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Capillary electrophoretic determination of amino acids with indirect absorbance detection

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Abstract

Methods for the capillary electrophoretic (CE) analysis of a mixture of twenty common amino acids with indirect absorbance detection were developed. The suitability of nine background electrolytes (BGEs) was investigated. The effects on the CE separation of the analytes of the BGE, pH, and various additives were evaluated. Metal cations and cationic surfactants were used as buffer additives either to decrease or to reverse the electroosmotic flow in order to improve the resolution. *p*-Aminosalicylic acid and 4-(N,N-dimethyl)aminobenzoic acid are best suited as the carrier buffers and background absorbance providers as they have effective mobilities closer to the mobilities of most amino acids at alkaline pH. The CE separation of 17–19 amino acid peaks could be achieved in 20–40 min. The performance of CE in various BGEs and the influence of pH, divalent metal ions and cationic surfactants are discussed.

1. Introduction

The detection method in the capillary electrophoretic (CE) determination of amino acids is of considerable interest as it dictates whether the determination of amino acids can be routinely performed in most commercial instruments. The most widely used detection method for amino acids developed in the past is via pre- or post-column derivatization of the analytes with fluorescent probes [1–3] and detection by measuring fluorescence or laser-induced fluorescence. The fluorescence method has the advantage of high detection sensitivity, reaching attomolar mass detection limits. However, the derivatization process can be very time consuming and requires considerable additional work. The process also changes the native electrophoretic mobility of analytes. Alteration of the electrophoretic mobility of the analyte is dependent on the nature and reactive group of chemical modifying agents.

The detection of underivatized amino acids can also be accomplished by indirect methods in CE similar to those employed in liquid chromatography. In capillary zone electrophoresis (CZE), indirect fluorescence detection has been developed for a variety of analytes, including amino acids [4,5]. For the best detection limits, often a laser light source is required. Amperometric and refractive index gradient detection methods for the CE of amino acids have also been demonstrated [6,7]. Another very attractive alternative is by indirect absorbance detection, as most commercial CE systems are equipped with a UV–Vis absorbance detector and a wide variety of background absorbers are available.

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Indirect UV detection in CZE has become popular recently, and has been applied to the detection of a wide variety of analytes, *e.g.*, organic acids and bases, inorganic anions and metal cations. However, only very few studies have been made on the CE of amino acids based on indirect UV detection. Foret *et al.* [8] reported the use of benzoic acid or sorbic acid as background absorbing co-ion [or background electrolyte (BGE)] for separating organic anions including some dicarboxylic amino acids. Bruin *et al.* [9] discussed theoretical and experimental considerations for indirect detection methods in general and reported the indirect CE determination with UV detection of seven amino acids using salicylate as the BGE at pH 11.0. Ma *et al.* [10] reported the use of indirect UV detection in the CE separation of polyamines and some basic amino acids with quinine sulphate as the BGE. Quinine sulphate could also be used for indirect fluorescence detection as its quantum yield is high.

In this work, we investigated the potential use and suitability of nine BGEs for the CE determination of amino acids with indirect UV detection. The BGEs studied were sorbic, salicylic, benzoic, nicotinic, phthalic, *p*-aminosalicylic (PAS), *p*-aminobenzoic (PAB), 4-(N,N′-dimethylamino)benzoic (DMAB) and 7-amino-4-hydroxy-2-naphthalenesulphonic (AHNS) acids. The type and concentration of the BGE and pH all play a role as they influence the separation behaviour of amino acids in CE. Metal cations (Mg^{2+}, Zn^{2+} and Cu^{2+}) and long-chain cationic surfactants were employed as buffer additives either to decrease or to reverse the electroosmotic flow (EOF) in order to improve the resolution. The roles that BGEs, pH and buffer additives play in the CE separation are discussed.

2. Experimental

2.1. Chemicals

BGEs and twenty common amino acids were obtained from Sigma. All other chemicals were of analytical-reagent grade from several suppliers. Doubly deionized water prepared with a Milli-Q system (Millipore, Bedford, MA, USA) or doubly deionized, distilled water was used exclusively for all solutions. Metal cation additives were chloride salts.

Buffers and pH adjustment

Sorbic, nicotinic, benzoic, phthalic, DMAB and AHNS acids and sodium salts of salicylic, PAS and PAB acids were prepared as 0.01 *M* stock standard solutions. These were diluted to 5 or 10 m*M* of BGE and the pH was adjusted as required, by adding aliquots of 1 *M* NaOH, from 10 to 11.2, depending on the experiments, as specified in the figures. As the buffer had a fairly high pH, if exposed to air the pH could be lowered by dissolution of CO_2, hence the vial must be capped tightly immediately after use. The original pH could be maintained for 3–4 days. The pH of the buffer was checked periodically and readjusted if necessary. For the surfactant experiments, dodecyltrimethylammonium bromide (DTAB), tetradecyltrimethylammonium bromide (TTAB) and cetyltrimethylammonium bromide (CTAB) were employed. Surfactant solutions of 10 m*M* were prepared, containing 10 m*M* BGE, and then diluted to the desired concentration.

