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Selectivity control in the non-aqueous capillary electrophoretic separation of amino acids

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Abstract

The separation of dansylated amino acids and underivatized amino acids in non-aqueous electrolytes was evaluated with direct and indirect UV detection. Different migration orders were achieved for dansylated amino acids in methanol compared to aqueous electrolyte systems. A reversed migration order was observed for some dansylated amino acids. Separation selectivity was different under acidic and basic conditions and was also a function of the solvation properties of the solvent. Underivatized amino acids were separated in basic and acidic electrolytes in methanol; different separation selectivities and, for some amino acids, a reversed migration order were also observed in these electrolyte systems. Analytical merits of the separation of both derivatized and underivatized amino acids were briefly evaluated; detection limits for dansylated amino acids were in the range of $2 \cdot 10^{-7}$–$4 \cdot 10^{-7}$ mol/l and, for underivatized amino acids, were $2 \cdot 10^{-6}$–$4 \cdot 10^{-5}$ mol/l. © 1997 Elsevier Science B.V.

Keywords: Selectivity; Buffer composition; Amino acids

1. Introduction

Amino acids are important in a wide range of samples, such as biological tissues and fluids, foods and industrial products, and their determination is often difficult. These compounds have been traditionally separated by liquid chromatography (LC) [1] and it took several years of intensive research to optimize the complete LC separation of all amino acids [2]. Since the introduction of capillary electrophoresis (CE) [3], more than 100 studies have been reported on the CE separation of amino acids, because of its high efficiency, low reagent consumption and fast analysis times. Most of these studies have been focused on detection, some on chiral separation and a few on separation selectivity. Amino acids have been separated in different pH and electrolyte systems by CE techniques such as capillary zone electrophoresis, capillary gel electrophoresis and micellar electrokinetic capillary electrophoresis [4–31]. In spite of these extensive studies on the separation of amino acids, significant changes in separation selectivity were not observed. Consequently, alternative techniques for selectivity control in CE could be useful for specific analysis problems.

A new approach for improvement in the CE separation selectivity of amino acids might be the use of non-aqueous electrolytes, since it is expected that amino acids will behave differently in terms of solvation and ion-interaction. The usefulness of non-aqueous solvents for CE separations has been shown in several recent publications that deal with the

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separation of different classes of compounds [32–52], where unique selectivities were often reported. These changes in separation selectivities (compared to water) are most likely to be due to the different physiochemical properties of organic solvents, such as dielectric constant (ε), donor number and solvating ability. The solvation of ions can also influence ion-interactions; the term ''ion-interaction'' is used in this paper to represent any ion association, ion-pairing or ion-cluster formation that may occur in non-aqueous systems [44]. For amino acids, similar changes in solvation and ion interaction may also produce different separation selectivities. In addition, it has been shown recently that the use of a non-aqueous solvent, such as acetonitrile, can improve fluorescence detection sensitivity by more than twenty times compared to that in aqueous systems [42]; this feature could be an advantage for the separation of fluorescent derivatives of amino acids. Other potential advantages of non-aqueous solvents include a wider range of acid–base properties, reduced sorption of hydrophobic substances onto capillary walls, enhanced solubility of insoluble compounds in water and reduced Joule heating. Furthermore, very recently, non-aqueous systems have been investigated for the chiral separation of amino acids using cyclodextrins [45], but this aspect is not examined in this report.

