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Direct and indirect chiral separation of amino acids by capillary electrophoresis

Hong Wan[1], Per E. Andersson, Anders Engström, Lars G. Blomberg*

Department of Analytical Chemistry, Arrhenius Laboratories for Natural Sciences, Stockholm University, S-106 91 Stockholm, Sweden

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Abstract

Two approaches to the chiral separation of racemic mixtures of amino acids by means of capillary electrophoresis have been evaluated. These were indirect separation of diastereomers formed by derivatization with (+)- or (−)-1-(9-fluorenyl)ethyl chloroformate and direct chiral separation after derivatization with 9-fluorenylmethyl chloroformate.

Separation conditions were optimized by the application of a full factorial design. For indirect separation, concentration of sodium dodecyl sulphate (SDS) and pH were the most important separation variables, and for direct separation, concentration of isopropanol (IPA), β-cyclodextrin and SDS were the most prominent factors affecting separation. The presence of IPA was a prerequisite for chiral recognition.

With regard to selectivity, efficiency, analysis time and ease of method development the best results were provided by the indirect method. It should be noted, however, that the success of this approach is based on the availability of a derivatization reagent in high optical purity.

1. Introduction

Capillary electrophoresis is a rapidly expanding area of analytical chemistry. In a number of publications, it has been demonstrated that this technique, in the form of electrokinetic chromatography (EKC), is suitable for chiral separations [1–9]. Two different approaches to achieve such a separation can be distinguished. These are direct chiral separation and indirect separation of diastereomers. In general, derivatization is performed prior to the introduction of the sample into the separation capillary. The purpose of such a derivatization is twofold: to improve separation and detection [10,11]. When chirality is maintained after the derivatization, direct chiral separation is attempted on separation systems providing chiral selectivity. Diastereomeric derivatives are separated on non-chiral systems. However, the formation of diastereomers can also be utilized as a mechanism for chiral recognition in the separation capillary [12]. Further, a few examples of chiral separation of underivatized amino acids have been described [13–15].

With the current techniques for chiral separation by capillary electrophoresis, derivatization is of crucial importance. High demands are

* Corresponding author.

[1] On leave from Lanzhou Institute of Chemical Physics, Chinese Academy of Sciences, Lanzhou 73000, China.

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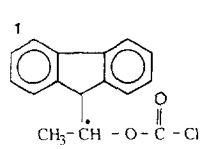
thereby made on the properties of the derivatization reagents. An ideal reagent should thus fulfil several requirements [16]. First, it should be stable and give rapid reactions in high yields at low temperatures and the reaction products should be sufficiently stable. Excess reagent or by-products from the reaction should not disturb the separation. Further, the reagent should be selective for the target analytes. In those cases where diastereomers are formed, the reagent must have a very high degree of chiral purity. A small impurity can lead to large errors, especially in the determination of enantiomeric excess (e.e.) [11]. Further, the reagent should contain or produce a strong chromophore or fluorophore. The reagent should be commercially available, and for chiral reagents, it is desirable that they are available in the L- as well as the D-form, so that elution orders can be reversed when required. In addition, the reagents should be inexpensive, although chiral reagents in high purity are inherently costly. A large number of derivatization reagents have been developed for application in connection with chiral separation in HPLC [10,11]. Many of these have been examined with regard to electrophoretic methods, but the performance of some promising reagents remains to be evaluated. In the present work, a reagent, FLEC, belonging to the latter group was tested for indirect chiral separation of D- and L-amino acids by micellar electrokinetic chromatography (MEKC).

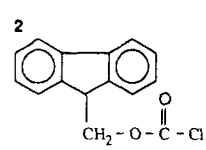
Several examples of the direct separation of chiral derivatives of D- and L-amino acids have been presented. Dansylation is here the most commonly applied derivatization method [15,17–25]. Eggum and Sørensen have discussed the merits of dansylation [26]. Other types of derivatization reagents include: naphthalene dicarboxaldehyde (NDA) [22,27,28], phenylhydantoin (PTH) [29–31] and 4-fluoro-7-nitrobenz-2,1,3-oxadiazol [32]. Several types of separation systems providing chiral selectivity have been applied for the separation of this type of derivatives. The selectivity may thus occur in the form of chiral mixed micelles, cyclodextrins, crown ethers and bile salts.