2.2. Apparatus

CE experiments were carried out in a fully automated Spectra Phoresis Model 1000 instrument (Spectra-Physics, San Jose, CA, USA) as described previously [11]. In most experiments except in the determination of the electrophoretic mobility of BGEs, the detector wavelength was fixed at the optimum value depending on the BGE used (see Table 1). In indirect detection, peaks in the electropherogram appeared originally as negative peaks but were inverted to positive peaks by using the vendor's software. The separation capillaries (bare fused silica) from Polymicro Technologies (Phoenix, AZ, USA) were 75 μm I.D. (365 μm O.D.) × 70 cm (63 cm to the detector) for the determination of the mobilities of BGEs and 75 μm I.D. (365 μm

O.D.) × 90 cm (83 cm to the detector) for the separation of mixtures of amino acids. UV–Vis absorption of the BGEs were measured with a Model U-2000 double-beam scanning spectrophotometer (Hitachi, Tokyo, Japan).

2.3. Electrophoretic procedures

Prior to first use, a new capillary was subjected to a standard wash cycle, and subsequent runs were carried out according to the established procedure [11]. Stock 10 m*M* solutions of amino acids were prepared in deionized water. Equal aliquots of each were mixed to obtain a mixture of twenty amino acids, each with a final concentration of 5×10^{-4} *M*. Sample injection was effected in the hydrodynamic (HD) mode for 1 s. The separation run was carried out at +20 kV constant voltage at 25°C constant temperature and with a current of 7–10 μA. All buffer solutions were filtered through 0.20-μm membranes and degassed under vacuum for 10 min. Between runs, the capillary was post-washed with deionized water for 5 min. As a daily routine, the capillary was prewashed in the following sequence: (a) 0.1 *M* NaOH, (b) deionized water, 10 min each at 60°C, (c) deionized water, 5 min at 25°C and (d) running buffer, 10 min at 25°C.

Peak identification for each analyte was carried out by spiking with known standards and the peaks with increased height were identified.

2.4. Electrophoretic mobility determination

Benzyl alcohol was added to the samples as a neutral marker for the electrophoretic mobility determination. The mobilities of various BGEs under the specified CE conditions were determined in the buffer containing 10 m*M* sodium borate and 10 m*M* sodium phosphate at pH 11.0. A mixture of all BGEs, 0.1 m*M* each in deionized water at pH 11.0, was injected in the HD mode for 1 s. The CE voltage applied was +15 kV. Detection was effected by rapid scanning of the absorbance from 200 to 350 nm, which allowed a positive identification of the background provider. The electroosmotic mobility (μ_{eo}, in $cm^2\ V^{-1}\ s^{-1}$) is calculated by the following equation:

$$\mu_{eo} = l_d l_t / (t_m V)$$

$$\mu_e = \mu_{obs} - \mu_{eo}$$

where l_d and l_t are the length of the capillary to the detector and the total length of the capillary, respectively, V is the running voltage and t_m the migration time of the neutral marker (benzyl alcohol). The electrophoretic mobility of the BGE, μ_e, is obtained by subtracting μ_{eo} from the observed mobility, μ_{obs}.

3. Results and discussion

3.1. General considerations for selecting BGE

In selecting a BGE suitable for CE, the mobility of the BGE and the molar absorptivity of the BGE in the wavelength region where the BGE and amino acids both absorb were taken into consideration. A BGE with a mobility matching those of the majority of the analytes would give a better separation and resolution. The absorbance of the BGE should be high and, ideally, should not overlap with those of the analytes. To avoid the absorbance from the aromatic amino acid residues, the wavelength region 265–285 nm should be avoided. In Fig. 1, a three-dimensional spectral scan of the CE of six BGEs is presented to show the absorption spectral characteristics of various BGEs. The optimum detection wavelengths are summarized in Table 1.

In order to maximize the negative charges carried by the analytes, the pH was set between 10 and 11.2. In this pH range, the amine group is unprotonated and all BGEs carry negative charges. The electrophoretic mobility (in $cm^2\ V^{-1}\ s^{-1} \times 10^3$) of amino acids (not counting the basic amino acids, Arg and Lys) varies from −0.100 (proline) to −0.440 (aspartic acid). The mobilities of the BGEs are in the range −0.249 to −0.329 (Table 1). Hence no single BGE could accommodate all amino acids with good res-

