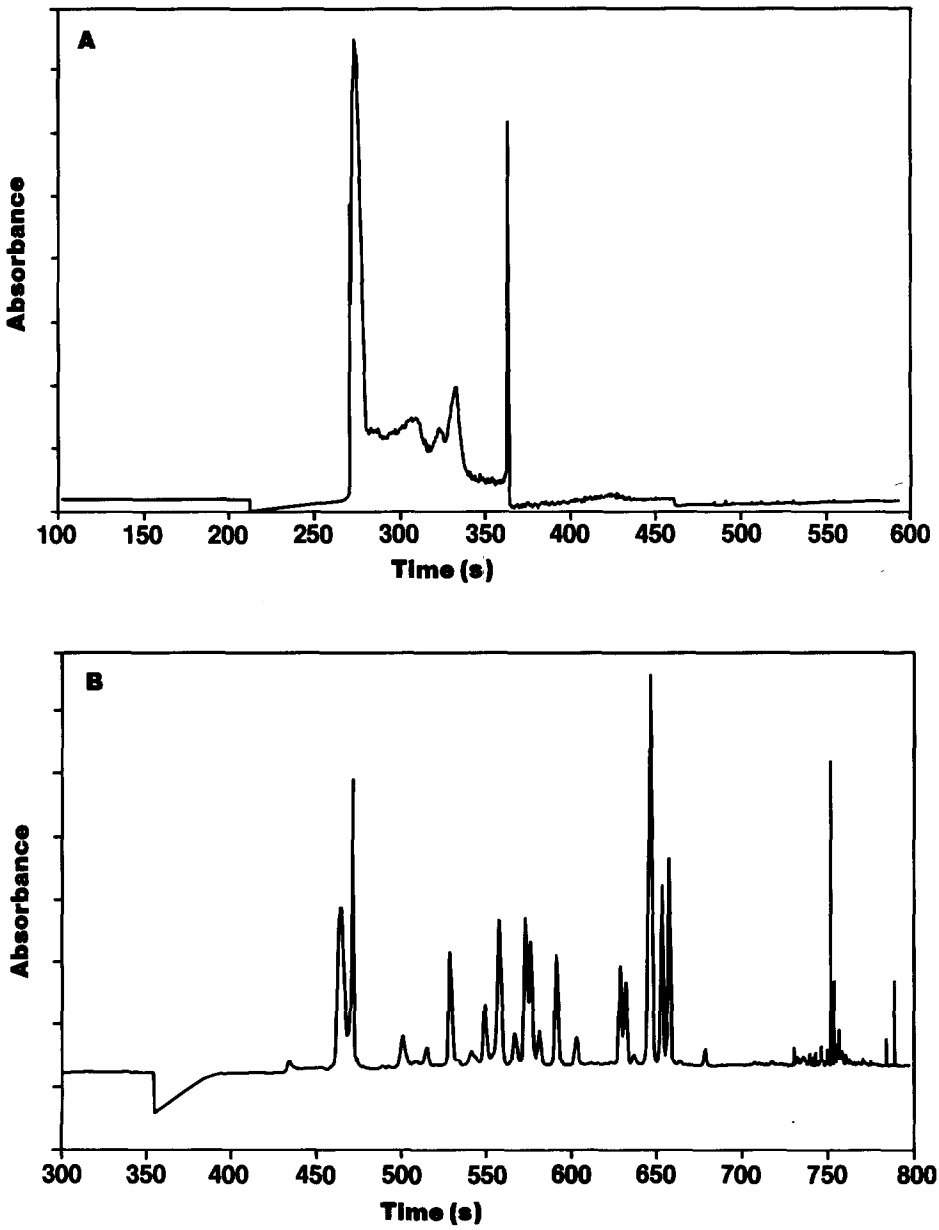

Fig. 3. Electropherogram of hGH digest in pH 8.1 tricine buffer. (A) 0.01 *M* tricine (condition A); (B) 0.1 *M* tricine (condition C).

effect of increased tricine concentration was the greatly improved efficiency and resolution (Fig. 3). The efficiency and resolution were improved by increasing the buffer concentration whether or not salt or morpholine was present (see Fig. 4; other data not shown). The increased efficiency was apparently due to the increased buffer capacity rather than ionic strength (see below). This result demonstrates the necessity of

