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Separation of precolumn-labelled D- and L-amino acids by micellar electrokinetic chromatography with UV and fluorescence detection

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Abstract

Micellar electrokinetic chromatography (MEKC) was examined for the separation of labelled D- and L-amino acids to permit rapid screening of protein amino acid enantiomers in microchemical analytical work. Precolumn chiral derivatization was performed using *o*-phthaldialdehyde/2,3,4,6-tetra-O-acetyl-1-thio-β-D-glucopyranose (OPA/TATG) reagent and the diastereomers formed were detected by UV or fluorescence detection. Optimization of separation buffer pH, ionic strength and surfactant concentration was carried out and was focused on the effective separation window available for the resolution of the amino acid derivatives. The effects of added organic modifiers, methanol, acetonitrile and tetrahydrofuran, on the relative retention of the derivatives were characterized for the purpose of fine tuning the separation selectivity. The resolution of the derivatives of the D- and L-forms of each protein amino acid was very high (mean value of $R_s = 14.3$, range 0.8–28), except for aspartic acid and glutamic acid, whose enantiomers could not be resolved at the alkaline pH studied. A separation of 34 D,L-amino acids in less than 5 min, is demonstrated with only a few peaks co-eluting.

1. Introduction

For the determination of amino acid enantiomers in microchemical analytical work, a microcolumn separation technique such as liquid chromatography (LC) in capillary format or capillary electrophoresis (CE) should be employed. Generally, a higher separation efficiency in a shorter analysis time can be provided by CE than by LC, which is why CE is more attractive to explore for the purpose of obtaining a method for rapid screening of complex samples, e.g., to resolve the D- and L-forms of all protein amino acids in a single run.

In CE, chiral resolution of amino acids can be accomplished basically in two ways, in the direct or the indirect way. The first approach involves the addition of a chiral selector such as cyclodextrins [1–3], crown ethers [4] or copper(II) complexes [5] to the separation buffer or a chiral surfactant such as digitonin [6], N-dodecyl-L-valinate (SDVal) [6–9], glycyrrhizic acid (GRA) [10], β-escin [10] or bile salts [11]. Alternatively, a chiral selector can be incorporated into a

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stationary film coated on the inner surface of the separation capillary [12,13]. The use of chiral selectors in CE has recently been thoroughly reviewed by Terabe et al. [14]. Since only a few amino acids exhibit a useful response with UV–Vis absorption or fluorescence detection, most of the studies cited above on chiral selectors involve the separation of amino acids labelled with achiral reagents, e.g., dansyl chloride (DNS) [1,3,5,11], phenyl isothiocyanate (PITC) [6,8–10] or naphthalene-2,3-dicarboxaldehyde (NDA) [2]. The second approach to accomplish chiral resolution, the indirect way, is to utilize chiral labelling, whereby analytes are converted into derivatives with good detection properties and, because the derivatives are diastereomers, an achiral CE separation method can be used. A prerequisite, however, is that the reagent possesses a high optical purity in order to prevent more than one derivative being produced for each chiral analyte. In addition, racemization during derivatization must be avoided. Of several reagents earlier employed for chiral derivatization of amino acids in LC, thoroughly reviewed elsewhere [15], few have so far been examined in CE. Both Marfeys' reagent, as used by Tran et al. [16] and 2,3,4,6-tetra-O-acetyl-β-D-glucopyranosyl isothiocyanate (GITC), as used by Nishi et al. [17], yield derivatives which are amenable to UV detection. Under alkaline conditions more than ten derivatized D,L-amino acids were resolved within 40 min using a sodium dodecyl sulfate (SDS) micellar separation buffer, i.e., separation by micellar electrokinetic chromatography (MEKC). *o*-Phthaldialdehyde (OPA) together with either N-acetylcysteine (NAC) or Boc-cysteine (BocC) was examined by Kang and Buck [18] as a chiral reagent for amino acids in CE. They demonstrated an MEKC separation of six derivatized D,L-amino acids within 30 min with an SDS micellar separation buffer at pH 9.5 containing 5.5% methanol and by using UV absorption detection at 340 nm. In addition, Houben et al. [19] examined OPA–NAC for the determination of the chiral purity of valine by MEKC with UV absorption detection.

In this study, micellar electrokinetic chromatography was examined for the separation of D- and L-amino acids precolumn-labelled with *o*-phthaldialdehyde/2,3,4,6-tetra-O-acetyl-1-thio-β-D-glucopyranose (OPA/TATG). TATG, which is a chiral thiol analogue of GITC, is an inexpensive, commercially readily available chemical which can be obtained in high optical purity. OPA/TATG was selected for this study partly because the derivatives formed with primary amino acids can be detected by UV absorption detection and by conventional and laser-induced fluorescence detection [20] and partly owing to our previous experience with this chiral reagent in LC [21]. In the course of optimizing the overall resolution of the amino acid derivatives in MEKC, separation parameters such as buffer pH, surfactant concentration and concentration of organic modifiers were varied. Our interest here was focused on the derivative retention window, i.e., the effective window available for the separation of labelled amino acids. It was observed that this window was affected in a slightly different way by added organic modifiers than was expected from the results of the MEKC retention window calculated in the traditional way from retention data for Sudan III and acetone. The utility of the MEKC method for the rapid screening of D- and L-amino acids in microchemical analytical work is briefly discussed.

2. Experimental

2.1. Apparatus

MEKC separations were performed using an automated CE instrument, a Model 2050 P/ACE System, from Beckman Instruments (Palo Alto, CA, USA), equipped with a UV absorption detector. Wavelength selection was made with exchangeable filters using a 254-nm filter during the general optimization of the separations and a 340-nm filter for selective detection of the OPA/TATG amino acid derivatives. Occasionally, a laboratory-built CE system was used together with a Shimadzu RF-535 LC fluorescence detector, modified for on-column detection with capillary columns. The excitation and emission wave-

lengths were 350 and 415 nm, respectively. A UG11 bandpass filter was attached to the slit on the excitation side to prevent unwanted stray light from reaching the capillary. Alignment of the capillary in the detector cell housing was made by flowing a 1 m*M* solution of salicylic acid in water–ethanol (95:5, v/v) while adjusting the capillary position to maximize the fluorescence signal.

A high-voltage power supply, Model 2462 (0–30 kV), from Bertan (Hicksville, NY, USA) was used to generate the high voltage over two platinum wire electrodes (Goodfellow Cambridge, Cambridge, UK), each positioned in separation buffer in the glass vials at the injection and detection end of the capillary, respectively. MEKC separations were carried out in both systems using untreated fused-silica capillaries of 50 μm I.D. and 192 μm O.D. from Polymicro Technologies (Phoenix, AZ, USA).