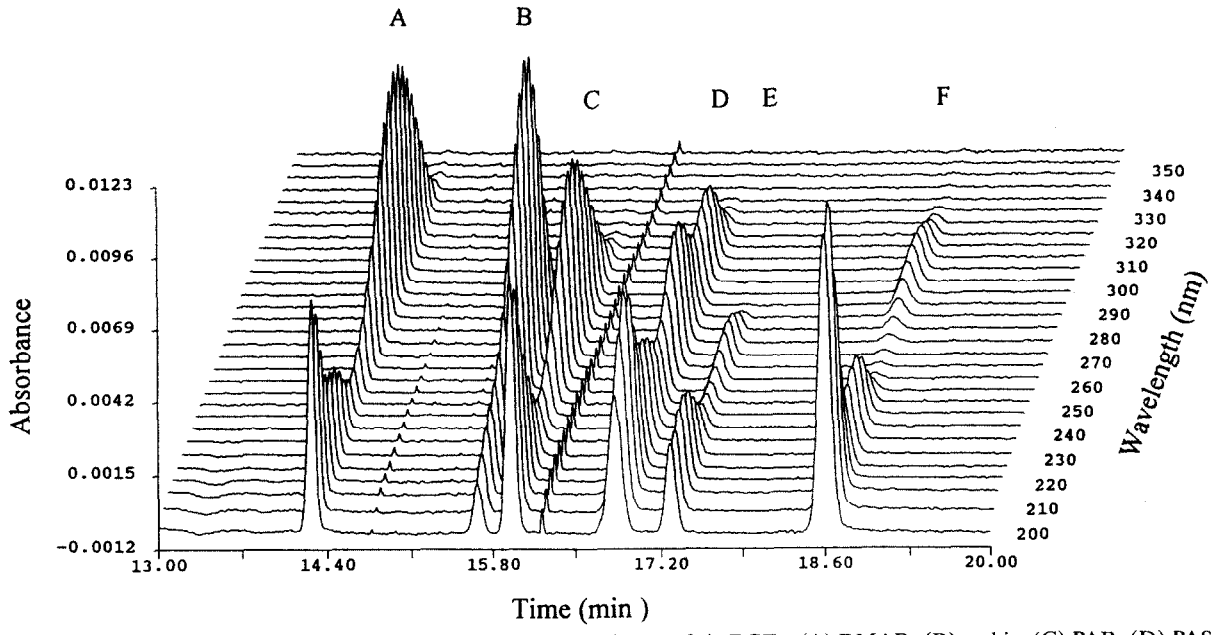

Fig. 1. Three-dimensional spectral view of CE separation of a mixture of six BGEs: (A) DMAB, (B) sorbic, (C) PAB, (D) PAS, (E) nicotinic and (F) salicylic acids. Conditions same as in the mobility determination (see Experimental for details).

olution. Our search with nine BGEs resulted in finding two BGEs that serve better than the others. Under optimum conditions, nineteen peaks could be resolved with at least seventeen peaks completely separated at the baseline level. Among the twenty amino acids, leucine (Leu) and isoleucine (Ile) have the closest structural and charge similarity, and hence are the most difficult to separate.

3.2. Separation of the amino acids in DMAB and PAS

Among the BGEs that were investigated, DMAB and PAS gave the best overall results. Fig. 2a shows the electropherogram of twenty amino acids in 10 m*M* DMAB at pH 11.0. Seventeen amino acid peaks are identified. The Arg peak merged with the system peak (the

Table 1
pK_a values, electrophoretic mobilities, electroosmotic velocities and detection wavelengths for various BGEs

BGE acid	pK_a	Mobility[a] ($cm^2\ kV^{-1}\ s^{-1}$)	μ_{eo}[b] ($cm^2\ kV^{-1}\ s^{-1}$)	Detection wavelength (nm)
Salicylic	2.94	−0.329	0.796	230
Nicotinic	4.82	−0.309	0.770	263
PAS	3.25	−0.301	0.766	266
Benzoic	4.19	−0.290		222
PAB	4.94	−0.285	0.756	266
Sorbic	4.77	−0.281	0.737	254
DMAB	6.03	−0.249	0.714	288

[a] See Experimental for details.
[b] Mean value ($n = 5$); relative standard deviation <0.2%.