Chiral separation can, as mentioned above, be achieved on the basis of the formation of diastereomeric interactions in the column. This approach is applied in ligand-exchange electrophoresis and when crown ethers are present in the buffer. When the chiral Cu(II)–aspartame complex was included in the background electrolyte, as many as 14 out of 18 dansyl amino acids could be resolved [33].

A few applications of the separation of diastereomers have been published. First, Tran et al. used 1-fluoro-2,4-dinitrophenyl-5-L-alanine amide (Marfey's reagent) for the formation of amino acid diastereomers [34]. After derivatization with 2,3,4,6-tetra-O-acetyl-β-D-glucopyranosyl isothiocyanate (GITC), Nishi et al. [35] were able to separate 19 D,L-amino acids; GITC was employed also by Lurie [36]. Van der Wal et al. prepared diastereomers by derivatization with *o*-phthalaldehyde (OPA) and N-acetylcysteine [37]. Similarly, Kang and Buck derivatized amino acids with (OPA) and either N-acetyl-L-cysteine or N-*tert.*-butoxycarbonyl-L-cysteine [38]. Derivatization with (+)-diacetyl-L-tartaric anhydride has been presented [39]. The advantages of the diastereomer approach are that optimization is simple and rapid and that the MEKC technique, in general, provides higher efficiency than direct chiral separation methods where complexation with the chiral selector often results in slow kinetics.

The aim of the present work was, in general terms, to compare the pros and cons of direct and indirect separation of racemic mixtures of amino acids (AAs) by capillary electrophoresis. For that purpose, comparison has been made, after optimization, of the separation of racemic amino acids after their derivatization by two chemically similar reagents, FLEC (**1**) and FMOC (**2**). For the determination of D- and L-amino acids, these reagents fulfil most of the above mentioned requirements.





It has been demonstrated that the application of statistical techniques for the optimization of

the separation conditions in capillary electrophoresis is a fruitful approach [40–44], and in the present work, a full factorial design was employed for the optimization of the separation conditions.

2. Experimental

2.1. Apparatus

A Prince (Lauerlabs, Emmen, The Netherlands) electrophoresis instrument equipped with an on-column UV detector, CV^4 (ISCO, Lincoln, NE, USA), operated at 254 nm and a high voltage power supply, CZE 1000 R, 0–30 kV (Spellman High Voltage Electronics, Plainview, NY, USA) was used. Uncoated fused-silica capillary tubing (Polymicro Technologies, Phoenix, AZ, USA) 360 μm O.D. and 25 μm I.D. in different lengths was utilized. Samples were introduced by pressure. Chromatograms were recorded with an ELDS 900 laboratory data system (Chromatography Data System, Kungshög, Sweden).

2.2. Reagents

The amino acids were obtained from Sigma (St. Louis, MO, USA). Sodium dodecyl sulphate (SDS) was obtained from Fluka (Buchs, Switzerland), (+)- and (−)-1-(9-fluorenyl)-ethyl chloroformate (FLEC) and 9-fluorenylmethyl chloroformate (FMOC) from Eka Nobel (Bohus, Sweden). Hydroxypropyl-β-cyclodextrin (HP-β-CD) was synthesised according to Armstrong et al. [45]. Other reagents for preparation of buffers were of analytical grade. Buffers were prepared daily. The buffers were filtered through a 50 μm porous filter (Supelco, Bellefonte, PA, USA) before use.

2.3. Derivatization procedures

Derivatization of amino acids with (+)-FLEC and (−)-FLEC was as described earlier [46]; 200 μl of 10 m*M* FLEC was thus added to 200 μl of 3 m*M* amino acid in 0.2 *M* borate buffer (pH 9.0). This mixture was kept for 2 min, then extracted with 0.5 ml pentane to remove excess of reagent. After dilution, ten times with water, the sample was ready for introduction. Derivatization of amino acids with FMOC was as for FLEC.

2.4. Conditions

When changing buffers, the capillary was first rinsed with 0.1 *M* NaOH for 5 min, then with water for 10 min and it was finally conditioned with buffer for 30 min before sample introduction. Between sample introductions, the capillary was rinsed with buffer for 5 min. The separation capillary was thermostatted at 25°C. Optimization computer program was Codex (AP Scientific Service, Sollentuna, Sweden). Abbreviations: amino acids are indicated by three-letter symbols according to current standards [26].