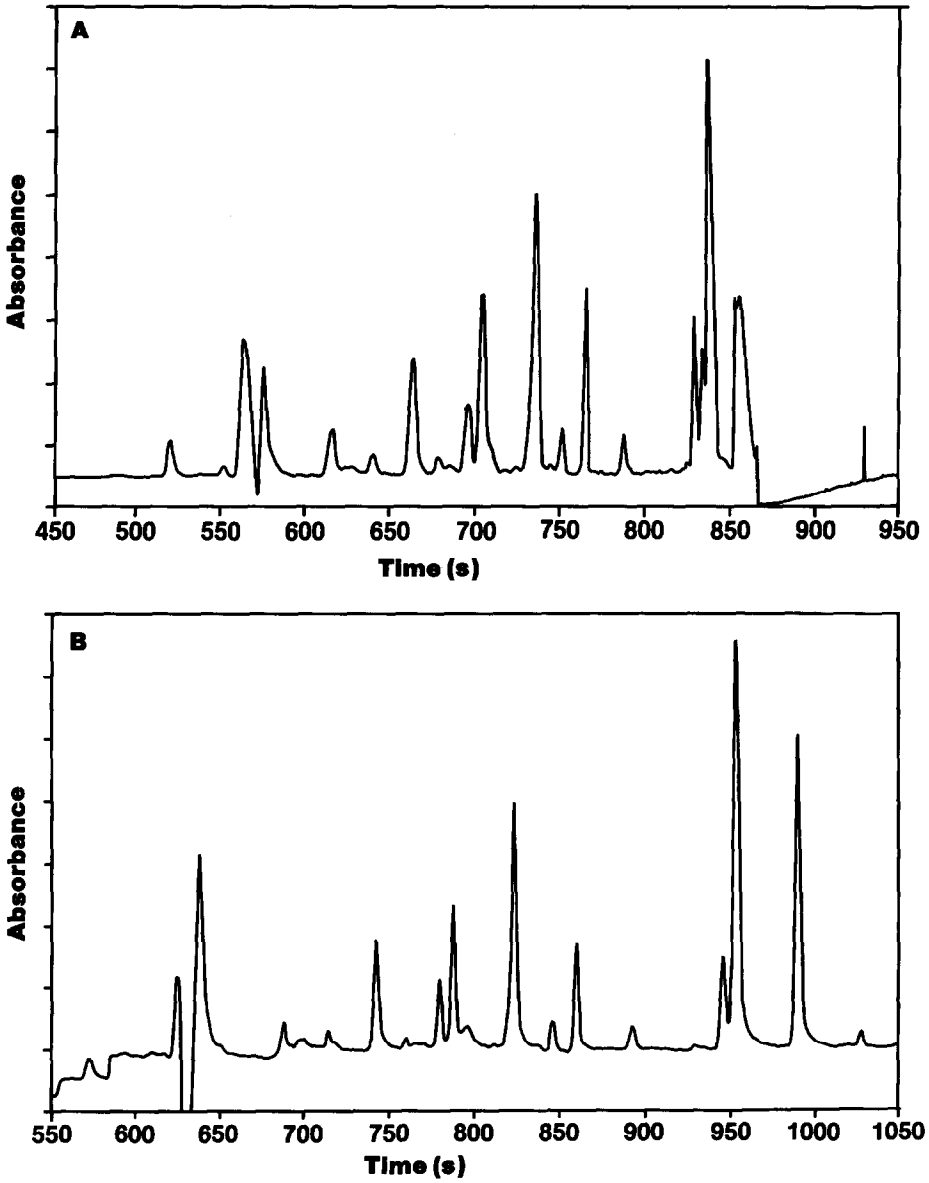

Fig. 4. Electropherogram of hGH digest in pH 8.1 tricine buffer. (A) 0.01 *M* tricine, 0.04 *M* sodium chloride, and 0.045 *M* morpholine (condition J); (B) 0.1 *M* tricine, 0.04 *M* sodium chloride, and 0.045 *M* morpholine (condition R).

maintaining a large excess of buffer to prevent distortions in the electric field gradient and pH within the sample zone and the resulting loss of resolution. Based on data for this system, at least 0.05 *M* tricine (*i.e.*, buffer capacity about 300 times the analyte concentration) is required for optimum resolution.

