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SEPARATION OF PRECOLUMN *ortho*-PHTHALALDEHYDE-DERIVATIZED AMINO ACIDS BY CAPILLARY ZONE ELECTROPHORESIS WITH NORMAL AND MICELLAR SOLUTIONS IN THE PRESENCE OF ORGANIC MODIFIERS

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SUMMARY

Isoindole derivatives of amino acids, formed through a well-established reaction with *o*-phthalaldehyde, have been effectively resolved by capillary zone electrophoresis and detected fluorimetrically. Relative retention of the formed derivatives is strongly influenced by added detergents and organic modifiers. Analytical reproducibility has also been assessed.

INTRODUCTION

o-Phthalaldehyde (OPA) has been commonly used as a precolumn derivatization agent for the high-performance liquid chromatographic (HPLC) analysis of amino acids[1–5]. In the presence of a reducing agent, such as 2-mercaptoethanol or ethanethiol, OPA reacts specifically with primary amine groups to form highly fluorescent substituted isoindoles. The advantages offered by OPA include its relatively specific and strong fluorescence, rapid reaction with amino acids, and ease of use. However, a major drawback is the instability of OPA-derivatized amino acids, which has reduced its utility in separation methods which require relatively long analysis times. The development of a rapid, efficient, and sensitive separation system is seen as a needed improvement in the area of amino acid analysis.

Capillary zone electrophoresis (CZE) has been developed[6,7] as an effective, high-efficiency separation technique for both small ions[6–11] and macromolecules[12]. To extend the application of CZE to the analysis of neutral species, Terabe *et al.*[13] introduced a variant which is based on the differential distribution of sample species between a mobile phase (electroosmotically pumped) and a micellar phase, formed by adding a surfactant which is retarded due to the electrophoretic effect. This technique has also been termed "micellar electrokinetic capillary chromatography" (MECC) by Burton *et al.*[14] Both CZE and MECC combine the desirable features of high separation efficiency (often on the order of 10^5 theoretical plates) and short analysis times.

In the present study, we explore possibilities for the separation of primary amino acids as their OPA derivatives by CZE, using both a normal solution and a micellar solvent system. Investigations dealing with the stability of the derivatized products,

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reproducibility of the electrophoretic profile, linearity, and the effects of pH and organic modifiers on retention characteristics are described. The application of this method to the separation and analysis of a protein hydrolysate is also demonstrated.

EXPERIMENTAL

Apparatus

A CZE system, similar to that described by Jorgenson and Lukacs[7], was constructed in-house. Fused-silica capillaries (Polymicro Technologies, Phoenix, AZ, U.S.A., 50–100 cm × 50 μm I.D., 187 μm O.D.) were suspended between two electrodes, immersed in reservoirs filled with the same buffer solution, all contained within a safety interlock box. The high voltage was provided by a d.c. power supply delivering 0–30 kV (Spellman High-Voltage Electronics, Plainview, NY, U.S.A.). Fluorescence detection was performed with a Model FS950 Fluoromat detector (Kratos, Ramsey, NJ, U.S.A.), with an excitation wavelength of 365 nm and fluorescence emission measured with a 418-nm cut-off filter. An on-column optical cell was made by removing the polyimide coating on a short section of the fused-silica capillary.

Chemicals

All amino acids, proteins, and sodium dodecyl sulfate (SDS) were purchased from Sigma (St. Louis, MO, U.S.A). Ethanethiol and OPA were obtained from Fluka (Ronkonkoma, NY, U.S.A.). Methanol and tetrahydrofuran (THF) were of HPLC grade. Sodium acetate and boric acid were of analytical-reagent grade.

Capillary cleaning and injection procedures

To maintain a high surface charge density on the inner surface of the capillary and create a strong electrokinetic flow, capillary cleaning procedures are necessary[12]. The capillary was initially flushed with distilled water for *ca.* 30 min, followed by 1.0 *M* KOH and 0.1 *M* KOH for 30 min each. The capillary was then rinsed thoroughly with distilled water and equilibrated with the buffer solution to be used for the separation. All cleaning procedures were carried out by using a vacuum pump to draw the liquid through the capillary.

Sample injection was accomplished by hydrodynamic introduction. The high-voltage end of the capillary was dipped into the sample vial, which was raised to a level higher than the other end of the column and maintained for a certain length of time. The end of the capillary was then returned to the buffer reservoir, and high voltage was applied to start the electrophoretic run.