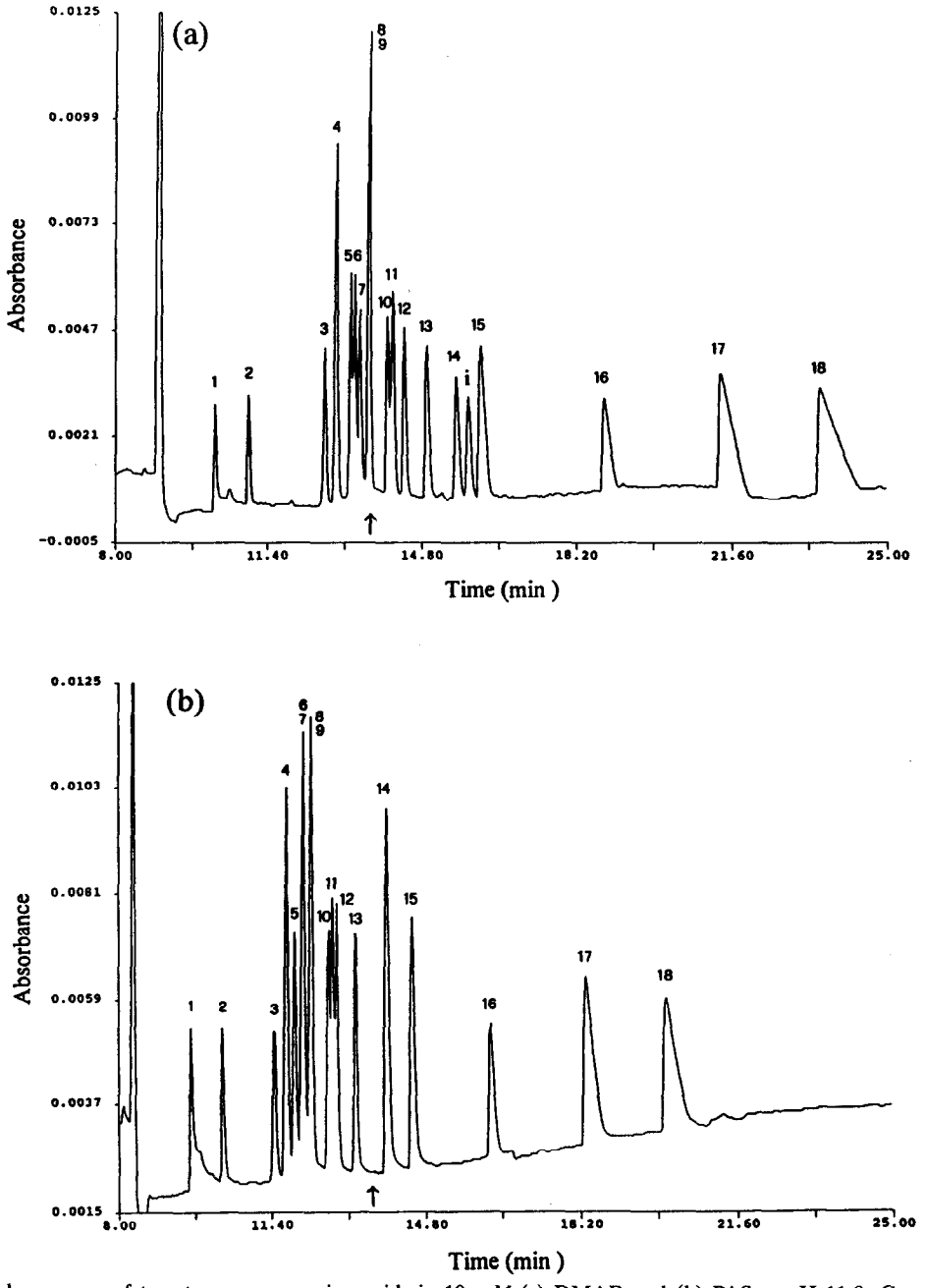

Fig. 2. Electropherograms of twenty common amino acids in 10 m*M* (a) DMAB and (b) PAS at pH 11.0. Concentrations of amino acids, 0.5 m*M* each. Migration order (peaks): 1 = Lys; 2 = Pro; 3 = Trp; 4 = Leu, Ile; 5 = Phe; 6 = Val; 7 = His; 8 = Met; 9 = Gln; 10 = Ala; 11 = Thr; 12 = Asn; 13 = Ser; 14 = Gly; 15 = Tyr; 16 = Cys; 17 = Glu; 18 = Asp. Peak i is the internal marker (benzoic acid). Arg is merged with the system peak (the first peak). The upward arrows indicate the migration positions of the BGEs.

leading peak). The migration order for the twenty amino acids is as follows: (0) Arg, system peak, (1) Lys, (2) Pro, (3) Trp, (4) Leu, Ile (not resolved), (5) Phe, (6) Val, (7) His, (8) Met, (9) Gln, (10) Ala, (11) Thr, (12) Asn, (13) Ser, (14) Gly, (i) internal marker, benzoic acid, (15) Tyr, (16) Cys, (17) Glu and (18) Asp. Peaks are labelled in this way throughout the text and figures.

In DMAB, Met and Gln (peaks 8 and 9) could not be separated; peaks 5–7 and peaks 10 and 11 could not be baseline resolved. Leu and Ile could not be separated at all in any of the BGEs. The electropherogram of the mixtures in PAS is presented in Fig. 2b. The migration pattern is similar to that in DMAB, but the profiles are different in the region where the peaks (peaks 5–12 from 12 to 14 min) are most congested and difficult to resolve. In PAS, only sixteen peaks are resolved. Phe (peak 5) could be separated but Val and His are merged (peaks 6 and 7). Met and Gln (peaks 8 and 9) still could not be separated. The separation among Ala, Thr and Asn (peaks 10–12) is worse.