Other effects due to the increased tricine concentration need to be discussed. First, the higher ionic strength reduces the zeta potential which causes a lower electroosmotic flow velocity. (Other effects due to a higher ionic strength include a reduced thickness of the double layer, and changes in the dielectric constant and viscosity[19]). Therefore, the analyte effectively sees a reduced charge on the capillary surface at high buffer concentrations. The increased buffer concentration also competes for ion-exchange sites on the capillary as discussed in the next section. Thus, increasing the buffer concentration will minimize wall interactions by two mechanisms, a decrease in the zeta potential and saturation of ion exchange sites. Minimizing the wall interaction will allow analyte species that interact with free silanols (at low buffer strengths) to move at rates more consistent with their inherent electrophoretic mobility (*e.g.*, size and charge). Third, it is possible that at high ionic strengths, the charge on the analyte is more effectively shielded from the electrostatic field by its surrounding ionic environment. Finally, zwitterions such as tricine should not directly interact with the sample to produce species of different charge[6].

Sodium chloride (ionic strength adjustor) optimization

Earlier work by Jorgenson[15] and Lauer and McManigill[6] suggested that one source of wall interaction was due to exposed silanols that act as cation-exchange sites for positively charged regions of the analyte. This type of interaction could be minimized by the addition of inert salts (to increase the ionic strength) or other cations (to compete for the exchange sites). Initial experiments in our laboratory suggested that resolution of the digest fragments might be dependent upon the ionic strength. Thus, the effect of ionic strength was investigated by the addition of 0–0.04 *M* salt.

The total ionic strength, including contributions from the buffer and morpholine, is given in Table II (range of 2 to 79 m*M*). At 0.01 *M* tricine and 0.04 *M* sodium chloride, the current was about 36 μA (condition E) compared to 3 μA without sodium chloride (condition A). Note that increased salt concentration affects the double layer structure and reduces the electroosmotic flow in a manner analogous to that produced by increased buffer concentrations (electroosmotic flow velocity 1.6 mm/s compared to 2.4 mm/s for conditions E and A, respectively). In addition to the changes in double layer structure, the sodium ion should compete with the sample for cation-exchange sites on the silica surface.

In this systematic study, the overall resolution was poorer in the presence of salt, as apparent in Fig. 5 compared to Fig. 3B (other data not shown). Based on our observations, it was concluded that there was no obvious advantage from increasing the ionic strength by the addition of sodium chloride.

Morpholine (mobile phase additive) optimization

In RP-HPLC, mobile phase additives are frequently employed to minimize the interaction of cationic analytes, especially amines, with exposed silanols. When used in CZE, these additives will have a similar effect in coating the free silanols. However, they have the additional major effect of modifying the interfacial double-layer struc-

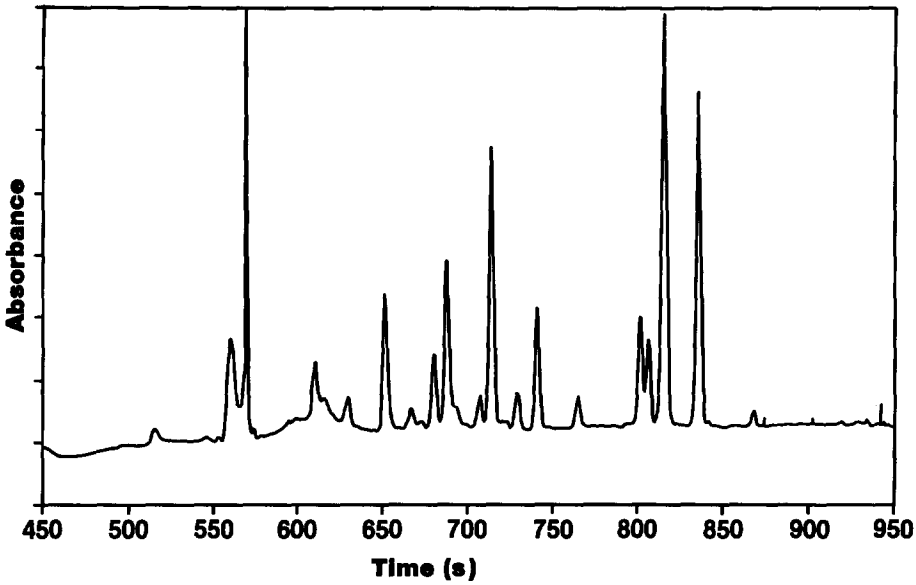

Fig. 5. Electropherogram of hGH digest in pH 8.1 tricine buffer, 0.1 *M* tricine and 0.04 *M* sodium chloride (condition M).