Derivatization and protein hydrolysis procedures

The OPA–ethanethiol derivatizing solution was prepared by dissolving 5 mg of OPA in 0.45 ml of methanol. To this solution, 5 μl of ethanethiol and 50 μl of borate buffer (0.4 *M* boric acid solution adjusted to pH 9.50 with 1.0 *M* KOH) were added and mixed. This solution should be protected from light and freshly prepared prior to each use.

Stock solutions of each individual amino acid were prepared in a phosphate buffer (pH 7.00) at a concentration of 10 mg/ml. A mixture of amino acids was

prepared by mixing equal volumes of the stock solution of each amino acid. A solution of 2-aminoethanol at a concentration of 1 mg/ml was also prepared and included in the mixture as an internal standard. All solutions were kept frozen when not in use.

Protein samples were hydrolyzed by placing 20 μl of protein solution (1.0 mg/ml) into a hydrolysis tube and adding 0.5 ml of constant-boiling HCl. The tube was sealed under vacuum after 30 min and heated at 110°C for 24 h. The solution was lyophilized and the residue was dissolved in 50 μl of distilled water just prior to derivatization.

Derivatization of the amino acids was performed by mixing an aliquot of amino acid standard solution or protein hydrolysate solution (2–5 μl) with an appropriate volume of internal standard solution, 0.4 *M* borate buffer, and 2–5 μl of the OPA–ethanethiol derivatizing solution. The mixture was allowed to stand at room temperature for 1–2 min prior to injection. Approximately 2–20 nl of sample solution were injected for analysis.

RESULTS AND DISCUSSION

OPA, in the presence of either 2-mercaptoethanol or ethanethiol, reacts rapidly with primary amino acids to form highly fluorescent thio-substituted isoindoles[15,16]. In our initial experiments, OPA–2-mercaptoethanol was used to derivatize the amino acids. However, the fluorescence response obtained following separation of the amino acids by CZE was very weak. The instability of fluorescent products in the OPA–2-mercaptoethanol derivatization reaction has been reported[1,15], and it is known that the substituted isoindole decays readily to an ethylene sulfide polymer and 2,3-dihydro-1*H*-isoindol-1-one. It is possible that the heat generated by the applied high voltage during a CZE separation could accelerate the degradation of the OPA–2-mercaptoethanol fluorescent products, leading to the weak signal. In place of 2-mercaptoethanol, we used ethanethiol in the OPA derivatization reactions and found that the stability of the fluorescent product was considerably improved.

Fig. 1 shows an electropherogram of the OPA–ethanethiol derivatives of four amino acids and 2-aminoethanol, used as an internal standard. We initially used a ternary solvent system of aqueous buffer, methanol and THF, similar to that commonly used in reversed-phase HPLC. A mixture of 0.05 *M* sodium acetate, 15% methanol and 1% THF was found to give good resolution of the OPA-derivatized amino acids, seen in Fig 1. The stability of the derivatized components was ascertained by measuring the peak heights from successive electropherograms, obtained over a period of several hours. It was observed that the derivatized products are stable for *ca.* 5 h, exhibiting no significant decrease in the fluorescence response. This stability, coupled with the feature of short analysis times for CZE, makes it possible to put the same sample through several electrophoretic runs.

Reproducibility of the electrophoretic profile of OPA-derivatized amino acids was investigated to assess the value of CZE for quantitative analysis. The precision of the method was determined by injecting the mixture of amino acid standards used in Fig. 1 five times under the same experimental conditions. The ratio of retention time of the amino acids relative to the internal standard was calculated for each amino acid, and peak heights were measured. The calculated values for the mean, standard deviation (S.D.) and relative standard deviation (R.S.D.) are listed in Table I. Analysis of the data indicates that the average R.S.D. for the retention time ratio is less than 2%, while the R.S.D. for the peak-height ratio is 1.3–4.8%.