2.5. Evaluation procedures

Resolution was calculated according to

$$R_s = \frac{1.18\,\Delta t}{w_{(0.5)1} + w_{(0.5)2}} \quad (1)$$

where Δt is the difference in migration times and $w_{(0.5)1}$ and $w_{(0.5)2}$ are the peak widths at half peak height.

According to Terabe et al. [47] the separation factor, α, in MEKC is

$$\alpha = \frac{k'_2}{k'_1} = \frac{t_2 - t_0}{t_1 - t_0} \times \frac{1 - t_1/t_{mc}}{1 - t_2/t_{mc}} \quad (2)$$

where t_1, t_2, t_0 and t_{mc} are the migration times of the first and second analyte peak, the aqueous phase and the micelles respectively, and k' is the capacity factor. The micelle time is thus necessary for the calculation of α. However, the determination of t_{mc} is uncertain in cases when the buffer contains organic solvents [48] or cyclodextrin [23]. Therefore, the first term on the right hand side of Eq. 2 was utilized as a measure of selectivity. When applied to MEKC, this expression is always $< \alpha$, however, for

EKC, Eq. 2 is in accordance with the definition of α.

3. Results and discussion

3.1. Optimization

In CE, a number of variables has to be considered when optimal conditions are sought. Several of these are interrelated, and a univariate approach to the optimization, thus trying to optimize one variable at a time, will, in general, not result in optimal conditions. Further, non-linear models are often required to describe resolution as a function of the separation variables. For these reasons, a multivariate approach has to be adopted for the optimization [49].

As a first step in the optimization process, it is beneficial to perform a screening experiment, where a relatively large number of variables are examined concerning their significance. It is of interest, of course, to gain a maximum of information from a minimum number of experiments. In order to achieve this, an experimental design should be applied. Several types of designs are available, which could be adopted, and the choice of design depends on the number of variables involved and how detailed the information is to be. A full factorial design is a good choice when the number of variables are four or less. When more than four variables are of interest, a fractional factorial design is applicable. With more than fifteen variables, a Plackett–Burman design is the preferred choice. However, with Plackett–Burman, a linear model is assumed to be satisfactory, and in CE, non-linear models are, as mentioned above, often desired [49].

Of an experimental design, the most critical part is the selection of the low and high limits of the design. A good knowledge of the separation system is generally needed in order to apply the proper limiting values. In a situation where the high and low limits have been selected above and below optimal, the experiment will be misleading as to in which direction the optimum will be found.

3.2. Derivatization reagents

Comparing the analysis of some differently derivatized amino acids by capillary electrophoresis, Albin et al. [50] maintained that FMOC offered the greatest utility with respect to separation efficiency and concentration detection limit when using fluorescence detection with a xenon lamp as light source. Instrumentation for fluorescence detection, suitable for capillary electrophoresis, has, however, not been available to us, and therefore detection was by UV in the present work.

Absorptivity of primary amines derivatized with different chiral reagents was compared at five wavelengths by Houben et al. [37]. The highest absorption was in the low-UV region, but derivatization with Marfey's reagent resulted in relatively high absorption also at 340 nm. A drawback of Marfey's reagent is, however, that a relatively high temperature or a prolonged time is required for the reaction. FLEC showed good absorptivity at 254 nm, and this wavelength was selected for the present work, thus avoiding interferences. Other merits of FLEC include high optical purity, >99.0 e.e. according to the manufacturer, availability in the (+) and (−) forms, rapid reaction at room temperature, no racemization during reaction and the formation of stable derivatives [46,51].