3.3. Effects of Mg^{2+}, Zn^{2+} and Cu^{2+} as buffer additives

In order to improve the resolution of amino acids in the most congested region (time span between 12 and 14 min.), means to decrease the EOF was sought. Decreasing the EOF would allow the analytes more time to be resolved. Previous studies [12,13] have shown that the addition of metal ions leads to better separations and improves the number of theoretical plates in the micellar CE of oligonucleotides and in the separation of sulphonate and sulphate surfactants. Addition of metal cations could neutralize the negative charges on the bare silica capillary wall, thus decreasing the EOF. However, these cations also form complexes with the BGEs (formation constant $Cu^{2+} > Zn^{2+} > Mg^{2+}$). Because the formation constant of Mg^{2+} is lower, the extent of complex formation with BGE is less. Mg^{2+} is most effective in decreasing the EOF, as shown in Fig. 3. The extent of the decrease in EOF increases as the concentration

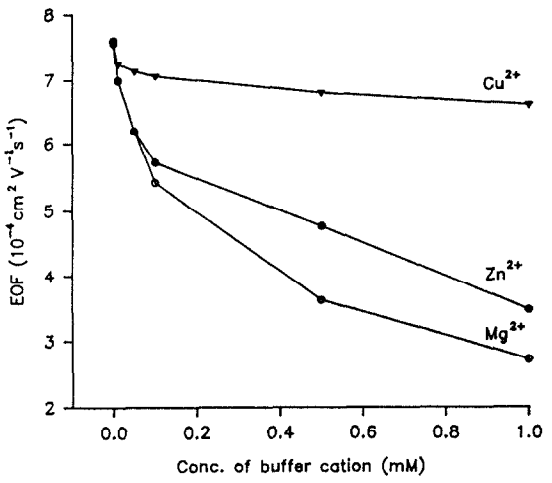

Fig. 3. Effect of divalent metal cations on EOF in 10 m*M* PAS at pH 11.0.

of cation is raised. However, concentrations of cation higher than 0.1 m*M* result in a significant increase in the overall separation time. The optimum Mg^{2+} concentration is about 0.05 m*M*. When the Zn^{2+} concentration is >0.1 m*M*, precipitates will form. Note that complex formation of metals with BGEs also results in alterations of the charge and mobility of the BGE. Of the three metal ions studied, Cu^{2+} is the least suitable.

The electropherogram in 10 m*M* PAS in the presence of 0.05 m*M* Mg^{2+} is presented in Fig. 4. In comparison with Fig. 2b, the addition of Mg^{2+} improves the separation of the peaks in the congested region, particularly for Ala, Thr and Asn (peaks 10–12). However, Val and Phe (peaks 5 and 6) are merged as one peak, and so are Met and Gln (peaks 8 and 9). Note also that the overall separation time is about 8 min longer for the mid-region, and is double for the last three peaks owing to the decrease in EOF.

3.4. Influence of pH

A major factor affecting electrophoretic separations in almost every kind of CE is pH. Its effects are manifested in several ways: (1) in ionizing analytes and BGEs and affecting their mobilities, (2) in altering charges on the capillary

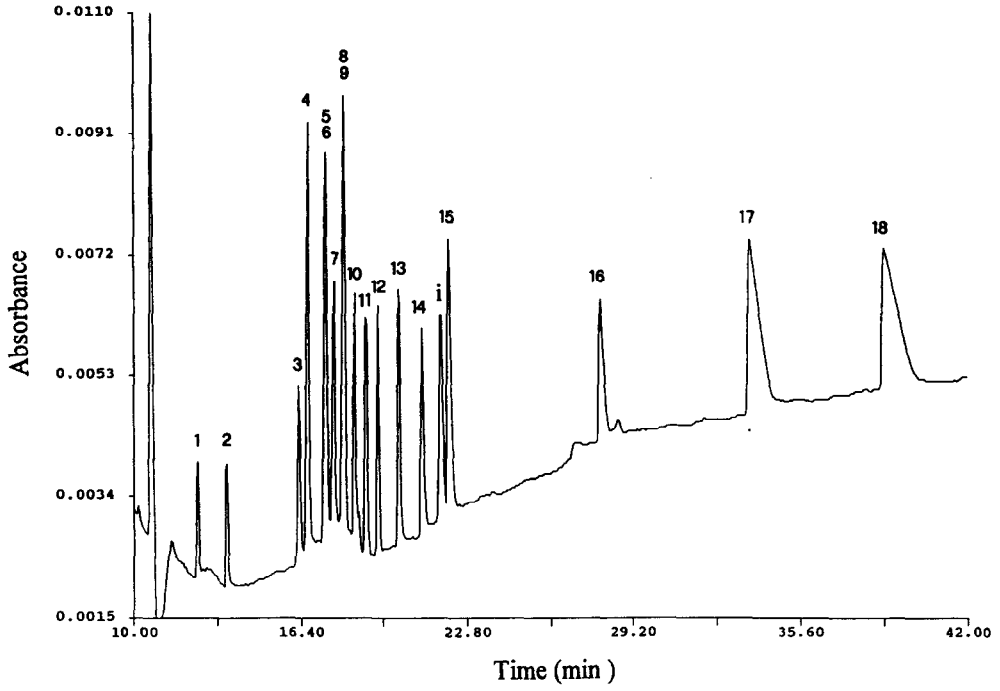

Fig. 4. Electropherogram of twenty common amino acids in 10 m*M* PAS–0.05 m*M* Mg^{2+} at pH 11.1.