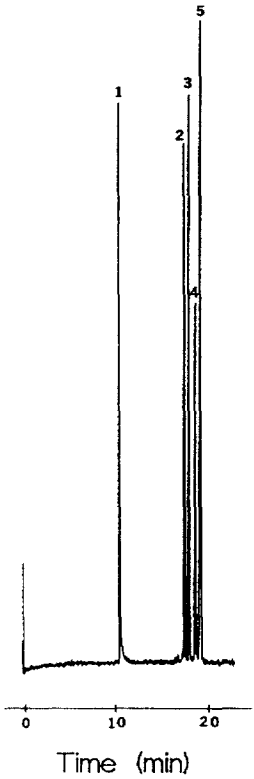

Fig. 1. Electropherogram of OPA–ethanethiol derivatives of four amino acids and an internal standard. Peaks: 1 = 2-aminoethanol (internal standard); 2 = serine; 3 = alanine; 4 = glutamine; 5 = asparagine. Buffer, 0.05 *M* sodium acetate buffer (pH 9.50)–15% methanol–1% THF; capillary, 100 cm × 50 μm I.D. (70 cm to detector); injection time, 2 s; applied voltage, 30 kV.

Presently, two sample introduction techniques are widely used in CZE: the electromigration technique[7] and hydrodynamic suction[10]. The exact amount of sample solution introduced by either technique is not easily determined, but both methods rely primarily on the time of injection. It has been found[17] that the electromigration technique results in some sample discrimination, and the hydro-

TABLE I
REPRODUCIBILITY OF RETENTION TIME AND PEAK HEIGHT

	Peak-height ratio (n=5)			*Retention ratio (n=5)*		
	Mean	*S.D.*	*R.S.D. (%)*	*Mean*	*S.D.*	*R.S.D. (%)*
2-Aminoethanol	1.00	0.00	0.00	1.00	0.00	0.00
Serine	1.14	0.06	4.8	2.03	0.02	1.17
Alanine	1.24	0.05	4.1	2.13	0.03	1.46
Glutamine	0.95	0.02	2.4	2.25	0.04	1.73
Asparagine	1.38	0.02	1.3	2.33	0.04	1.71

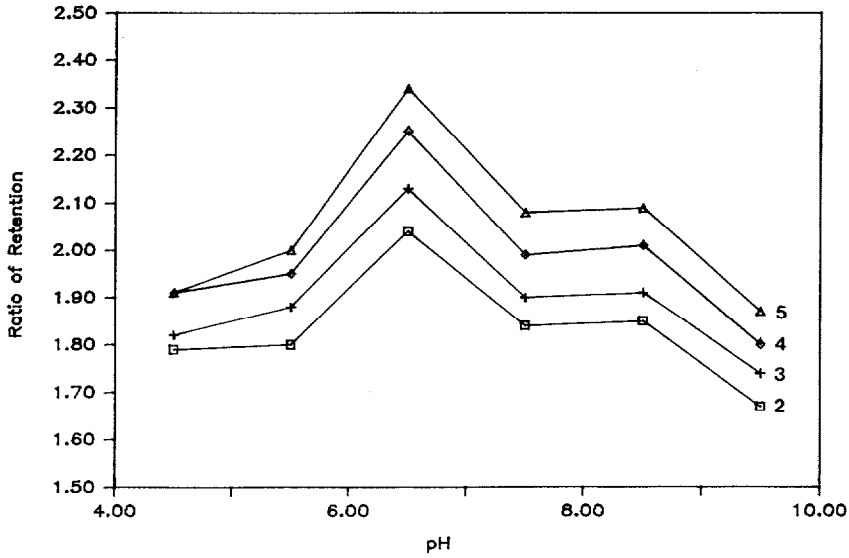

Fig. 2. Dependence of retention ratio on pH. Conditions as for Fig. 1, except for the applied voltage (25 kV). The numbers correspond to the solutes in Fig. 1.

dynamic injection method generally gives more reproducible results[18]. Therefore, the hydrodynamic injection technique was employed throughout this study. The estimated volume of sample solution injected is 2–20 nl, which corresponds to 1.25–12.5 pmol of each amino acid in our study. However, this does not represent the detection limit, which is estimated to be less than 300 fmol of each amino acid. Better reproducibility for both qualitative and quantitative analysis will require more precise control of injection time and further improvement in sample introduction techniques.

An important factor in both CZE and MECC systems is the pH of the buffer solution, since it directly influences the electrophoretic mobility of the solutes and the electroosmotic flow of the buffer solution. The effect of pH on the retention ratio of the OPA derivatives of serine, alanine, glutamine, and asparagine was investigated, as shown in Fig. 2. All four amino acids show the same trends of retention, except at pH 4.50 where both glutamine and asparagine have the same migration time. It is of interest to note that a maximum retention time for these four amino acids was observed at a pH near 7. The migration of charged species in a buffer solution depends simultaneously upon the electrophoretic mobility of the solutes themselves and the electroosmotic flow of the buffer solution. The effect of pH is reflected in a change of the effective charge on the species and the double-layer formed between the capillary wall and buffer solution, which in turn influences the zeta potential and the electroosmotic flow.