Amino acids are normally separated in CE either as free or derivatized amino acids. Derivatization of amino acids has been used in most of the CE separations reported, primarily to enhance detection by laser florescence [4–14] or UV absorbance [15–17]. Most derivatization methods used in CE were originally developed for liquid chromatography to enhance detection sensitivity, but in CE, their importance can also be associated with improved separation possibilities. In general, these tagging agents are bound to the nitrogen of an amino group and, consequently, they influence the pK_a values of amino acids. The most common derivatized amino acids used in CE include dansylated amino acids (Dns-amino acids) [23], phenylthiohydantoin amino acids [13], 2,3-naphthalene dialdehyde amino acids [53], 4-(dimethylamino)azobenzene-4′-sulfonyl chloride amino acids [54], fluorescein isothiocyanate amino acids [55], *o*-phthaldialdehyde amino acids [9] and 9-fluorenylmethylchloroformate amino acids [56]. Only a few studies have been reported on the CE separation of underivatized amino acids [24–31], which is probably due to the fact that available detection techniques are not sensitive enough to determine free amino acids in many types of samples. However, since derivatization methods are time-consuming, the separation of underivatized amino acids needs to be considered as an alternative approach when the concentration of amino acids in the sample is fairly high (i.e. $>10^{-5}$ mol/l).

In this study, the potential usefulness of non-aqueous CE has been investigated for the separation of underivatized and dansylated amino acids. Dansylated amino acids were chosen as test solutes because they are commonly used and could also be detected by UV absorbance (214 nm). Underivatizied amino acids (amino acids) were also examined with both indirect and direct UV detection. Experimental factors for selectivity adjustment, such as the separation medium (methanol, acetonitrile and ethanol) and the electrolyte, were evaluated. The analytical merits of these non-aqueous systems were also studied briefly.

2. Experimental

2.1. Instrumentation

A Quanta 4000 (Waters Chromatography, Milford, MA, USA) CE unit was used for UV detection (214 nm). Capillaries were 75 μm I.D. and 370 μm O.D. (Polymicro Technologies, Phoenix, AZ, USA), with an end-to-end length of 50 cm and end-to-detection window length of 43 cm. Samples were injected hydrostatically by elevation of the sample vials to 10 cm for 5 s, unless specified otherwise. The applied voltage for both separation and electroosmotic measurements was 20 kV (either positive or negative). Detection of amino acids was done with both direct and indirect UV absorbance at 214 nm. Benzyl alcohol was used as a neutral marker for the electroosmotic flow measurements. Capillaries were washed with electrolyte for 1 min between runs, by purging under vacuum (15 mm Hg).

2.2. Chemicals

All sample solutions were prepared from deionized water that was double distilled (Corning, Mega-

Pure System, MP-6A and D2, NY, USA). Tetrabutylammonium hydroxide (TBAOH), tetraethylammonium hydroxide (TEAOH) (25%, w/v, in methanol) and tetramethylammonium hydroxide (TMAOH) were purchased from Sigma (St. Louis, MO, USA). Acetic acid, perchloric acid, hydrochloric acid, methanol, ethanol and acetonitrile were purchased from BDH (Toronto, Canada). Dimethylbenzylamine (DMBA) was purchased from Aldrich (Milwaukee, WI, USA). Stock solutions of all amino acids (Sigma, 0.01 mol/l) and dansylated amino acids (Dns-amino acids; Pierce, Rockford, IL, USA; 1000 ppm) were prepared in water, and standard solutions were diluted to the desired concentration with water. As observed in our previous studies [37], the use of aqueous samples did not influence the separation. All electrolytes used in the separation of Dns-amino acids and amino acids were prepared by the careful addition of concentrated acid to the base, which was already dissolved in the solvent. All electrolytes were filtered through a 0.2-μm nylon-66 membrane syringe filter (Cole-Parmer, Chicago, IL, USA) immediately prior to use. The Dns-amino acids used were dansylated norleucine (Dns-Nle), glycine (Dns-Gly), alanine (Dns-Ala), threonine (Dns-Thr), glutamic acid (Dns-Glu), isoleucine (Dns-Ile), leucine (Dns-Leu), asparagine (Dns-Asn), proline (Dns-Pro), hydroxyproline (Dns-Hyp) and tyrosine (Dns-Tyr). The free amino acids used in this study were diphenylalanine (Dph), tyrosine (Tyr), phenylalanine (Phe), histidine (His), tryptophan (Trp), alanine (Ala), lysine (Lys), isoleucine (Ile), threonine (Thr) and citrulline (Cit).