surface, thus affecting the EOF, and (3) in changing the extent of complexation between the BGE and metal ion additive. Fig. 5 is a plot of pH *vs.* the electrophoretic mobilities for ten selected amino acids in 10 m*M* PAS–0.05 m*M* Mg^{2+} in the pH range 10.0–11.2. In this range, the change in mobility is associated with the pK_2 or pK_3 of the analytes. In general, the α-amino groups of aliphatic amino acids have larger p*K*s and they are more sensitive to pH. They exhibit a sharper slope in the plot. They are also the analytes in the most congested region, and therefore are the most difficult to separate. At pH 10.3, nineteen peaks could be identified, but the peaks of Met and Gln (peaks 8 and 9) and of Ser and Tyr (peaks 13 and 15) are only partially resolved (Fig. 6). Note that the migration order for peaks 3–10 changes significantly. The last three peaks are also delayed by about 30 min owing to the decrease in EOF.

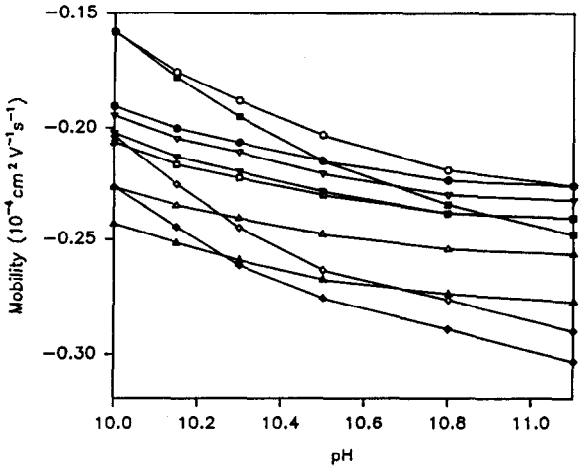

Fig. 5. Effect of pH on the electrophoretic mobility of selected amino acids in 10 m*M* PAS–0.05 m*M* Mg^{2+}. ○ = Val; ▽ = His; □ = Gln; △ = Thr; ◇ = Gly; ● = Phe; ▼ = Met; ■ = Ala; ▲ = Ser; ◆ = Tyr.

3.5. *Influence of cationic surfactants on CE*

To improve the resolution further, the influence of some cationic surfactants on CE was investigated. Surfactants affect the CE separation mainly by dynamically controlling the EOF [14]. There have been several reports on im-

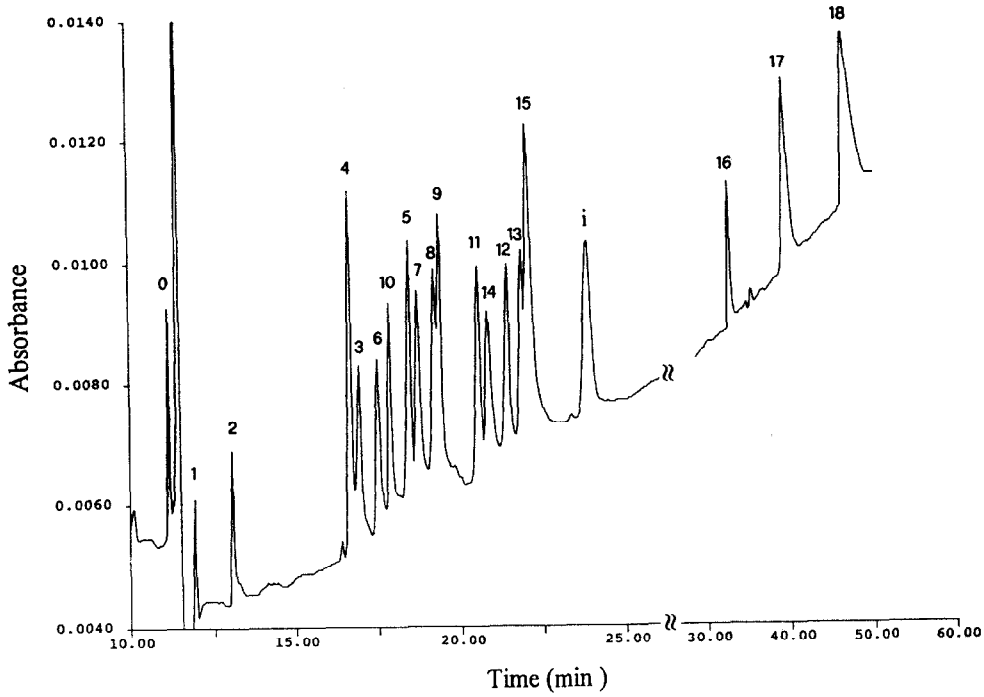

Fig. 6. Electropherogram of twenty common amino acids in 10 m*M* PAS–0.05 m*M* Mg^{2+} at pH 10.3. The leading peak (0) is Arg, separable from the system peak.