The effect of pH on solute migration in a CZE system can also be seen in Fig. 3. These results indicate that pH has very little effect on the electrophoretic mobility of solutes. In contrast, the electroosmotic flow coefficient is more sensitive to pH because of the dependence of double-layer formation on the local electrostatic force. A buffer near pH 7 may result in a decrease of the zeta potential, thereby slowing the

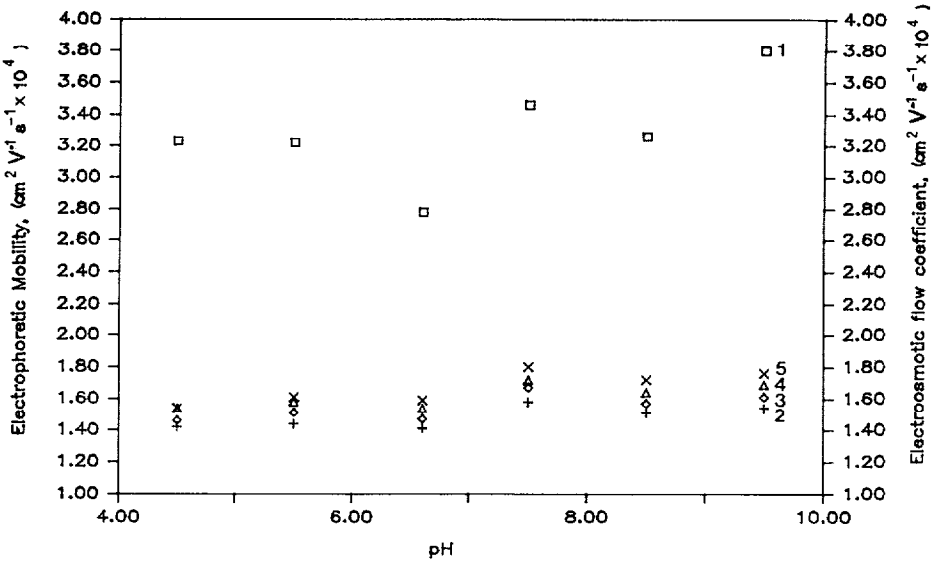

Fig 3. Dependence of electrophoretic mobility and electroosmotic flow coefficient on pH. Conditions and numbers as in Fig. 1. The points for 2-aminoethanol (□) correspond to the electroosmotic flow coefficient (right axis). The points for the remaining components correspond to the electrophoretic mobility (left axis).

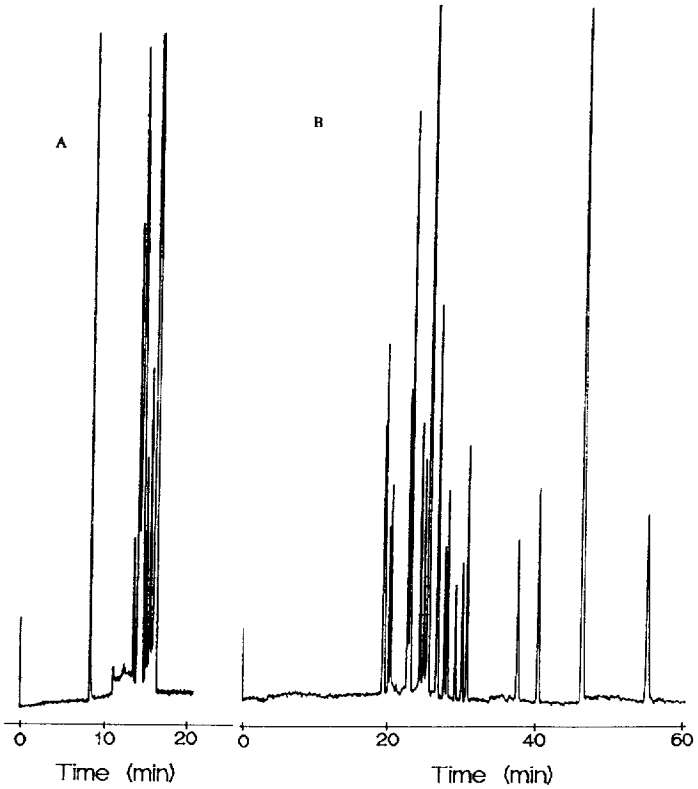

Fig. 4. Electropherogram of a mixture containing eighteen OPA amino acids and an internal standard. Buffer, 0.05 *M* sodium acetate buffer (pH 9.50)–15% methanol–1% THF: (A) without addition of SDS; (B) with addition of SDS (0.05 *M*). Injection time, 15 s. The remaining conditions are as for Fig 1.