2.2. Chemicals and reagents

Acetonitrile and methanol of HPLC grade were obtained from Rathburn Chemicals (Walkerburn, UK). *o*-Phthaldialdehyde (OPA), 2,3,4,6-tetra-O-acetyl-1-thio-β-D-glucopyranose (TATG), D- and L-amino acids, sodium dodecyl sulfate (SDS), boric acid, anhydrous sodium tetraborate (borax) and sodium hydroxide were all purchased from Sigma (St. Louis, MO, USA). Deionized water was obtained from a Milli-Q system (Millipore, Bedford, MA, USA).

2.3. Procedures

In order to vary the pH of the borate buffers used in the separations, while holding the ionic strength constant, and vice versa, they were prepared according to Bates and Bower [22]. Appropriate volumes of stock solutions of 40 m*M* borax and 0.1 *M* NaOH were mixed and subsequently diluted with deionized water to yield the desired pH and ionic strength. For example, borate buffer of pH ca. 9.5 and ionic strength 40 m*M* was prepared by mixing 37.5 ml of the borate stock solution with 10.6 ml of the sodium hydroxide stock solution and diluting to 100 ml with deionized water. This gave a measured buffer pH value of 9.50–9.55 at ambient temperature, which corresponded to a variation in temperature of ca. ±3°C as calculated from the dependence of pH on temperature for the borax–NaOH buffer system [22]. Measured pH values are quoted for all experiments. The MEKC separation buffers were prepared by dissolving specified amounts of SDS in a small portion of the respective borate buffer. Subsequently, the selected organic modifier, acetonitrile or methanol, and thereafter the rest of the borate buffer were added to the solution to yield the specified concentrations. Finally, the separation buffers were mixed in an ultrasonic bath for 5 min prior to use.

New fused-silica capillaries were pretreated by flushing with 1 *M* NaOH for 10 min followed by rinsing with deionized water and finally with the separation buffer. Between each run in a consecutive series of samples, the capillary was rinsed with 0.1 *M* NaOH, deionized water and separation buffer for 1 min each. Moreover, before each analytical separation, the capillary was equilibrated with the separation buffer by electrokinetic pumping for 2 min.

Acetone and Sudan III were used as markers for the electroosmotic flow and the micellar flow, respectively. A standard solution of these markers (ca. 2% and ca. 18 μM, respectively) was prepared by adding 30 μl of a stock solution of Sudan III (ca. 900 μM) in acetone to 1.5 ml of the separation buffer. Standard solutions of D- and L-amino acids (pH ca. 7) were prepared from 2.5 m*M* stock solutions in 0.1 *M* HCl and neutralized with 0.1 *M* NaOH followed by dilution with deionized water. To make peak identification easier during the MEKC optimization, amino acids were grouped in four standards with 8–10 D- and L-amino acids in each. To control the precision during the measurements, D-methionine was used as a reference compound in each standard. The chiral OPA/TATG reagent solution was prepared by dissolving 8 mg of OPA and 44 mg of TATG in 1 ml of methanol. The borate buffer used for derivatization was prepared from 0.4 *M* boric acid and adjusted to pH 9.5 by adding ca. 1.2 ml of 10 *M* NaOH.

Derivatizations were carried out in a 1-ml glass reaction vial, from which the injection into the CE system was later made. A 75-μl volume of amino acid enantiomer standard solution (100 μM each of the selected D- and L-amino acids) was mixed with 15 μl of borate buffer and then 15 μl of the OPA/TATG reagent were added. The reaction was allowed to proceed for 6 min before the derivatized sample was injected. On the automated CE instrument, injections were made hydrodynamically. Using a separation capillary with a total length of 27 cm (19 cm to the detection window), the injection time on the instrument was set to 2 s (pressure ca. 0.5 p.s.i.). With the laboratory-built instrument, injections were made by gravity. The injection end of the capillary, when placed in the glass vial containing the sample, was elevated 6.5 cm above the level of the detection end of the capillary. An injection time of 30 s was used for a capillary with a total length of 80 cm (44 cm to the window).

3. Results and discussion

Separation of a complex mixture of the derivatives of the protein D- and L-amino acids in a single run where twice as many peaks must be resolved simultaneously, compared with achiral separations of amino acids, is not trivial. Although the exquisite separation power of CE has been elegantly demonstrated, e.g., in separations of biopolymers in gel-filled capillaries where up to 300–400 peaks have been resolved [23–25], there have been few studies on the separation of large sets of small molecules such as amino acids. However, this is not surprising when considering the limits to MEKC resolution, as can be derived from general CE theory. In MEKC the basic equation for resolution between two closely eluting solutes when similar retention factors are assumed, $k'_1 \approx k'_2$, is given by [26,27]

$$R_s = \frac{\sqrt{N}}{4} \cdot \frac{(\alpha - 1)}{\alpha} \cdot \frac{k'_2}{1 + k'_2} \cdot \frac{1 - (t_0/t_{MC})}{1 + (t_0/t_{MC})k'_1} \quad (1)$$

where t_0 is the retention time of the aqueous phase, t_{MC} the retention time of the micellar phase, N the plate number and α the selectivity factor. The retention factor, k', is given by

$$k' = \frac{(t_R - t_0)}{t_0[1 - (t_R/t_{MC})]} \quad (2)$$

where t_R is the retention time of the solute. It should be noted that Eq. 2 is valid only for neutral solutes. However, in most practical cases it can be used to calculate "effective" k' values also for ionized analytes [28], as has been done in this study. Assuming no change in separation efficiency and selectivity, Eq. 1 indicates that the resolution can be optimized by altering the retention factor and the elution range. This MEKC retention window (MRW) over which the analytes are distributed is given by the ratio

$$MRW = t_{MC}/t_0 \quad (3)$$

It should be noted, as pointed out by Rasmussen and McNair [29], that despite the high plate numbers commonly generated in MEKC, for example, 150 000 plates would only give a resolution comparable to conventional HPLC (16 000 plates at $k' = 10$). That comparison was based on the assumption that a similar selectivity is obtained in both systems. The peak capacity, n, i.e., the maximum number of peaks that can theoretically be resolved within the retention window, is given by [28,30]

$$n = 1 + \frac{\sqrt{N}}{4} \cdot \ln\left(\frac{t_{MC}}{t_0}\right) \quad (4)$$

Although the maximum number of peaks that can be resolved in an MEKC system with $N =$ 150 000 is thus well over 100, the peak capacity in practice is much lower, as in conventional HPLC. The best overall resolution that can be attained in MEKC in a separation of a large set of analytes is therefore, as in conventional HPLC, also strongly dependent on the separation selectivity. In addition, in MEKC how well the entire retention window can be utilized for resolving a given set of analytes is of equal importance. CE separations of complex mixtures of amino acid derivatives have recently been compiled by Skocir et al. [31]. Commonly 14–23 amino acid derivatives have been completely or

almost completely resolved within a time frame of 12.5–75 min. This should be compared with a set of the protein D- and L-amino acids, which requires the resolution of more than 30 peaks.