3.3. Diastereomeric separation of FLEC-AAs

For the separation of amino acids derivatized with FLEC, two key factors, pH and SDS concentration were optimized by full factorial design as mentioned above. The applied low factors were: pH 7.8 and SDS concentration 20 m*M*. The high factors were: pH 9.2 and SDS concentration 80 m*M*. For the design, 10 experiments, including two centre points, were performed utilizing a mixture of 4 different FLEC(−)-AAs (Thr, Ile, Val and Phe). The results obtained from optimization indicated that a high pH was necessary for the chiral resolution

of these 4 FLEC-AAs and that the different AAs showed optimal chiral separation at different SDS concentrations. For example, the first eluted analyte, Thr, had a relatively high optimal SDS concentration, ca. 60 m*M*, Fig. 1A. In contrast, for the last eluted amino acid, Phe, the SDS concentration should be as low as possible, Fig. 1B. This could be explained by the fact that Phe is more hydrophobic and the partition into the micelle phase would thus be more extensive for this amino acid. As a consequence, a lower SDS concentration should be needed. A buffer containing an intermediate concentration of SDS, 20 m*M*, and a high pH, 9.2, was thus selected in order to facilitate separation of a maximum number of FLEC(−)-AAs. As a result, 11 of 19 FLEC(−)-AAs examined were baseline separated. The amino acids, Arg, Asp, Cys, Glu, Lys, Pro, Trp, Tyr were not resolved. Much longer migration times were observed for these amino acids; thus, a buffer concentration of 20 m*M* SDS was too high for chiral separation of these amino acids.

When an organic modifier, acetonitrile (ACN), was added to the buffer, the separation of early eluted AAs such as Ser, Ala and Thr

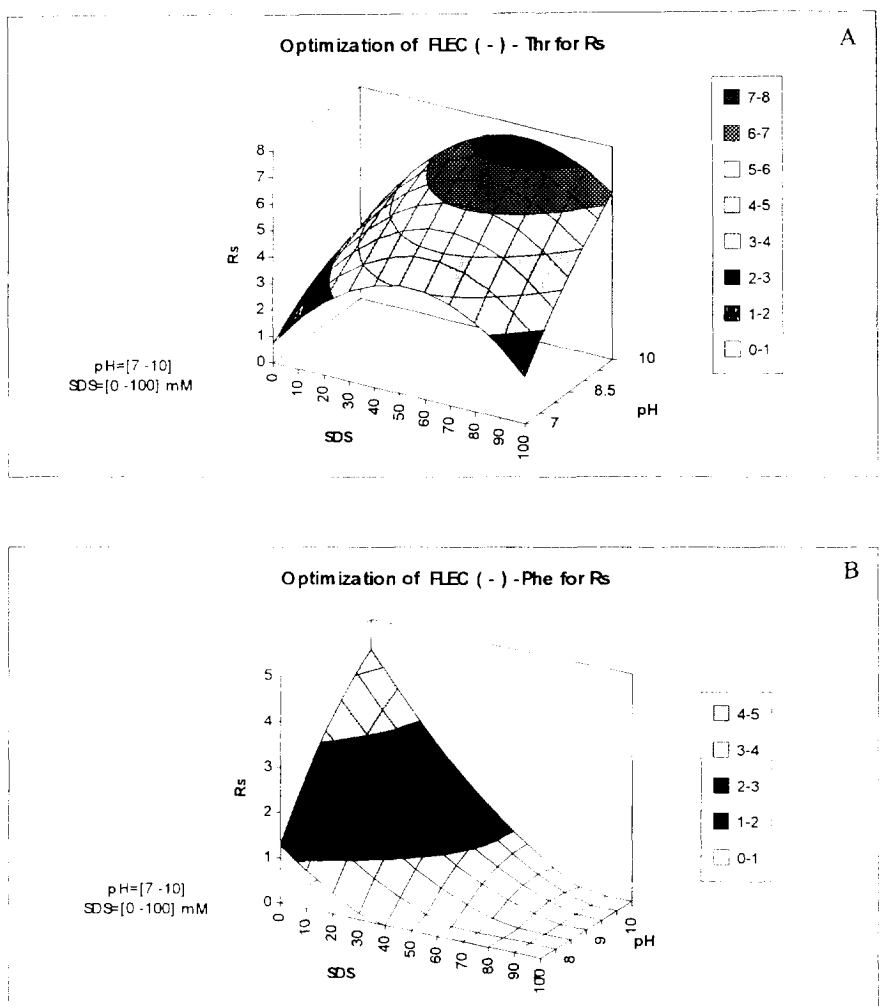


Fig. 1. Three-dimensional peak resolution surface for amino acids derivatized with (−)-FLEC. A = threonine; B = phenylalanine, as a function of the SDS concentration and the pH.