provements of resolution in CE separations of catecholamines [15], inorganic anions [16], urea herbicides, alkylbenzenes and phenylalkyl alcohols [17] by the addition of cationic surfactants. The effects of three cationic surfactants, DTAB, TTAB and CTAB, are illustrated in Fig. 7. Surfactants with longer straight alkyl chains, e.g. CTAB and TTAB, are more effective in reversing the EOF. Complete reversal of the EOF is achieved with 0.05 m*M* CTAB or 0.1 m*M* TTAB. On the other hand, 1 m*M* DTAB would be required to obtain a similar decrease in EOF. At these concentrations, the surfactants are well below their critical micelle concentrations (CMC). Above the CMC, the EOF is less affected by the concentration of the surfactant. The electropherogram of a mixture in 10 m*M* PAS in the presence of 0.25 m*M* DTAB at pH 10.9 is displayed in Fig. 8. Seventeen analyte peaks could be detected. However, Phe and Val (peaks 5 and 6), and Met and Gln (peaks 8 and 9) could not be separated. In DTAB, the baseline is flatter and the overall separation time span is about 3 min shorter. In 0.05 m*M* CTAB, the EOF is completely reversed. The CE separation of amino acids could be performed with

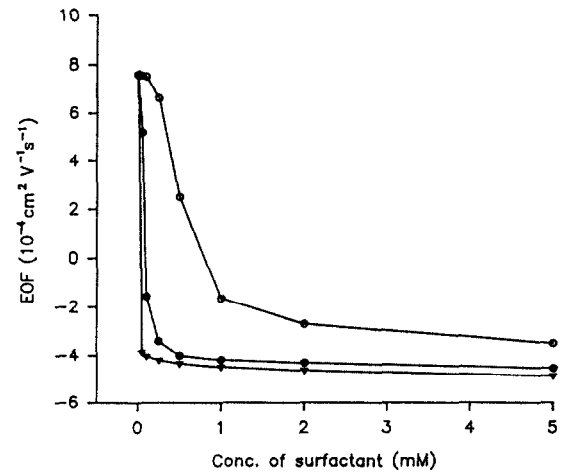

Fig. 7. Effect of cationic surfactants on EOF in 10 m*M* PAS at pH 11.0. ○ = DTAB; ● = TTAB; ▽ = CTAB.

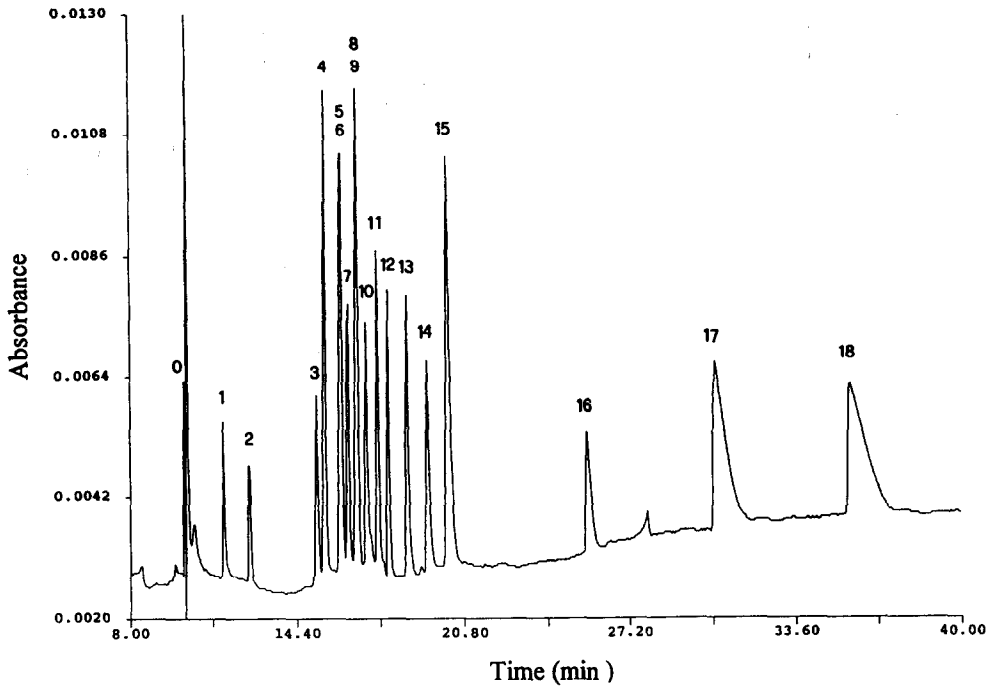

Fig. 8. Electropherogram of twenty common amino acids in 10 m*M* PAS–0.25 m*M* DTAB at pH 10.9. The leading peak (0) is Arg.

reversed polarity (applying a negative voltage); the analytes would then run toward anode. The migration order of all analytes is reversed completely. The negatively charged Asp and Glu (peaks 18 and 17) come out first (Fig. 9). Two advantages could be realized by running with reversed polarity: (1) the separation time span is significantly shorter and (2) acidic analytes exhibit sharper and more symmetric peaks. However, the separation for peaks 5–9 is only partial.