3.1. MEKC separation of OPA/TATG-amino acid enantiomers

The chiral OPA/TATG reagent reacts with amino acids in analogy with other OPA/thiol chiral and achiral reagents that are commonly employed for the precolumn derivatization of primary amines, particularly in LC [15]. The reaction, depicted in Fig. 1, is completed at alkaline pH within a few seconds for most amino acids with the exception of threonine [20]. A feature of the bulky thiol TATG is that the diastereomers formed are relatively stable, no derivative breakdown being observed after 1.5 h. Compared with amino acid derivatives formed with the common achiral reagent OPA/β-mercaptoethanol, the OPA/TATG–amino acid derivatives are more hydrophobic but still highly soluble in aqueous solutions. Further, in comparison with the other two chiral thiols examined so far together with OPA in capillary electrophoresis, NAC and BocC [18,19], TATG gives derivatives which are slightly more hydrophobic (results not shown).

In this study, the aim was to obtain simultaneously a good resolution of both the hydrophilic and hydrophobic OPA/TATG derivatives and to balance this goal with a reasonable total analysis time. The work was carried out mainly in the traditional way through optimization of one experimental parameter at a time. By varying the separation buffer pH, ionic strength, surfactant concentration and concentration of organic solvent, we tried, in each experiment, to spread the analytes evenly over as large a part of the separation window as possible. The degree of utilization of the retention window was judged visually when examining the electropherograms. In addition to the common measure of the MEKC retention window, t_{MC}/t_0 (MRW), in this study we also defined another measure for the purpose of describing the effective window available for separation of the OPA/TATG–amino acid derivatives. This derivative retention window (DRW) is given by by the ratio

$$DRW = t_L/t_F \quad (5)$$

where t_L and t_F denote the retention times for the last- and first-eluting derivatives, respectively. This is a measure which has hardly been discussed in connection with the optimization of the overall resolution in MEKC separations.

During the initial CE experiments, when no micellar system was used, only a few OPA/TATG–amino acid derivatives could be resolved in a single run at alkaline pH. When the anionic surfactant SDS was added to the separation buffer, to provide for hydrophobic interactions, the enlargement of the derivative retention window, DRW, available for separation of the derivatives was a factor of 2–2.5. During optimization, the SDS concentration was varied between 10 and 100 mM in seven steps using 40 mM borate buffer at pH 9.5. Over this concentration range the derivative effective retention factor, k', increased almost linearly. The correlation coefficient (r) for five selected amino aid derivatives, L-serine, D-alanine, L-isoleucine, D-methionine and L-arginine, ranged between 0.999 and 0.992. From the equation for the

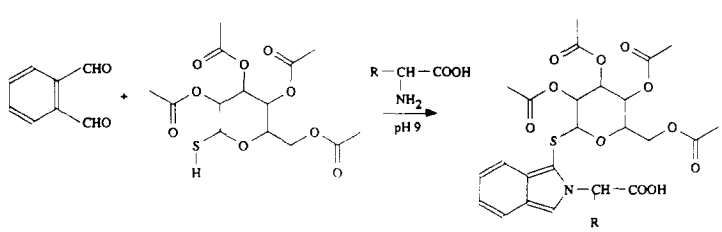

Fig. 1. Reaction of amino acids with the chiral OPA/TATG reagent.

linear dependence of k' on SDS concentration, the critical micelle concentration (*cmc*) can be estimated. In MEKC the retention factor at low micelle concentrations is related to the SDS concentration by [26,32]

$$k' = P_{MW}V([\text{surfactant}] - cmc) \quad (6)$$

where P_{MW} is the partition coefficient of the derivative into the micelles and V is the molar volume of the surfactant. The intercept on plotting k' vs. [SDS] gave a mean value for *cmc* of 2.1. As expected, this value is lower than the *cmc* of 8 m*M* for SDS in pure water (25°C) and agrees well with other *cmc* values reported for SDS in the presence of buffer [32–36]. The best overall resolution of the derivatives was obtained with an SDS concentration between 40 and 50 m*M*; the derivatives were then most evenly distributed over the entire MEKC retention window. At 45 m*M* SDS the values of the *MRW* and *DRW* were 3.66 and 2.97, respectively. The increase in *MRW* with increase in SDS concentration was paralleled by an increase in *DRW*, such that the two showed a constant ratio at all SDS concentrations examined. Although higher SDS concentrations gave some improvement in the resolution between the most hydrophilic derivatives, this could not be balanced by the achievement of a reasonable analysis time because of the drastic increase in retention time for the most hydrophobic derivatives at higher SDS concentrations.

3.2. Dependence of MEKC retention and resolution on pH and ionic strength

The effect of pH on derivative retention and resolution was investigated using a borate buffer with an ionic strength of 40 m*M* and an SDS concentration of 45 m*M*. Between pH 8.5 and 10 no major changes in the elution order of the derivatives were observed. This is what would be expected, considering that the amino acids have their primary amine function(s) derivatized with OPA/TATG, and in the MEKC separation predominantly behave as common weak organic acids, which at this alkaline pH are fully deprotonated. A point here regarding time optimization of the separation is that a higher buffer pH gave a small decrease in retention times without having any large effect on the overall resolution. This was due to a slightly higher electroosmotic flow at high pH, where both t_0 and t_{MC} are similarly affected, with a ca. 3% decrease between pH 9.3 and 10.0. It should be noted that this is mainly a pure pH effect, since the buffers were prepared to have a constant ionic strength while varying the pH. The matter of covariance of ionic strength with change in pH and its effect on the electroosmotic flow has been discussed in detail by Vindevogel and Sandra [36]. In contrast to that study, where the separation buffer concentration was given as its boric acid equivalent, our values of buffer ionic strength also take into account the sodium hydroxide concentration [22]. They observed a slight decrease in electroosmotic flow whereas we observed a slight increase. Moreover, in our study a slight decrease in *MRW* and *DRW* with increase in pH was observed and a pH of 9.5 was chosen as a compromise for further studies. In addition, this separation buffer pH is compatible with the amino acid sample, which after derivatization has a pH just above 9. The choice of pH may also be justified by a higher precision in retention times obtainable at high pH in MEKC, as has been reported by Skocir et al. [31].

The effect of ionic strength on resolution was investigated with a borate buffer of pH 9.5 containing 50 m*M* SDS. As with pH, varying the ionic strength (30–65 m*M* in four steps) did not give any major changes in the elution order of the derivatives. However, both the *MRW* and *DRW* increased with increasing ionic strength to give values of 4.63 and 3.68, respectively, for the 65 m*M* borate buffer. An important aspect in the choice of ionic strength is to have a sufficient buffer capacity in order not to induce excessive peak broadening. During optimization, the variation in plate number for a certain derivative was less than 20% within the respective pH and ionic strength ranges examined. Thus, our changes in buffer pH and ionic strength had only minor effects on separation efficiency. Another aspect of buffer ionic strength is to use as dilute a

separation buffer as possible, since high buffer concentrations gives high currents and accordingly may cause problems with Joule heating. This is particularly important in time optimization, when high voltages are desired to accelerate the separation. In addition, the buffer concentration should match the ionic strength of the injected derivatized sample.