2.3. Procedures.

Capillaries were conditioned by washing them with methanol for 1 h, followed by another 2 h with the separation electrolyte, using a 10-cm height differential; overnight, capillaries were stored in methanol. All glassware was rinsed with a chromic/sulfuric acid solution followed by water and acetone and then dried in an oven. The number of theoretical plates was calculated from $N=5.54(t_R/w_{0.5})^2$, where t_R is the migration time of the analyte and $w_{0.5}$ is the peak width at half the peak height. Electroosmotic mobilities were calculated from, $\mu_{eo}=L1/(t_{eo}V)$, where μ_{eo} is the electroosmotic mobility, t_{eo} is the migration time for the neutral marker (in s), V is the separation voltage (in volts), L is the length of the capillary and 1 is the injection-to-detection length (in cm). The electrophoretic mobilities (μ_{ep}) of ions were calculated from, $\mu_{ep}=\mu_{eo}+L1/(t_{ep}V)$ where t_{ep} is the migration time for the analyte. Detection limits were defined as the concentration required to give peak heights of twice the peak-to-peak noise. The linearity of calibration curves was evaluated from plots of sensitivity vs. concentration [57]. The sensitivity, S, was obtained from $S=(I-b)/C$, where I and C are the response and the concentration, respectively, and b is the Y-axis intercept obtained from the analysis of least-squares regression using the original response–concentration data [57].

3. Results and discussion

3.1. Separation of dansylated amino acids

In aqueous CE, Dns-amino acids are separated at a pH of ~7 because most Dns-amino acids have a net negative charge under neutral conditions and, hence, can be separated according to their electrophoretic mobilities. Our observed anodal migration (towards the positive electrode and detection point) of Dns-amino acids, against a counter electroosmotic flow, under basic, neutral and weakly acidic conditions (TEAO–acetic acid electrolytes in methanol) indicated that Dns-amino acids were also negatively charged in these methanolic electrolytes. Since cathodal electroosmotic flow was fairly rapid ($\sim 2\cdot 10^{-4}$ s^{-1}) under basic conditions, the migration of some Dns-amino acids took a long time (~50 min), due to analyte migration counter to the electroosmotic flow. Therefore, acidic conditions were chosen for the separation of Dns-amino acids. However, acidification of the electrolyte with a strong acid, such as HCl or $HClO_4$, led to fast anodal electroosmotic flow ($\sim -2\cdot 10^{-4}$ cm^2 V^{-1} s^{-1} [44], and this caused rapid migration and poor resolution. Consequently, the separation of Dns-amino acids was studied in weakly acidic electrolytes [tetraethylammonium acetate (TEAA) plus acetic acid] in methanol, which gave a moderate cathodal electroosmotic flow ($\sim 0.5\cdot 10^{-4}$ cm^2 V^{-1} s^{-1}); detection was via direct UV absorbance at 214 nm. Methanol was chosen because it had previously provided

different selectivities in the separation of inorganic anions [37] and cations [43]. Moreover, methanol has favorable properties, such as dielectric constant, viscosity, donor number and a useful UV range for detection.

A typical electropherogram of the separation of Dns-amino acids in methanol is shown in Fig. 1. As can be seen, all tested Dns-amino acids are baseline resolved in less than 23 min. The separation pattern observed in this non-aqueous solvent is unique compared to a number of published aqueous separations of Dns-amino acids [4–8,15–17]. For instance, Dns-Nle is the fastest Dns-amino acid in this non-aqueous system, but it migrates after a number of Dns-amino acids in aqueous systems [5]. To further compare the selectivity in methanol with that in aqueous systems, the effect of the addition of water to the separation was examined, and the results