3.6. *Separation of amino acids in other BGEs*

The use of salicylic acid as the BGE has been reported [9], but the mobility is fairly high (*i.e.*, more negative in value) and its absorptivity at 230 nm is only moderate. When CE is performed in salicylate buffer (electropherogram not shown), only the peaks of the slowly migrating Cys, Glu and Asp are improved by shortening the migration time and narrowing the peak width. Similar results were obtained with nicotinic acid. Hence they are not suitable as BGEs for the complete separation of all amino acids with indirect detection. Phthalic acid is not a good BGE if used alone, but could be mixed with a less mobile (towards the anode) BGE to improve the peak width for the acidic residues. AHNS is a larger molecule and may interact strongly with the analytes and the capillary wall, resulting in peak broadening and baseline drift. Another BGE that has been studied is PAB. Similarly to PAS, CE performed in PAB gives a good separation of at least seventeen peaks (electropherogram similar to Fig. 2b; data not shown). However, PAB is not stable in alkaline solution; a faint brown colour appears in the buffer solution after applying a high voltage. Although sorbic acid has a high molar absorptivity and the CE resolution appears to be as good as with PAS, the sensitivity appears to be only half that in PAS. Therefore, judged from

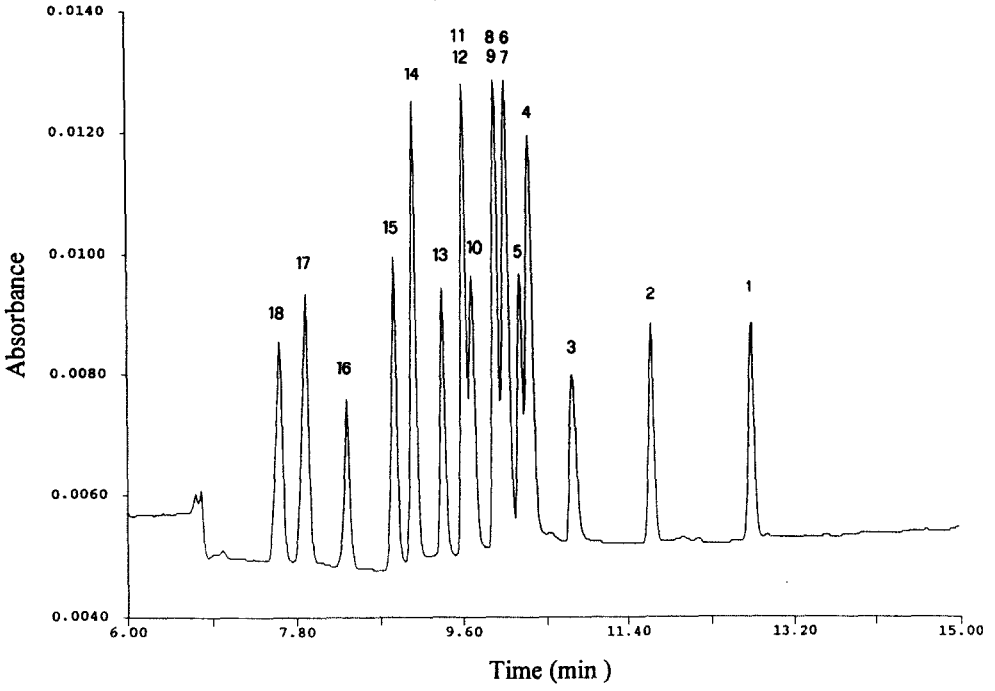

Fig. 9. Electropherogram of twenty common amino acids in 10 m*M* PAS–0.05 m*M* CTAB at pH 11.0 running with reversed polarity (towards the anode).

the overall performance, PAS and DMAB appear to be the best.

4. Conclusions

The effective CE separation and detection of 17–19 peaks for twenty acids could be achieved using commercial CE instruments with indirect UV absorbance methods. Of the nine BGEs studied, PAS and DMAB (10 m*M*) appear to be best suited for the analysis, running at pH 10.3–11.2. Resolution is also improved by the addition of Mg^{2+} (0.05 m*M*), DTAB (0.25 m*M*) or CTAB (0.05 m*M*) as buffer additives.

Acknowledgement

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