ture and reducing the zeta potential. Thus, they will reduce the electroosmotic flow as well as change the nature of the exposed surface. The effect of morpholine, a common additive for RP-HPLC, was investigated in CZE separations over a concentration range of 0 to 0.045 *M*. As mentioned above, morpholine will also increase the ionic strength and buffer capacity.

Addition of morpholine produced a lower electroosmotic flow and higher current as expected. For example, the current with 0.01 *M* tricine and 0.045 *M* morpholine was about 27 μA (condition H) compared to 3 μA without morpholine (condition A); the corresponding electroosmotic flows were about 1.3 mm/s and 2.4 mm/s, respectively. Both values are similar to that produced by changing the tricine concentration to 0.1 *M* in the absence of morpholine. However, morpholine also produced different resolution for the fragments (see Fig. 6 compared to Fig. 3A). Without any direct evidence, one can only speculate that it is either reducing the interaction of the analyte with the wall (as desired) and/or that it is changing the charge of the species in solution. In either case, there seems to be some advantage with using small amounts of morpholine (about 0.02 *M*), especially at low buffer concentrations.

Mobile phase interactions at pH 8.1

Additional mobile phase compositions were examined to validate the above observations and to uncover any interaction of variables. For example, condition Q (Fig. 7) represents a major increase in buffer concentration from condition H (Fig. 6) and shows improved resolution. This result is consistent with that expected from extrapolation of Figs. 3 and 4 as well as for other cases (data not shown) where the

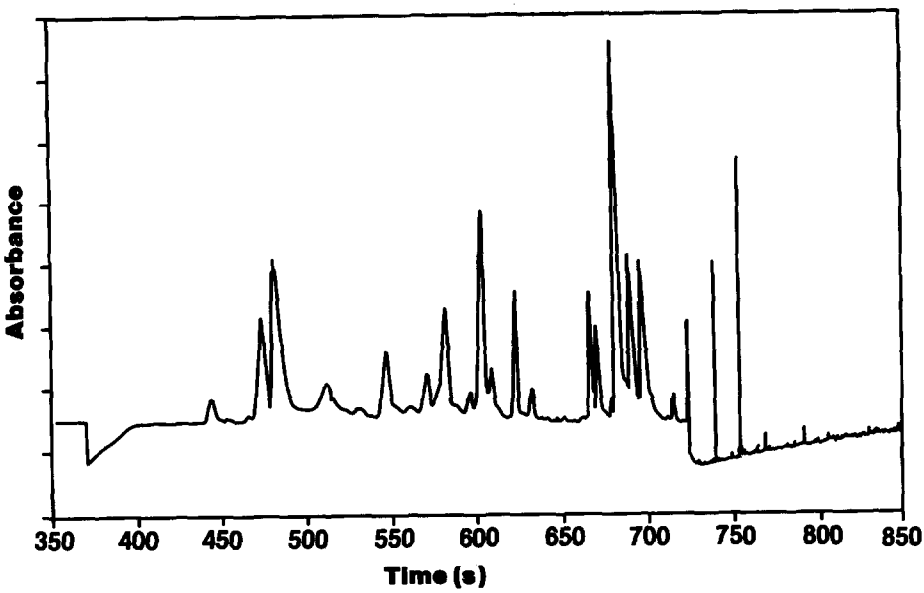

Fig. 6. Electropherogram of hGH digest in pH 8.1 tricine buffer, 0.01 *M* tricine and 0.045 *M* morpholine (condition H).