Table 1
Diastereomeric separation of FLEC(−)-AAs by MEKC

Amino acid	0% ACN				10% ACN			
	t (min)[a]	R_s	$\alpha >$	N/m ($\times 10^5$)	t (min)[a]	R_s	$\alpha >$	N/m ($\times 10^5$)
Ala	5.39	2.71	1.038	8.80	6.18	2.67	1.036	11.0
Arg	10.42	0	1	14.1	14.57	2.09	1.015	12.1
Cys	10.16	0	1	14.3	14.41	4.90	1.038	11.1
His	5.95	0.96	1.012	4.67	6.11	0	1	2.73
Ile	7.03	3.86	1.045	8.16	7.23	8.19	1.100	10.4
Leu	7.51	2.05	1.020	10.6	7.53	7.02	1.086	9.11
Lys[b]	–	–	–	–	5.01	4.76	1.159	6.91
Met	6.81	1.54	1.017	8.76	6.83	4.18	1.052	10.7
Nleu	7.68	3.62	1.035	9.00	7.79	9.23	1.100	11.0
Nval	6.49	3.98	1.047	9.40	6.76	6.40	1.082	10.8
Phe	8.26	2.71	1.021	13.2	8.60	8.84	1.107	8.44
Ser	5.18	3.33	1.040	9.11	5.94	2.03	1.029	9.60
Thr	5.32	5.70	1.080	9.64	5.92	4.00	1.064	9.29
Trp	10.01	0	1	13.4	10.86	2.01	1.019	12.1
Tyr[c]	–	–	–	–	6.97	1.23	1.023	5.22
Val	6.03	6.66	1.083	8.29	6.48	8.02	1.108	10.5

Conditions: buffer; 20 m*M* borate–15 m*M* phosphate (pH = 9.2) containing 20 m*M* SDS; separation column, 62.5 cm × 25 μm I.D. (effective length 45 cm); applied voltage, 25 kV; current, 9.0 μA; 8.2 μA for 10% ACN
[a] Migration time for the D form.
[b] 10 m*M* SDS and 30% ACN.
[c] 10 m*M* SDS and 50% ACN.

was less satisfactory, Table 1. This is because the optimal SDS concentrations required for Ser, Ala and Thr should be higher than 20 m*M* and thus, addition of ACN decreased the resolutions. However, the separation of later eluted AAs, Met, Ile, Leu, Nleu and Phe, was improved, Table 1. When 10% ACN was included in the buffer, Arg and Trp were resolved. After decreasing the SDS concentration to 10 m*M*, maintaining ACN at 10%, a small decrease in resolution, cf. Table 1, was observed for Trp (R_s = 1.30), and Arg (R_s = 1.33) with a seriously tailing peak. Further increasing the ACN concentration to 15% resulted in an improved separation of Trp (R_s = 1.70) and also a good peak shape for Arg (R_s = 1.05).

The amino acids Tyr and Lys were separated at 10 m*M* SDS when ACN concentrations were increased to 30% and 50%, respectively, Table 1. The application of these buffers resulted in a decrease in the number of theoretical plates, Table 1. This was most likely due to operation close to the critical micellar concentration (CMC). In the absence of any additives, the CMC for SDS in aqueous solutions at 25°C is 8.1 m*M* [52]. It has been demonstrated that, in the presence of a phosphate–borate buffer, micelle formation takes place at lower SDS concentrations, 5 m*M* [53]. However, it is also known that the CMC is increased in the presence of high concentrations of organic solvents [52].

The amino acids Pro, Glu and Asp could not be separated in this type of buffer, which is probably due to poor efficiency for Pro and electrostatic repulsion interactions for Glu and Asp. In addition, it was observed that the FLEC(−)-His derivative was unstable at room temperature since apparent changes in migration times or even the absence of a peak were observed a few hours after derivatization.