3.3. Dependence of MEKC retention, resolution and selectivity on organic modifier

Addition of organic solvents as modifiers to the separation buffer was examined both for the purpose of widening the retention window and for fine tuning the separation selectivity. The effect of methanol and acetonitrile on the MEKC and derivative retention windows is shown in Fig. 2. An increase in organic solvent concentration from 0 to 12% gave an increase in *MRW* by factors of 2.0 and 2.8 for methanol and acetonitrile, respectively. Despite the difference in effect on *MRW* between the two solvents, it is noteworthy that the increase in size of the effective retention window for derivative separation (*DRW*) was similar, a factor of 1.8 for both methanol and acetonitrile. Hence, although the *MRW* value can be raised from just below 4 to above 10 by the addition of acetonitrile, the *DRW* is less affected (from 2.3 to 4.2). If we look in detail at the effect of organic modifier concentration between 0 and 12%, the decrease in electroosmotic flow is larger with methanol than with acetonitrile, a factor of 1.5 and 1.3, respectively. The electroosmotic flow is therefore slightly higher with acetonitrile than with methanol added to the buffer, a factor of 1.15. This is mainly due to a larger effect on the ζ-potential from methanol compared with acetonitrile [37] and emanates to a large extent from the change in the MEKC separation buffer viscosity and dielectric constant. In addition, the net velocity of the SDS micellar phase was less affected by methanol than by acetonitrile for the same reason. The viscosity increases with increasing concentration (0 to 12%) of organic solvent by factors of 1.35 and 1.12, respectively, for methanol and acetonitrile. It should be noted that the

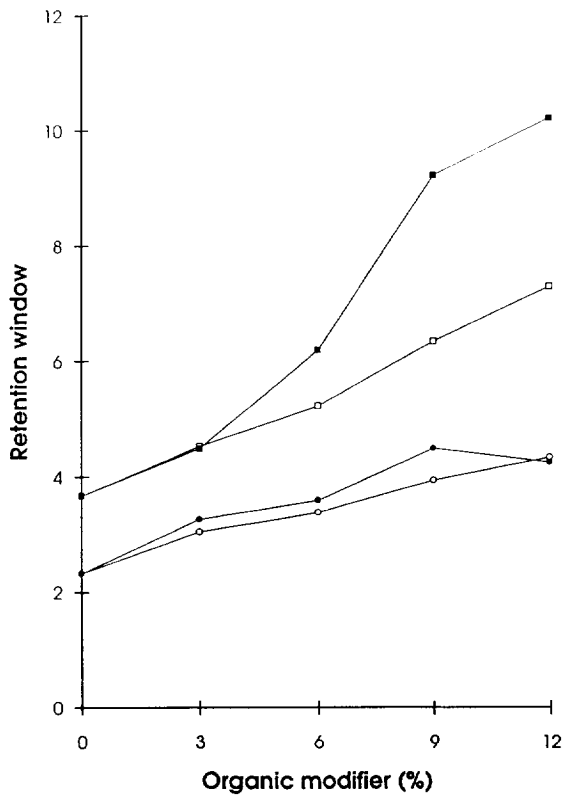

Fig. 2. Retention window dependence on concentration of organic modifier: effect of (□) methanol and (■) acetonitrile on MEKC retention window (t_{MC}/t_0) measured as the ratio of the migration time for Sudan III (t_{MC}) to the migration time for acetone (t_0) and effect of (○) methanol and (●) acetonitrile on OPA/TATG–amino acid derivative retention window (t_L/t_F) measured as the ratio of the migration time for the last-eluting derivative (t_L) to the migration time for the first-eluting derivative (t_F).

change in retention for the first-eluting derivative was in fact the same as the change in electroosmotic flow, a factor of 1.5 and 1.3 with methanol and acetonitrile, respectively. Thus, the change in retention with addition of organic modifier to the buffer for the most hydrophilic derivatives is due mainly to the change in electroosmotic flow. However, with increasing organic concentration the change in retention of the last-eluting derivatives to the retention of Sudan III is significantly larger with acetonitrile than with methanol, a factor of 1.5 and 1.1, respectively. Hence the most hydrophobic de-

rivatives are more strongly retained in the aqueous phase than in the micellar phase with acetonitrile as organic modifier, indicating its higher solvent strength. This resembles the effect of organic modifier on the partitioning of the same OPA/TATG–amino acid derivatives between an aqueous mobile phase and a hydrophobic stationary phase in reversed-phase HPLC [20]. In conclusion, the combination of these two effects (change in electroosmotic flow and solute partitioning between the aqueous buffer–micellar phase) is therefore the likely explanation of why the effective window for derivative retention is actually no larger for acetonitrile than for methanol.

As expected, within the concentration range 0–12%, there was in the main a linear dependence of log k' on organic solvent concentration (negative slope). The correlation coefficient for the four selected amino acid derivatives, D-alanine, D-methionine, L-arginine and L-ornithine, ranged between 0.990 and 0.845 for methanol and between 0.991 and 0.831 for acetonitrile. In addition to the effect of the organic modifier on retention and resolution, there was also a significant increase in plate number with the addition of methanol or acetonitrile to the buffer. This effect was obtained in spite of the fact that the OPA/TATG–amino acid derivatives are highly soluble in aqueous eluents. The increase was most pronounced for late-eluting hydrophobic derivatives, with a ca. 50% higher plate number, but still significant for early-eluting hydrophilic derivatives with a 15–20% increase in N. Initially, tetrahydrofuran was also investigated as an organic modifier in the MEKC separation of the OPA/TATG–amino acid derivatives. In contrast to methanol and acetonitrile, there was no increase in separation efficiency with the use of tetrahydrofuran. Rather, a slightly lower plate number was observed, 80–90% of that obtained with the aqueous SDS buffer solely. This is not surprising considering that tetrahydrofuran has a much lower dielectric constant than acetonitrile and methanol ($\varepsilon = 7.6$, 37.5 and 32.7, respectively), and therefore in general has a lower ability to disperse electrostatic charges via ion–dipole interactions. No further experiments were conducted with tetrahydrofuran.