electroosmotic flow and the rate of migration of the solutes. A higher pH results in a shorter analysis time, without a significant loss of resolution. A further advantage of using a high pH is the resultant dynamic deactivation of the capillary wall[12], which serves to decrease the adsorption of solutes on the capillary surface. At high pH, the strong electrostatic repulsion between the negatively charged species and the negatively charged capillary walls results in very sharp peaks and high resolution. A pH of 9.50 was found to be optimal for our studies.

Although pH plays an important role in the migration of charged species in CZE, adequate resolution of complex mixtures of OPA-derivatized amino acids is difficult to achieve without further modifications of the buffer system. The addition of SDS, initially utilized for the solubilization and CZE separation of neutral compounds[13], was found to be very effective for improving the resolution of the OPA derivatives. Fig. 4 shows two electropherograms of a mixture containing eighteen OPA-derivatized amino acids, both obtained under the same experimental conditions, except for the addition of SDS. A dramatic improvement in the resolution is noted with the SDS-modified buffer system. The addition of SDS creates a micellar phase within the mobile phase of the buffer. The improved resolution can then be attributed to the effects of distribution of the amino acids between the mobile and micellar phases. In addition, the use of SDS results in an increased dynamic deactivation effect due to the formation of large, negatively charged micelles, which are repelled from the capillary walls. Also, the column capacity is increased with the addition of SDS because of the migration of the micelles in opposition to the direction of the electroosmotic flow. Each of these factors likely plays a role in enhancing the resolving power. It is also evident from Fig. 4 that the internal standard, 2-aminoethanol, which is eluted first

TABLE II
RETENTION PARAMETERS OF OPA DERIVATIVES OF PRIMARY AMINO ACIDS

Peak number	*Amino acid*	*Retention time (min)*	*Retention ratio*
1	Glutamine	16.9	0.425
2	Threonine	17.3	0.435
3	Serine	17.6	0.442
4	Histidine	18.6	0.467
5	Alanine	19.4	0.486
6	Glycine	19.7	0.494
7	Valine	20.4	0.513
8	γ-Aminobutyric acid	20.8	0.522
9	Methionine	21.4	0.533
10	Taurine	21.8	0.548
11	Isoleucine	23.0	0.578
12	Tryptophan	23.9	0.601
13	Leucine	25.2	0.633
14	Lysine	27.6	0.693
15	Glutamic acid	30.2	0.753
16	Arginine	37.4	0.940
17	2-Aminoethanol (internal standard)	39.8	1.00

when SDS is not added to the buffer, migrates more slowly than most of the OPA amino acids when SDS is added. This is due to the fact that OPA 2-aminoethanol is more neutral and hydrophobic, and hence is more easily solubilized by the micellar phase. The slower migration of the micellar phase then results in its longer retention time.

Although the use of a sodium acetate buffer proved satisfactory for the electrophoretic separation of simple mixtures of OPA amino acids, its buffering capacity is limited at the high pH necessary for optimal resolution. For this reason, another quaternary solvent system, consisting of borate buffer, methanol, THF and SDS was employed and found to be very effective in separating all derivatized amino acids. The buffering capacity of the borate buffer is significantly higher than that of the