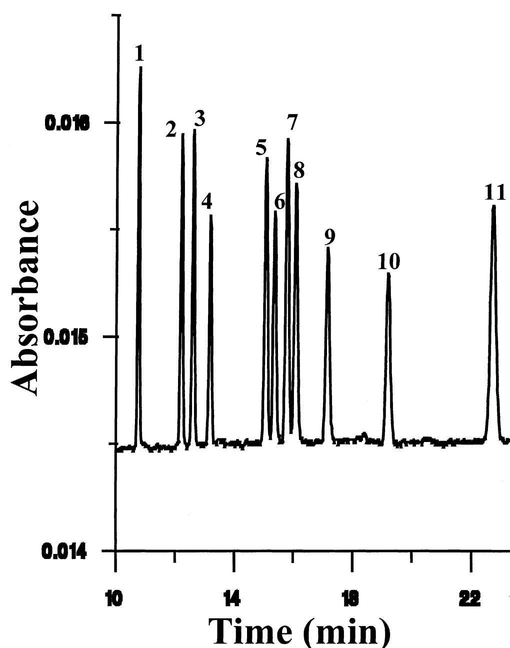

Fig. 1. Separation of dansylated amino acids in methanol. Experimental conditions: electrolyte, 0.015 mol/l TEAA–0.015 mol/l acetic acid in methanol; 50 cm capillary (75 μm I.D.) with an injection end-to-detection point length of 43 cm; separation voltage, −20 kV; sample concentration, $2\cdot10^{-5}$ mol/l; direct detection at 214 nm; Peak identification: (1) Dns-Nle, (2) Dns-Gly, (3) Dns-Ala, (4) Dns-Thr, (5) Dns-Glu, (6) Dns-Ile, (7) Dns-Leu, (8) Dns-Asn, (9) Dns-Pro, (10) Dns-Hyp and (11) Dns-Tyr.

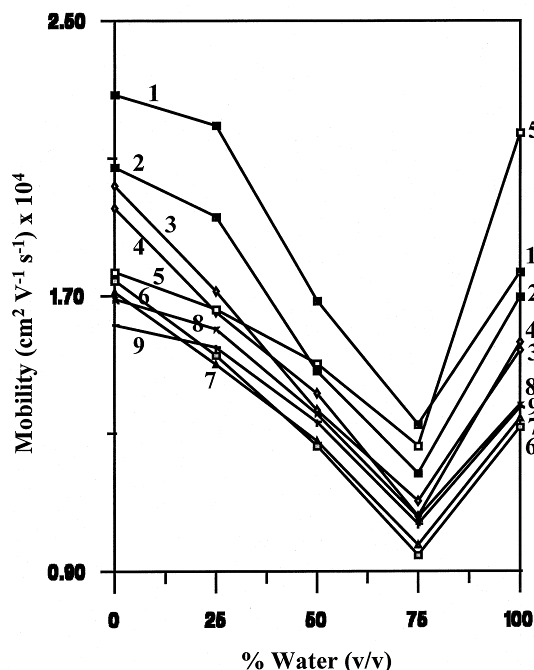

Fig. 2. Effect of the addition of water to a methanol electrolyte on the separation of dansylated amino acids; other conditions are the same as for Fig. 1.

are shown in Fig. 2. The overall trend shown in the mobility changes of Dns-amino acids upon the addition of water is expected because of the changes in ε/η values for these solvent systems [37]. However, the addition of water also altered the migration order of some Dns-amino acids. For instance, Dns-Glu is the fastest Dns-amino acid in the water system, while it is the fifth analyte among the Dns-amino acids tested. The faster mobility of Dns-Glu is most likely due to dissociation of the second acidic functional group. In addition, the migration order of Dns-Ile and Dns-Leu is reversed in water; they are also the slowest Dns-amino acids in water of the Dns-amino acids tested, probably because of their stronger solvation properties.