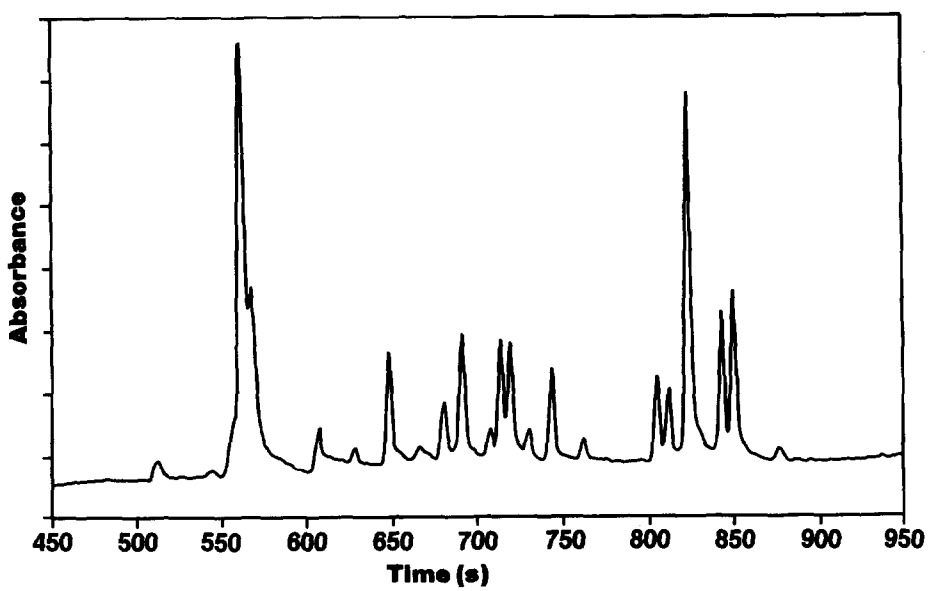

Fig. 7. Electropherogram of hGH digest in pH 8.1 tricine buffer, 0.1 *M* tricine and 0.045 *M* morpholine (condition Q).

tricine concentration was increased while the concentrations of the other components were kept approximately constant.

Similar comparisons can be made for the effect of added salt from condition H to condition J and from condition Q to condition R (Figs. 6 to 4A and 7 to 4B, respectively). No obvious advantage from increased sodium chloride concentration is observed regardless of the tricine or morpholine concentration levels.

The effect from addition of morpholine at high buffer levels (condition C to condition Q; Fig. 3B to 7) or with high salt and high buffer levels (condition M to condition R; Fig. 5 to 4B) is less obvious. Inspection of the electropherograms indicates some differences in resolution but no definite trends.

The observation that the optimum separation is obtained with a mobile phase that contains a high buffer concentration, no salt, and possibly some morpholine, was confirmed by running several additional conditions. These include conditions T, N, and P (0.1 *M* tricine, no salt, and increasing amounts of morpholine), condition S (0.1 *M* tricine, no morpholine and 0.02 *M* sodium chloride, an intermediate salt concentration), and condition U (0.15 *M* tricine, an increased buffer concentration, with no sodium chloride or morpholine). These data indicated that condition N (0.1 *M* tricine, 0.02 *M* morpholine and no salt) produced the optimum separation (Fig. 8).

In summary, the results of the mobile phase optimization at pH 8.1 are as follows: (1) increasing either tricine, sodium chloride or morpholine concentrations increased the current and reduced the electroosmotic flow velocity (all three species increase the ionic strength; only tricine and morpholine contribute to the buffer capacity), (2) a high buffer capacity, about 300 times the concentration of the analyte, was required to maintain high efficiency and good resolution, (3) added sodium chlo-