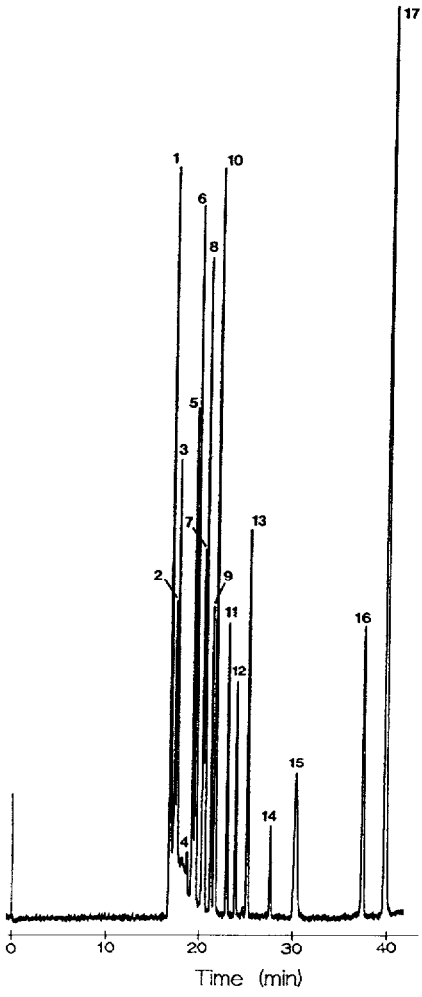

Fig. 5. Electropherogram of seventeen OPA amino acids. Buffer, 0.05 *M* borate buffer (pH 9.50)–15% methanol–2% THF–0.05 *M* SDS; capillary, 86 cm × 50 μm I.D. (53 cm to the detector); injection time, 10 s; applied voltage, 23 kV.

acetate buffer at pH 9.50, resulting in improved resolution. Fig. 5 shows an electropherogram obtained with this buffer system. The peaks in Fig. 5 are identified in Table II, which also lists the retention times and ratios of retention time to the internal standard for the individual components.

Organic modifiers play an important role in CZE separations, particularly for the enhancement of selectivity. Fig. 6 shows the effects of added methanol and THF on the separation behavior of OPA derivatives of glutamine, serine, alanine and glycine in a micellar system. Each of these amino acid derivatives exhibited an increase in

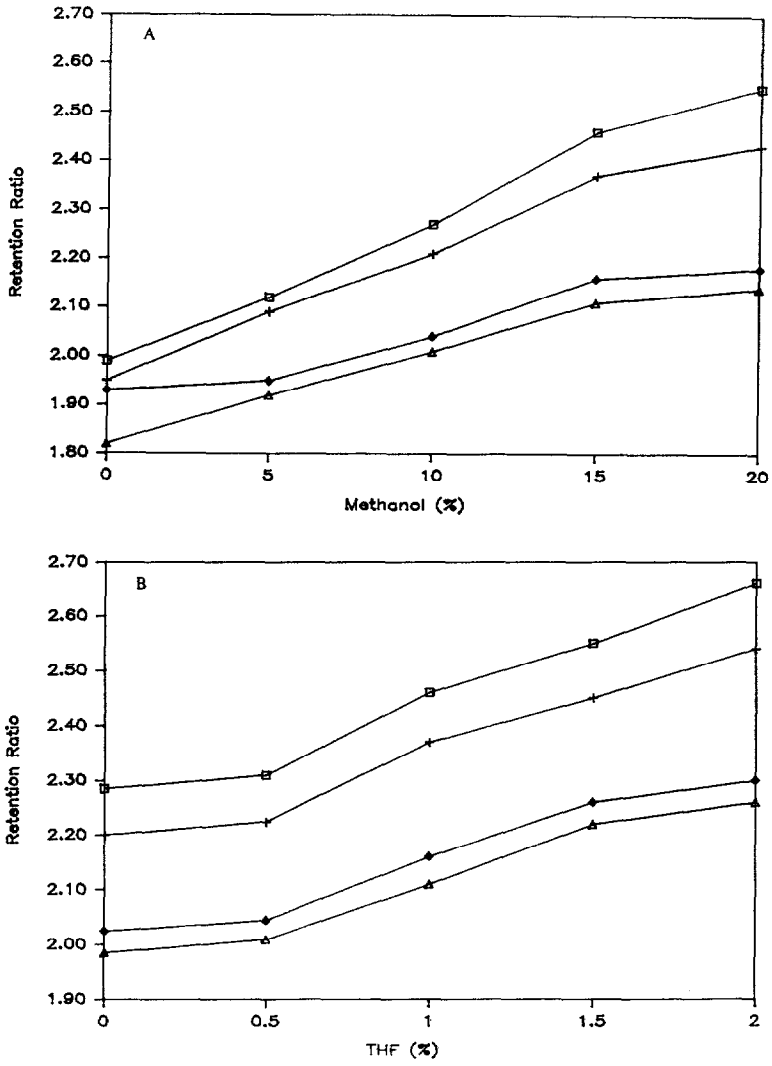

Fig. 6. Effects of organic modifiers on retention: □ = glutamine; + = serine; ◇ = alanine; △ = glycine. (A) Change in methanol concentration (volume percent) in 1% THF; (B) change in THF concentration (volume percent) in 15% methanol. The remaining conditions for both (A) and (B) are as for Fig. 5, except for the applied voltage (20 kV).