The effects of methanol and acetonitrile concentration on MEKC separation selectively for OPA/TATG–amino acid derivatives were examined and are shown in Figs. 3 and 4, where the retention is given as the relative retention which was obtained by normalizing the experimentally determined retention time with the retention time for the last-eluting derivative after correction for the time of the non-retained solute. In this way, a graphical overview is obtained of the relative position of the analytes within the effective retention window. Because of the large number of analytes, the data for each solvent are grouped in two separate figures. In general, methanol and acetonitrile gave similar overall elution patterns for the derivatives, although differences were obtained in selectivity on replacing one solvent with the other. In particular, addition of the organic solvents to the separation buffer has a larger effect on the relative retention for the hydrophobic than for the hydrophilic and acidic amino acid derivatives. Moreover, the effect of organic solvent added to the separation buffer on analyte retention is more similar for hydrophobic derivatives than for the hydrophilic derivatives when comparing methanol with acetonitrile. This is further illustrated in Fig. 5a and b by the results for the slope, k, for the OPA/TATG–D- and L-amino acids from a linear regression correlating log k' with organic modifier concentration. In the plots the derivatives have been classified in different groups based on the characteristics of the amino acid side-chain. Apart from the conformational difference between the derivatives of the respective amino acid D- and L-forms, the side-chain constitutes the structural difference between the analytes after derivatization of the primary amine function(s). The side-chain is therefore expected to play a major role in governing derivative retention.

The plots in Fig. 5 shows that with methanol as modifier there is a more coherent pattern than with acetonitrile. It is also shown, as discussed above, that acetonitrile has a larger effect on the distribution of the analytes between the micellar

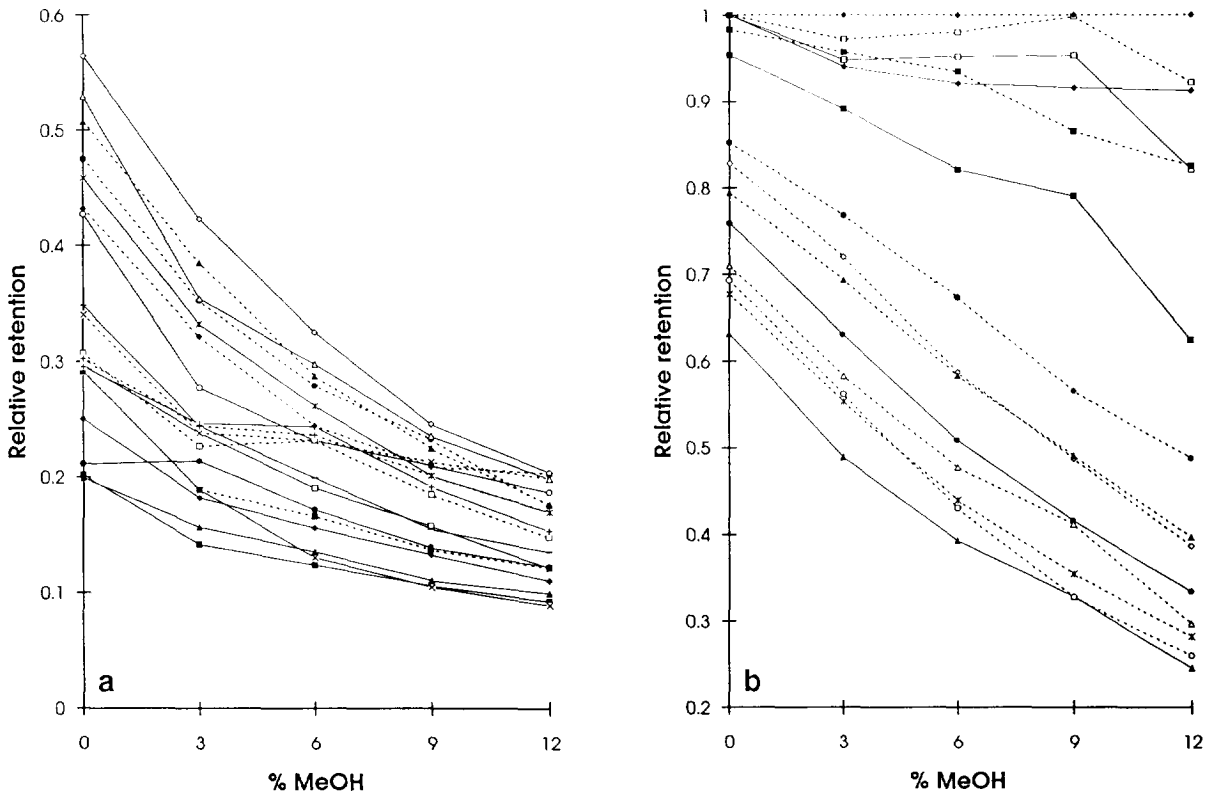

Fig. 3. Effect of methanol concentration on MEKC separation selectivity for OPA/TATG-D- and L-amino acid derivatives. Retention is given as a relative retention by normalizing experimental migration times to the migration time for the last-eluting derivative (t_L) and the electroosmotic flow as $(t_R - t_0)/(t_L - t_0)$. The data are grouped in two separate figures, (a) and (b), for illustration purpose only. The solid and dashed lines denotes the L- and D-form of amino acids, respectively. (a) ■ = Ser; □ = Glu; ◆ = Thr; X = His; ● = Ala; ○ = Trp; △ = Met; ▲ = Tyr; + = Asp; − = Gly; ∗ = Val; ◇ = Phe. (b) ■ = Arg; □ = Orn; ◇ = Phe; ● = Leu; ◆ = Lys; ▲ = Ile; ○ = Trp; △ = Met; ∗ = Val.

and the aqueous phases, particularly for the more hydrophobic derivatives. Considering that the separation buffer pH is well above the ionization constant for the basic amine of histidine (pK = 6.00 [38]), the variation in the slope within the group of basic amino acids is no larger than within the other groups of amino acid derivatives. When using acetonitrile as modifier, compared with methanol, the slightly larger spread in k values within most derivative groups emphasizes the greater effect of acetonitrile on the separation selectivity. In line with this is that more cross-overs in elution order were observed with acetonitrile than with methanol and that these changes were more frequent for the hydrophilic derivatives. This behaviour is in accordance with general observations for solute–micelle interactions in aqueous surfactant systems. Often solubilization of non-polar compounds can be considered as a simple partitioning between the micellar non-polar interior and the aqueous environment in the intermicellar solution, whereas polar compounds can participate in and influence the surfactant aggregation process [35,39]. Therefore, in spite of the fact that all micelles in the separation buffer are altered in the same way by a certain organic modifier, hydrophilic and hydrophobic OPA/TATG–amino acid derivatives may interact to different extents with the different parts of the micelles, thereby experiencing different chemical microenvironments during partitioning.