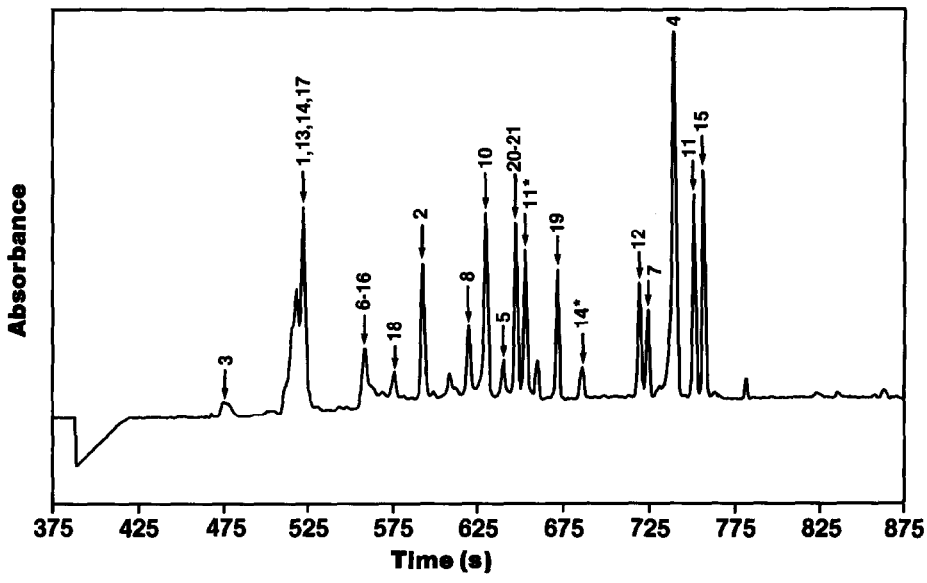

Fig. 8. Electropherogram of hGH digest in pH 8.1 tricine buffer, 0.1 *M* tricine and 0.02 *M* morpholine (condition N).

ride tended to decrease the resolution, and (4) small concentrations of morpholine improved the resolution.

Comparison of separation strategies

The initial separation at pH 8.0 (0.01 *M* tricine, 0.045 *M* morpholine, and 0.02 *M* sodium chloride) apparently has a high enough buffer capacity (25 m*M*) and ionic strength (38 m*M*) with an acceptable morpholine concentration to produce a good separation. However, the optimized separation (condition N, Fig. 8) is better for the mid-range and later eluting peaks with a minimal sacrifice in resolution for the three earliest eluting peaks. In contrast, the optimized separation is much better than the preliminary separation at pH 8.1 obtained in this work (Fig. 2) even though the change in mobile phase composition is relatively minor (replacement of 0.0343 *M* sodium chloride with 0.02 *M* morpholine). The optimized separation also is much better than that at pH 2.4 (Fig. 1), although the latter has not been optimized. Finally, it is important to note that the earlier eluting fragments (fragments 1, 13, 14 and 17) that are poorly resolved in the optimum separation are somewhat better resolved in the pH 8.0 system and are well resolved in the pH 2.4 system (although other peaks overlap).

Complete separation of all of the digest fragments was not achieved by any single elution condition in CZE. However, it appears that if two values of pH are used, then a more complete identification of all species in a complex mixture can be achieved. This is analogous to the use of two-dimensional techniques or coupled chromatographic conditions except that CZE is simpler since only the mobile phase composition needs to be changed. It is important to note that RP-HPLC does not achieve complete resolution of fragments even when a lengthy (1–2 h), complex gradient elution is used[22,26]. (Fragments 3, 5, 7 and 17 co-elute as do fragments 2 and 19; several other pairs of fragments closely elute.) As noted previously[11], CZE is a powerful complement to RP-HPLC for the characterization of the tryptic digest of hGH.

CONCLUSION

Adjustment of the composition of the mobile phase has been demonstrated to be crucial to obtaining optimum efficiency and resolution for the CZE separation of the tryptic digest of hGH. The optimum conditions will be achieved when the interaction of the analyte with the silica can be decoupled from the mobile phase pH so that the pH can be chosen to maximize the differences in charges between the species. A high buffer capacity appears to be one of the most important variables for this approach to succeed. However, mobile phase additives such as morpholine may be important, partly due to the reduction in electroosmotic flow in their presence. It is obvious that there is no simple set of rules that will produce the optimum separation, although a process similar to the one we have followed should be useful. The key variables were identified and the effects of changes in variables were found to be approximately additive.

For the tryptic digest of hGH, a mobile phase composition of 0.1 *M* tricine and 0.02 *M* morpholine at pH 8.1 appears to be optimum. This separation is slightly better overall than our previously reported data. Fragments that overlap under the optimum separation conditions may be separated by performing a second separation at a different pH.

ACKNOWLEDGEMENTS

We thank Drs. J. W. Jorgenson, R. M. Riggin, G. W. Becker and G. S. Sittampalam for helpful discussions and encouragement. We acknowledge ISCO, for the donation of a detector. We are grateful for the technical assistance given by D. S. LeFeber and P. A. Farb.