retention time as the amount of organic modifier was increased. However, the relative spacing between the components changes with increasing organic modifier, and this improves the selectivity of the separation. This effect is particularly evident in the case of methanol. In our investigations, we found that, when no methanol was added to the buffer system, serine and alanine could not be resolved, while glutamine and glycine were widely spaced. By using a buffer system containing 15% methanol, an evenly spaced baseline separation was obtained for the four components. The addition of organic modifiers was also found to enhance significantly the resolution and selectivity for more complex mixtures. In a mixture of seventeen amino acids, only fourteen peaks were resolved when no methanol was added to the buffer solution. With 5% methanol, sixteen peaks were observed, while with 15% methanol, all seventeen components were resolved (Fig. 5). Fig. 6B shows that the concentration of THF has a much smaller

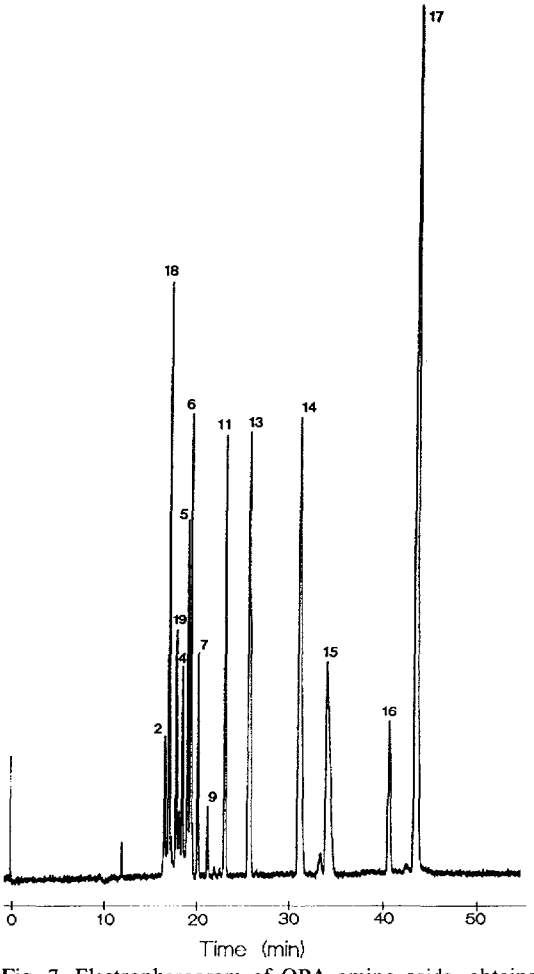

Fig. 7. Electropherogram of OPA amino acids, obtained from the total hydrolysis of cytochrome *c*. Conditions as for Fig. 5, except for the injection time (40 s). The numbers correspond to those in Table II, except that 18 is aspartic acid and 19 is tyrosine.

effect on the relative retention times of the four amino acids. However, it was found that THF is effective in improving the resolution of certain pairs of overlapping amino acids without altering the resolution of the remaining components of the mixture. In our study, an optimum quaternary solution system was found to consist of 0.05 *M* borate buffer, 15% methanol, 2% THF, and 0.05 *M* SDS. The effects of organic modifiers on CZE selectivity can be attributed to their influence on the capillary wall properties, which are related to the zeta potential, enhanced interaction between the solvent and solutes or the micelles and solutes, or a combination thereof.

The application of CZE to the amino acid analysis of a protein hydrolysate is shown in Fig. 7. Cytochrome *c* consists of fourteen different primary amino acids, which are all easily resolved in the quaternary solvent system previously discussed.

CONCLUSION

We have shown that CZE with both normal and micellar solutions and organic modifiers is an efficient and sensitive separation method for OPA-derivatized amino acids. The short analysis times are especially advantageous when dealing with such moderately stable analytes. The high resolution obtainable with CZE indicates a promising application to amino acid analysis in complex biological fluids. Additionally, the selectivity of the separation method can be readily modified with the use of micellar surfactants and organic modifiers. According to a recent report[19], the speed of analysis can be considerably improved by using shorter capillaries, 10 μm in I.D., and thus further improvements are likely in the future.

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