Previous results [37,43,44] showed that separation selectivity can be altered by a change in the physiochemical properties of the solvent, such as dielectric constant, donor number and hydrogen bonding. Consequently, the effect of a variation in solvent properties was examined briefly. The addition of ethanol, which has solvation properties similar to

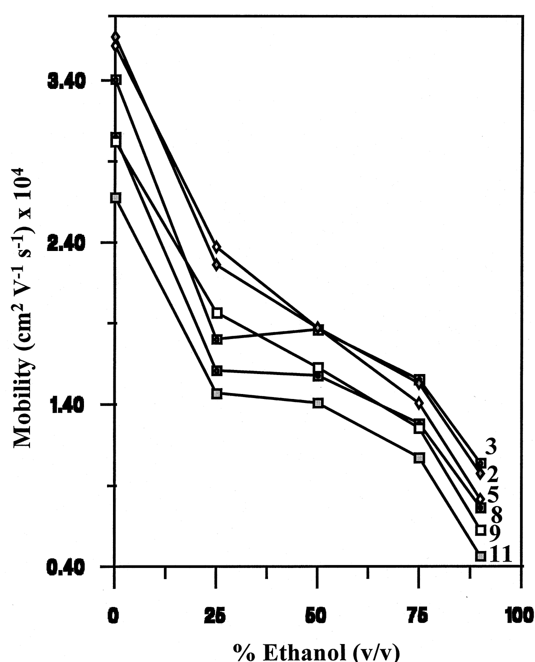

Fig. 3. Effect of the addition of ethanol to a methanol electrolyte on the separation of dansylated amino acids; other conditions are the same as for Fig. 1.

those of methanol, caused mobilities of Dns-amino acids to decrease rapidly (see Fig. 3), which is most likely due to the smaller value of ε/η for ethanol compared to methanol. In spite of the similarities between the two solvents, the separation selectivity also changed upon the addition of ethanol, as can be seen in Fig. 3. For instance, the migration order of Dns-Gly and Dns-Ala was reversed at a concentration of ethanol >50% (v/v) in the separation electrolyte, and the location of the Dns-Ala peak relative to the Dns-Gly and Dns-Glu peaks was reversed. The effect of acetonitrile, a solvent with considerably different properties, was also examined. On addition of 25% (v/v) of this aprotic solvent to the methanol electrolyte, the migration times of Dns-amino acids increased significantly. This change can be attributed to an increase in the electroosmotic flow and to possible promotion of ion interaction. However, since significant changes in the separation selectivity were not observed, the effect of acetonitrile was not studied further. Thus, changes in the composition of non-aqueous solvent systems can affect the migration order, and these studies suggest that protic solvent mixtures may be the most effective for manipulation of separation selectivity.

Since separation selectivity should vary with a change in the net charge of Dns-amino acids, the separation of Dns-amino acids was studied as a function of the amount of acetic acid added to a TEAOH electrolyte. The results in Fig. 4 show that, under basic conditions (concentration of acetic acid added <0.015 mol/l), the migration of Dns-Glu was the fastest because of the dissociation of two carboxylic acid functional groups, which gave it the highest anionic charge. In acidic conditions (concentration of acetic acid added >0.015 mol/l), however, its mobility decreased rapidly due to protonation of the carboxylic acid groups and had only the fifth fastest migration order among the Dns-amino acids tested; the migration order of Dns-Asn also changed relative to that of Dns-Pro (see Fig. 4). The effect of acetic acid was studied up to a concentration of 1.0 mol/l, but, as expected, beyond 0.03 mol/l (limit of X-axis in Fig. 4), only slight decreases in mobilities were observed; these de-