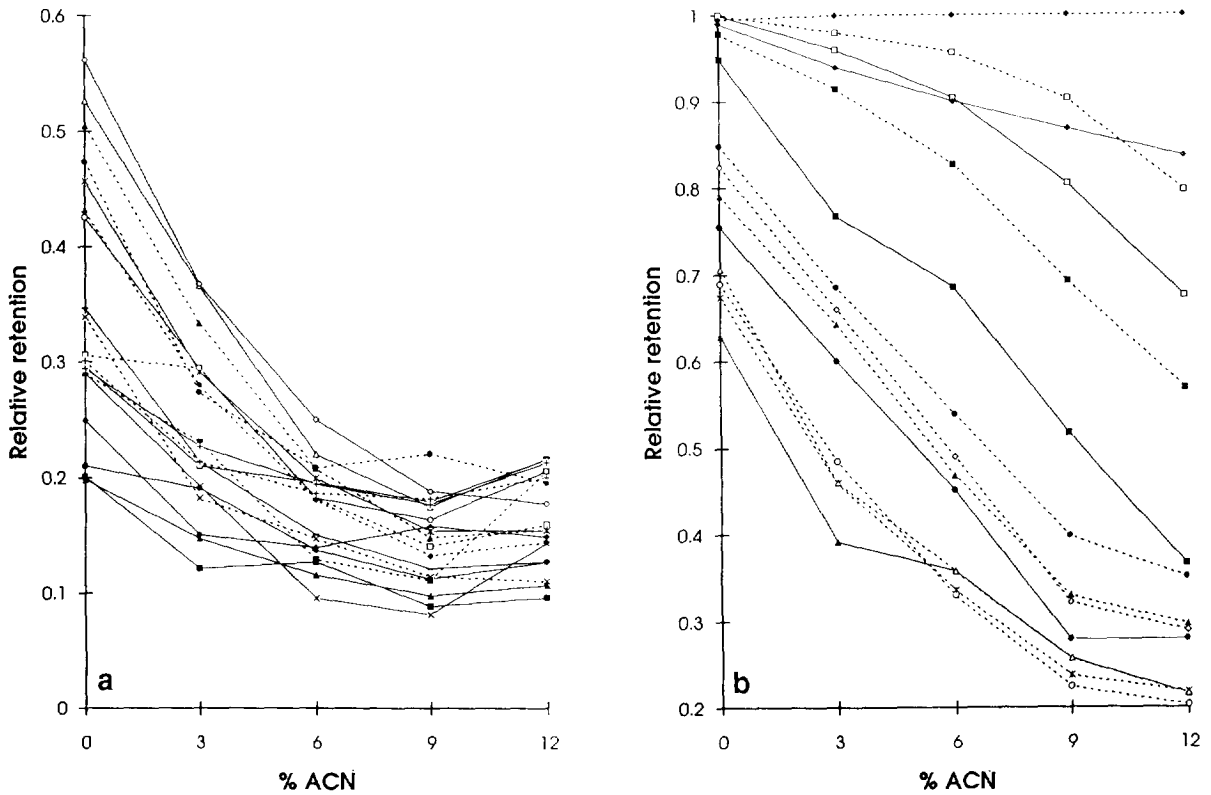

Fig. 4. Effect of acetonitrile concentration on MEKC separation selectivity for OPA/TATG–D- and L-amino acid derivatives. Retention is given as a relative retention by normalizing experimental migration times to the migration time for the last-eluting derivative (t_L) and the electroosmotic flow as $(t_R - t_0)/(t_L - t_0)$. The data are grouped in two separate figures, (a) and (b), for illustration purposes only. Symbols as in Fig. 3.

The changes in relative retention of a certain derivative, relative to the other derivatives, are more parallel for methanol than for acetonitrile. This is probably due to the fact that methanol is incorporated to a greater extent into the micellar structure in a lamellar configuration, in analogy with what has been reported for other alcohols in aqueous surfactant systems [35]. On the other hand, it is likely that acetonitrile to a larger extent modifies the surface of the micelles, in analogy with, e.g., octanenitrile in aqueous surfactant systems [35]. In addition, acetonitrile has a higher dielectric constant than methanol (see above) and therefore a greater ability to disperse the electrostatic charge of the anionic surfactant. Consequently, since polar compounds interact to a greater extent with the surface than with the interior of the micelles, it is likely that acetonitrile may induce more changes than methanol in the MEKC separation selectivity for the hydrophilic derivatives.

When optimizing the overall resolution in the separation of the OPA/TATG–amino acid derivatives, an organic solvent concentration in the range 3–6% was sufficient. The use of higher concentrations unfortunately tended to cluster the peaks at the beginning of the electropherogram, since almost all peaks exhibit a move towards the front in the derivative retention window. At this point it was of interest to examine whether an increase in surfactant concentration could be used as a means to improve the resolution further. However, at an acetonitrile concentration of 4% no significant increase in *DRW* (2.7 compared with 2.8) could be observed on increasing the SDS concentration