REFERENCES

1 J. W. Jorgenson, *Anal. Chem.*, 58 (1986) 743A–758A.
2 M. J. Gorden, X. Huang, S. L. Pentoney and R. N. Zare, *Science (Washington, D.C.)*, 242 (1988) 224–228.
3 S. W. Compton and R. G. Brownlee, *Biotechniques*, 6 (1988) 432–440.
4 A. G. Ewing, R. A. Wallingford and T. M. Olefirowicz, *Anal. Chem.*, 61 (1989) 292A–303A.
5 J. W. Jorgenson and K. D. Lukacs, *Science (Washington, D.C.)*, 222 (1983) 266–272.
6 H. H. Lauer and D. McManigill, *Anal. Chem.*, 58 (1986) 166–170.
7 J. J. Kirkland and R. M. McCormick, *Chromatographia*, 24 (1987) 58–76.
8 R. M. McCormick, *Anal. Chem.*, 60 (1988) 2322–2328.
9 P. D. Grossman, J. C. Colburn, H. H. Lauer, R. G. Nielsen, R. M. Riggin, G. S. Sittampalam and E. C. Rickard, *Anal. Chem.*, 61 (1989) 1186–1194.
10 R. G. Nielsen, G. S. Sittampalam and E. C. Rickard, *Anal. Biochem.*, 177 (1989) 20–26.
11 R. G. Nielsen, R. M. Riggin and E. C. Rickard, *J. Chromatogr.*, 480 (1989) 393–401.
12 J. Frenz, S. Wu and W. S. Hancock, *J. Chromatogr.*, 480 (1989), 379–391.
13 P. D. Grossman, K. J. Wilson, G. Petrie and H. H. Lauer, *Anal. Biochem.*, 173 (1988) 265–270.
14 P. D. Grossman, J. C. Colburn and H. H. Lauer, *Anal. Biochem.*, 179 (1989) 28–33.
15 J. W. Jorgenson, in J. W. Jorgenson and M. Phillips (Editors), *New Directions in Electrophoretic Methods, (ACS Symposium Series*, Vol. 335), American Chemical Society, Washington, DC, 1987, Ch. 13.
16 H. H. Lauer and D. McManigill, *Trends Anal. Chem.*, 5 (1986) 11–15.
17 X. Huang, W. F. Coleman and R. N. Zare, *J. Chromatogr.*, 480 (1989) 95–110.
18 G. O. Roberts, P. H. Rhodes and R. S. Snyder, *J. Chromatogr.*, 480 (1989) 35–67.
19 T. S. Stevens and H. J. Cortes, *Anal. Chem.*, 55 (1983) 1365–1370.
20 E. Grushka, R. M. McCormick and J. J. Kirkland, *Anal. Chem.*, 61 (1989) 241–246.
21 J. W. Jorgenson and K. D. Lukacs, *Anal. Chem.*, 53 (1981) 1298–1302.
22 G. W. Becker, P. M. Tackitt, W. W. Bromer, D. S. LeFeber and R. M. Riggin, *Biotechnol. Appl. Biochem.*, 10 (1988) 326–337.
23 H. A. Laitinen, *Chemical Analysis*, McGraw-Hill, New York, 1960, Ch. 3.
24 R. A. Mosher, D. Dewey, W. Thormann, D. A. Saville and M. Bier, *Anal. Chem.*, 61 (1989) 362–366.
25 M. M. Bushey and J. W. Jorgenson, *J. Chromatogr.*, 480 (1989) 301–310.
26 E. Conova-Davis, R. C. Chloupek, I. P. Baldonado, J. E. Battersby, M. W. Spellman, L. J. Basa, B. O'Connor, R. Pearlman, C. Quan, J. A. Chakel, J. T. Stults and W. S. Hancock, *Am. Biotechnol. Lab.*, 6 (May) (1988) 8–17.
27 B. Skoog and A. Wichman, *Trends Anal. Chem.*, 5 (1986) 82–83.
28 J. L. Meek and Z. L. Rossetti, *J. Chromatogr.*, 211 (1981) 15–28.