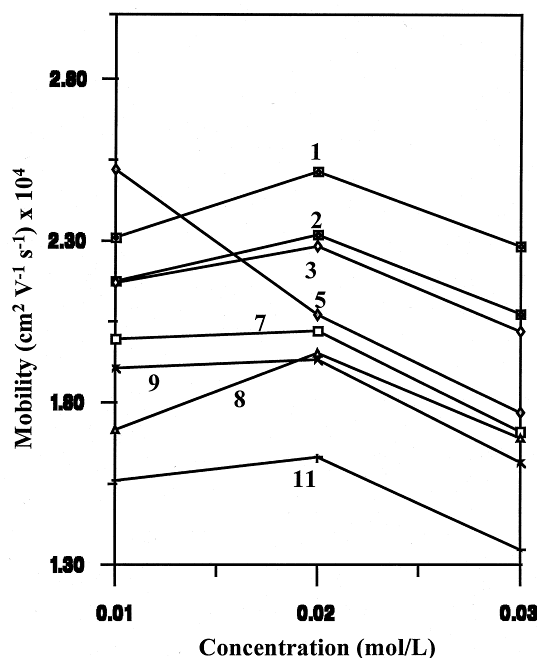

Fig. 4. Effect of the concentration of acetic acid in the electrolyte on the separation of dansylated amino acids; electrolyte, 0.015 mol/l TEAOH and acetic acid in methanol; other conditions are the same as for Fig. 1.

creases were most likely to be due to changes in the dielectric constant and viscosity of the separation medium.

3.2. Separation of free amino acids

The fact that only a few studies have been reported [24–31] on the separation of underivatized amino acids is most likely due to the lack of suitable detection techniques. In this work, the main purpose of which was to investigate separation selectivity in non-aqueous systems, two detection techniques were used; direct and indirect UV detection at 214 nm.

3.2.1. Separation and direct detection of free amino acids

The separation of five amino acids was examined in 0.01 mol/l TEAOH in methanol with direct UV detection at 214 nm. Under these conditions, amino acids were negatively charged and migrated towards the anode, while the electroosmotic flow was cathodal [44]. As shown in Fig. 5, all of the amino acids tested were baseline resolved in less than 20 min, but a number of these amino acids exhibit tailing peaks, which might be due to the ionic interaction of negatively charged amino acids with the counter-ion (TEA^+) [44]. The migration order of these amino acids is different from that of a number of published aqueous separations of amino acids [25,26]. For instance, the separation order was Trp, Phe, His and Tyr (among other amino acids) in the presence of 0.01 mol/l (*N*,*N*-dimethylamino)benzoic acid at pH 11.0 [26], which is different from that in a non-aqueous electrolyte (see Fig. 5). This change in selectivity is probably due to changes in solvation of the negatively charged amino acids in methanol. The above amino acids were also separated in 0.01 mol/l TMAOH in methanol (with a different counter-ion), but no changes in the separation selectivity or improvement in the peak shapes were observed.

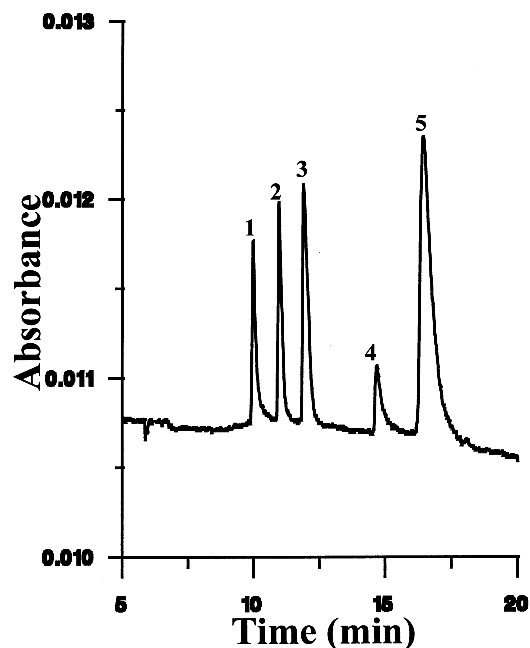

Fig. 5. Separation of underivatized amino acids in methanol. Experimental conditions: electrolyte, 0.01 mol/l TEAOH in methanol; 50 cm capillary (75 μm I.D.) with an injection end-to-detection point length of 43 cm; separation voltage, 20 kV; sample concentration, $2 \cdot 10^{-4}$ mol/l; direct detection at 214 nm; Peak identification: (1) Dph, (2) Tyr, (3) Phe, (4) His and (5) Trp.