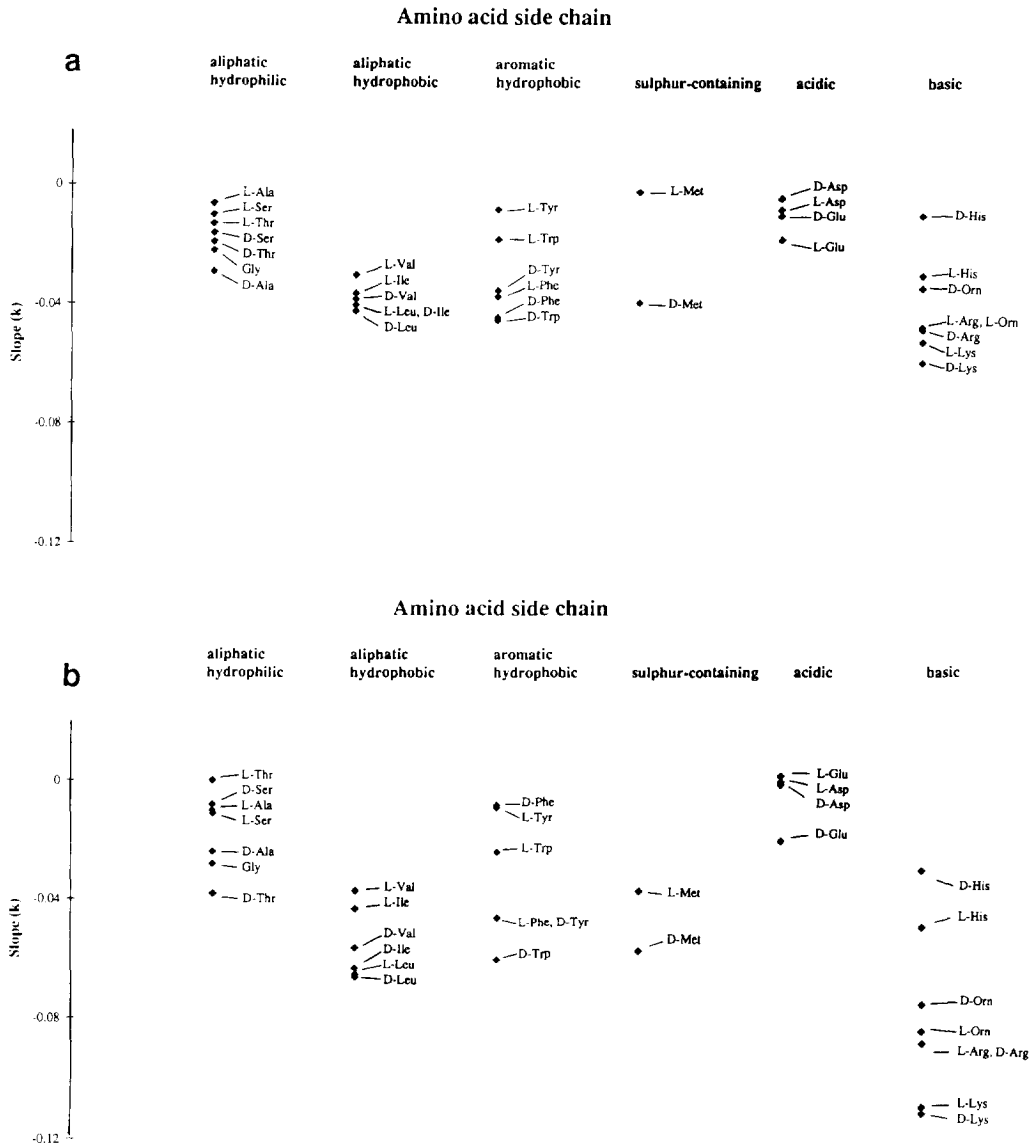

Fig. 5. Effect of organic modifier on OPA/TATG–amino acid derivative MEKC retention and selectivity. Plot of the slope k from linear regression of log k' vs. modifier concentration for data in Figs. 3 and 4. The derivatives are grouped based on the characteristics of their respective amino acid side chain. Data for (a) methanol and (b) acetonitrile.

from 45 to 55 mM. In fact, this change will only prolong the analysis time. Experimental data on the effective retention factor, k', selectivity factor, α, and resolution, R_s, for 14 OPA/TATG–D- and L-amino acid derivatives from an MEKC separation with 40 mM borate buffer (pH 9.5) containing 45 mM SDS and 4% acetonitrile are given in Table 1. The resolution of the derivatives of the D- and L-forms of a specific amino acid was generally very high (mean $R_s = 14.3$). This is predominantly a result of the high separation efficiency, mean plate number = 130 000, which corresponds to 680 000 plates/m. Notably, the value of the selectivity factor, α, was con-

Table 1
Experimental data on effective retention factor, selectivity and resolution for fourteen OPA/TATG–DL-amino acid derivatives as after MEKC separation

Amino acid	k'(L)	k'(D)	α	R_s
Ser	0.86	0.98	1.5	3.9
Thr	0.92	1.7	1.8	15
His	0.85	1.1	1.3	8.3
Trp	1.7	3.6	2.2	20
Tyr	0.92	1.7	1.9	18
Ala	0.98	1.7	1.7	12
Val	1.7	3.4	2.0	18
Ile	2.8	6.0	2.2	23
Phe	2.3	5.8	2.5	28
Leu	4.3	8.3	1.9	18
Met	1.9	3.4	1.8	17
Arg	14	37	2.6	12
Lys	47	410	8.6	6.8
Orn	82	98	1.2	0.79

Effective retention factor calculated from Eq. 2, selectivity factor $\alpha = k'_2/k'_1$ and resolution $R_s = 2(t_{R_2} - t_{R_1})/(w_{b_1} + w_{b_2})$. Conditions: Buffer, sodium borate buffer (pH 9.55, $I = 0.04$ M) containing 0.045 M SDS and 4% acetonitrile; untreated fused-silica capillary, 27 cm (19 cm to detector) × 50 μm I.D.; field strength, 405 V/cm (ca. 52 μA).

sistently slightly higher than that previously obtained in reversed-phase LC for the same derivatives and organic modifiers [20]. In addition, the same elution order between the amino acid L- and D-forms was obtained in the MEKC system as previously in HPLC, i.e., the L-amino acid derivative elutes before the D-amino acid derivative.

3.4. Time optimization

Several aspects must be considered in the course of time optimization in MEKC in order to arrive finally at a good compromise between overall resolution and analysis time. In addition to what has been discussed in previous sections, the length of the separation capillary is also an important parameter. As in conventional column chromatographic techniques, in MEKC the retention time for a solute, and consequently the analysis time, is a strong function of the capillary length ($t_R \propto L$), whereas resolution is a weak function ($R_s \propto \sqrt{N}$). The latter statement assumes that peak broadening is independent of capillary length and net velocity, which is not valid in general but, for the sake of giving guidelines in MEKC time optimization, can be used as an approximation. The influence of capillary length on both resolution and time optimization has hardly been discussed in the literature [40,41]. A practical limitation regarding capillary length is that most commercially available CE instruments only permit capillaries with a total length down to 25–30 cm. Another restriction may emanate from the detector electronics and timed control of hydrodynamic injection, which must be optimized for fast separations and the use of short separation capillaries. Moreover, the separation speed can be increased by the use of high voltages, but this will be limited by reaching too high a current, which causes problems with Joule heating deteriorating the performance. With the automated CE system used in this study, a 27-cm capillary could be mounted in the capillary cassette holder. The voltage was increased to give a field strength of 405 V/cm (ca. 52 μA), which is equal to a power per unit length of 2.1 W/m. This is close to the upper limit in terms of the heating effect reported by Sepaniak and Cole [42] for MEKC separations. An MEKC separation in less than 5 min of 34 D- and L-amino acids labelled with OPA/TATG is demonstrated in Fig. 6. Regarding the overall resolution of the amino acid derivatives, it is interesting to compare our results with the theoretical equations derived for MEKC optimization by Foley [41]. If no change in plate number and selectivity is assumed while optimizing the resolution at varying k', optimum resolution is obtained at

$$k'_{opt} = (t_{MC}/t_0)^{0.5} \quad (7)$$

Eq. 7 was derived for neutral solutes [41]. Although the retention factor of an acidic solute is the weighted average of the retention factor of its undissociated and dissociated forms, a separation buffer pH of 9.55 is well above the pK_a for the acidic derivatives. Hence, only minor effects of pH on retention for the majority of the derivatives are expected at this pH. This is also consistent with our findings, as discussed above. Therefore, Eq. 7 can be used to calculate an