Solvation and the ion interaction of cations in organic solvents are different from those for anions, thus, different separation selectivities are expected for the cationic amino acids that exist in acidic electrolytes. Consequently, the separation of these amino acids was examined in 0.01 mol/l tetraethylammonium perchlorate (TEAP) and 0.005 mol/l $HClO_4$ in methanol. As shown in Fig. 6, the migration order of these amino acids was almost reversed compared to that in the basic electrolyte. Since changes in either the counter-ion (Cl^- instead of ClO_4^-) or the co-ion (substitution of TEA^+ by TMA^+ and TBA^+ ions) did not alter the separation selectivity, the observed change in migration order can be attributed mainly to the differences between anion and cation solvation. The use of TEAA–acetic acid instead of TEAP–$HClO_4$ or tetraethylammonium chloride (TEAC)–HCl caused co-migration of all of these amino acids along with the neutral marker. This might be due either to strong ion-interaction formation of positively charged amino acids with acetate ions or to a lack of protonation of amino acids in the acetic acid electrolyte. It was also noticed that the detection sensitivity for some of these amino acids in the TEAP–$HClO_4$ electrolyte was increased by 2–3 fold compared to that in basic electrolytes.

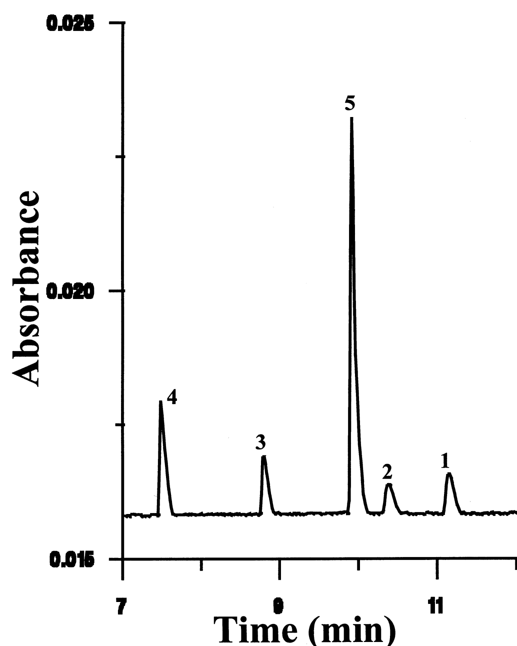

Fig. 6. Separation of underivatized amino acids in methanol. Experimental conditions: electrolyte, 0.01 mol/l TEAP and 0.005 mol/l $HClO_4$ in methanol; other conditions are the same as for Fig. 5.

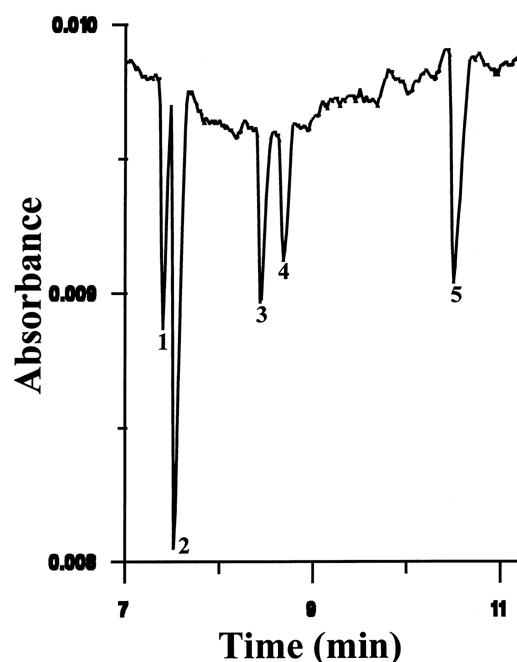

Fig. 7. Separation of underivatized amino acids in methanol. Experimental conditions: electrolyte, 0.01 mol/l DMBA–0.018 mol/l HCl in methanol; 50 cm capillary (75 μm I.D.) with an injection end-to-detection point length of 43 cm; separation voltage, 20 kV; sample concentration, $2 \cdot 10^{-4}$ mol/l; indirect detection at 214 nm; Peak identification: (1) Ala, (2) Lys, (3) Ile, (4) Thr and (5) Cit.