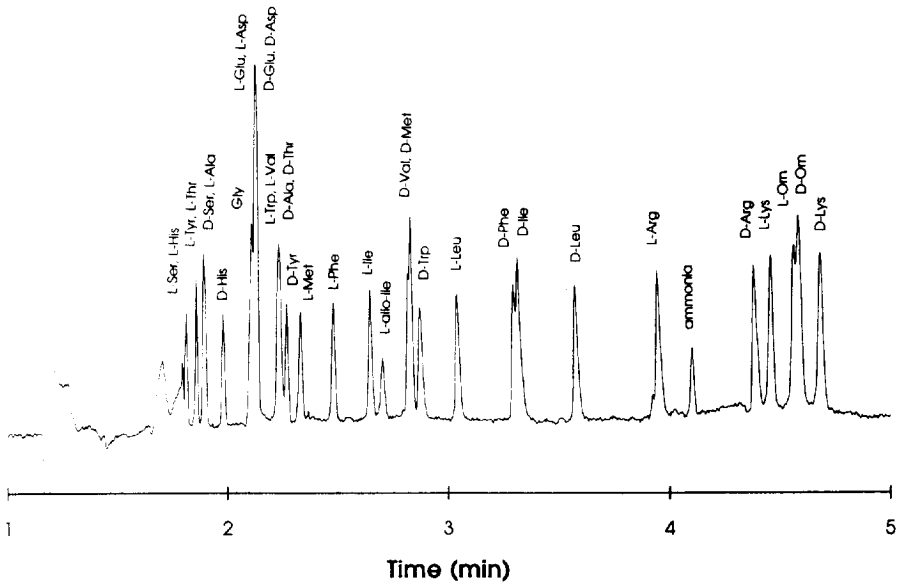

Fig. 6. MEKC separation of OPA/TATG–D,L-amino acid derivatives. Conditions: buffer, sodium borate buffer (pH 9.55, $I = 0.04$ M) containing 0.045 M SDS and 4% (v/v) acetonitrile; untreated fused-silica capillary, 27 cm (19 cm to detector) × 50 μm I.D.; field strength, 405 V/cm (ca. 52 μA); hydrodynamic injection (2 s at 0.5 p.s.i.); UV detection at 340 nm.

approximate theoretical value of the optimum retention factor for comparison with our experimental data. Calculating k'_{opt} for the separation in Fig. 6 gives a value of 1.99 for the retention factor where optimum resolution is obtained. In spite of the fact that the OPA/TATG–amino acid derivatives cover a wide k' range in that separation (0.85–410, see Table 1), it is interesting that the MEKC retention window is centred so that the derivative eluting in the middle has a k' between 3 and 5. Recalling that the k' scale is logarithmic if aligned with the t_R scale, our result for the experimental MEKC optimization of the retention of OPA/TATG–amino acid derivatives is in the vicinity of the theoretically predictable optimum.

3.5. Microchemical analysis

Although an in-depth evaluation of the utility of the present MEKC method for microchemical analysis is beyond the scope of this study, some results of interest for that purpose are given here. When using the automated CE system and UV detection, the mass limit of detection (MLOD) for the OPA/TATG–amino acid derivatives was similar for detection at 254 nm with detection at 340 nm. Injection of a 3.6-nl samples (257 fmol) of L-leucie, labelled with OPA/TATG, was detected after MEKC separation with a signal-to-noise ratio (S/N) of 23 and 20, respectively (noise measured peak-to-peak). Extrapolating to $S/N = 3$ gives MLODs of 33 and 38 fmol, respectively. This is equal to a concentration limit of detection (CLOD) of ca. 10 μM. When performing UV detection at the lower wavelength both the derivatives and also the excess of reagent, i.e., OPA, are detected. Fortunately, most of the peaks emanating from the reagent elute in front of the derivatives; however, some co-elution occurs between the reagent and the first-eluting derivatives, which is why detection at 340 nm should be used in quantitative and qualitative analytical applications. The MLOD when using the laboratory-built CE instrument with the modified HPLC fluoroscence detector was 64 fmol ($S/N = 3$), which is just above that obtained for UV detection with the automated CE system. Although the optical configuration of the HPLC fluorescence detector is not entirely perfect for on-capillary detection, fluorescence is more selective

than UV absorption for the detection of the OPA/TATG-labelled amino acids.

In addition, the method detection limit may be significantly improved if laser-induced fluorescence detection is utilized with excitation with either a helium–cadmium laser (325 nm) [20] or an argon ion laser (351–364 nm) [21]. In order to take full advantage of the present method for the rapid separation of labelled D- and L-amino acids, methods for rapid sample pretreatment should be available. For example, in studies of amino acid racemization in peptides and proteins, a rapid hydrolysis procedure is desirable. Regarding the chiral labelling of the amino acid enantiomers, the derivatization time needed should be minimized and, moreover, for the analysis of nanolitre sample volumes the derivatization reaction should preferably be carried out on-column in the separation capillary to avoid any sample dilution. Such studies are in progress [43].

4. Conclusions

Through optimization of one MEKC separation parameter a time we have obtained a method which permits the rapid screening of OPA/TATG-labelled D- and L-protein amino acids for use in microchemical analytical work. The method is versatile since the diastereomers formed can be detected by both UV and fluorescence detection, and laser-induced fluorescence detection may be employed if ultra-high sensitivity is required. An advantage with this chiral labelling is the very high resolution of the derivatives of the D- and L-form of each amino acid achieved. This high resolution is obtained simultaneously in a single run for all amino acids. This should be compared with the use of achiral labelling in combination with chiral additives in the separation buffer, e.g., cyclodextrins, which commonly gives good resolution only for a partial group of a large set of amino acids in a single MEKC run. It may be argued that a more dedicated method for MEKC optimization might have given the same result but saving experimental effort. Unfortunately, there is still a lack of a comprehensive theory of MEKC resolution quantitatively relating simultaneously efficiency, selectivity, capacity factor, retention window and analysis time and, moreover, which is applicable to both ionic and non-ionic solutes and can also take into account changes in analyte–micellar interactions when using organic modifiers. Dedicated strategies presented for MEKC separations [31,32,36,44,45] are commonly based on a global approach for optimization of resolution and the retention window, since several experimental parameters are interactive and cannot be optimized in isolation from each other. So far, however, such studies have usually only involved the simultaneous optimization of a limited number of the many experimental parameters of interest in MEKC: separation buffer pH, ionic strength, surfactant concentration, type of buffer, type of surfactant, surfactant counter ion, type and concentration of organic modifiers, voltage, temperature, capillary length, etc. Besides, before a global optimization can be successful, a number of initial experiments must be carried out to arrive at a preliminary system in order to permit proper judgements regarding the experimental design. However, optimization in the traditional way as carried out here is justified from the need to characterize the detailed behaviour of separation buffer modifiers on analyte retention and selectivity. This is essential to permit fine tuning of the separation conditions and is of particular importance when resolving complex mixtures such as large sets of amino acids. To the best of our knowledge, a separation of 34 D,L-amino acids in less than 5 min, with only a few peaks co-eluting, has not previously been demonstrated. For the purpose of gaining further insight into the MEKC separation selectivity, a multivariate analysis of the presented retention data, using various solute structural descriptors, will be the subject of a subsequent report.

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