3.2.2. Indirect detection of free amino acids

Indirect UV detection may not be a suitable technique for the determination of low concentrations of amino acids in some samples, but for those in which the analysis times are more important than low detection limits, indirect UV detection might be useful. Consequently, the separation of amino acids was studied in 0.01 mol/l DMBA and 0.018 mol/l HCl in methanol by indirect UV absorbance at 214 nm. DMBA was chosen as an indirect UV reagent because its mobility in the protonated form matches that of most of the amino acids. Acidic conditions were used because these permit the easy manipulation of the electroosmotic flow (EOF) by changing the solvent's properties [44], and also because DMBA must be protonated for indirect detection. A typical electropherogram obtained in methanolic electrolyte with indirect UV detection is shown in Fig. 7. These results show the possible application of non-aqueous solvents for the separation of free amino acids without any derivatization. Comparisons with other published results for the separation of amino acids is not possible because most have been reported for basic conditions.

3.3. Analytical measurements

Calibration curves were determined for both Dns-amino acids and amino acids, and the analytical data are summarized in Table 1. The separation electrolyte for Dns-amino acids was 0.015 mol/l TEAA plus 0.015 mol/l acetic acid in methanol; the separation electrolytes for amino acids were 0.01 mol/l TEAP plus 0.005 mol/l $HClO_4$ in methanol for direct UV and 0.01 mol/l DMBA plus 0.018 mol/l HCl in methanol for indirect UV. Detection was at 214 nm for both direct and indirect UV absorbance. Over a 3-h period, evaporation of methanol was in the range of 1–3%, and this evaporation produced no

Table 1
Analytical data from the measurements of derivatized and free amino acids

Analytical data	Dns-amino acids	Amino acids[a] (direct UV)	Amino acids (indirect UV)
Detection limit[b] (mol/l)	$2\cdot10^{-7}$–$4\cdot10^{-7}$	$2\cdot10^{-6}$–$1\cdot10^{-5}$	$9\cdot10^{-6}$–$4\cdot10^{-5}$
Concentration range (mol/l)	$2.3\cdot10^{-7}$–$6.5\cdot10^{-5}$	$5\cdot10^{-5}$–$5\cdot10^{-4}$	$5\cdot10^{-4}$
R^2	0.998–0.99990	0.996–0.9997	0.996–0.9998
Response factor variation[c]	2–17%	7–19%	8–20%
Peak efficiency (plate count)	60 000–140 000	10 000–70 000	40 000–60 000
Migration time (% R.S.D.[d])	1–2%	1–2%	2%

Experimental conditions:
[a]Direct detection data corresponds to both basic and acidic conditions and other data correspond to acidic conditions.
[b]Detection limits at a signal-to-noise ratio of two with a 15-s hydrodynamic injection at an elevation of 10 cm.
[c]Maximum range for change in the response factor.
[d]Repeatability of migration times for three injections over a 3-h period.

noticeable effects on the separations. As shown in Table 1, the detection limits for Dns-amino acids were at least ten-times lower than those for free amino acids, and this clearly demonstrates the advantages of derivatization for detection. The linearity ranges for Dns-amino acids were larger than those for amino acids, and the response factors for Dns-amino acids were more stable. The peaks for Dns-amino acids were more symmetrical than those for free amino acids and had higher peak efficiencies. Peak shapes for amino acids in acidic conditions were better than those in basic solution; separation efficiencies were in the range of 30 000–70 000 in acidic electrolyte and 10 000–40 000 in basic electrolyte.

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