These results suggest that it is difficult to separate all AAs under the same conditions, since it is evident that partition coefficients and interactions differ too much. However, by op-

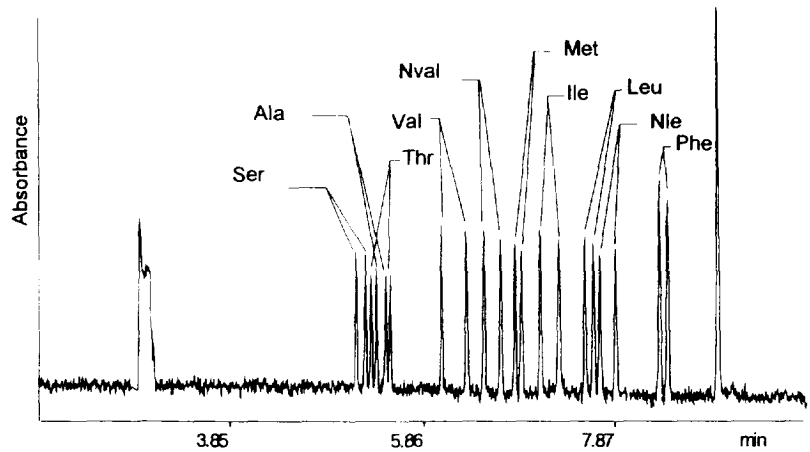


Fig. 2. Chromatogram, UV detection, of 10 amino acids derivatized with (−)-FLEC. Separation capillary, fused silica; 350 μm O.D., 25 μm I.D., total length 62.5 cm, effective length 45 cm; background electrolyte, 20 m*M* borate–15 m*M* phosphate (pH = 9.2), 20 m*M* SDS; applied voltage, 25 kV; current, 9.0 μA; temperature, 25°C.

timization, baseline separation of a mixture of 10 AAs has been achieved in one run in less than 10 min, Fig. 2. The D-form was eluted first when the AAs had been derivatized with (−)-FLEC. Reversal of elution order was, as expected, easily realized with (+)-FLEC. In order to be able to separate a wider range of amino acids in one run, the application of a mobile phase gradient would be necessary. Systems for the performance of such gradients have been presented by Sepaniak and co-workers [54,55].

3.4. Enantiomeric separation of FMOC-AAs

For the separation of FMOC-AAs, β-CD and HP-β-CD have been evaluated as chiral selectors. First, separation was attempted with HP-β-CD as chiral selector. In the capillary zone electrophoresis-mode (CZE), 7 FMOC-AAs (Thr, Phe, Leu, Met, Val, Nval and Nleu) were separated using a buffer containing 2.5 m*M* HP-β-CD and 100 m*M* phosphate at pH 6.0, Table 2. It was observed that an increase in buffer ionic strength resulted in enhanced resolution for these FMOC-AAs, but efficiencies were decreased. For instance, FMOC-Phe had a resolution of 0.87 and plate numbers of 5.69×10^5/m in a buffer containing 25 m*M* phosphate; but a resolution of 1.29 and plate numbers of 3.13×10^5/m in a buffer containing 100 m*M* phosphate. The decrease in efficiencies observed when increasing the ionic strength may be due to the decrease in electroosmotic flow. Further, since the selectivity was increased, an increase in analyte–CD interactions may be suspected, and this could also lead to band broadening. The Joule heating was 1.37 W/m. Taking into account that there was an active cooling of the capillary and that the diameter was only 25 μm, it may be concluded, on the basis of published data [53,56,57], that band broadening due to thermal effects ought to be quite small in this case. It was recently considered that the interaction between analyte and CD is impeded by buffer salts and water molecules, which would result in low enantioselectivities [58]. However, the present results demonstrate that buffer salts may have a positive effect on the chiral selectivity.

Separation of 4 FMOC-AAs under these conditions is shown in Fig. 3. Surprisingly, no chiral separation was observed when HP-β-CD was employed in MEKC, even though a wide range of conditions was investigated. The parameters pH, HP-β-CD and SDS concentrations were thereby varied. Further, the presence of three different organic modifiers, methanol, ACN and

Table 2
Separation data for FMOC-AAs and FLEC-AAs

Amino acid	Separated AAs	Method[a]	Migration time (min)	Selectivity α	Resolution	Plate height (μm)
FMOC-[d]	Ile	1	11.81	1.031	1.53	2.8
	Leu	1	12.26	1.023	1.24	3.6
	Met	1	12.88	1.025	1.31	3.8
	Nleu	1	12.41	1.020	0.94	4.0
	Nval	1	11.83	1.018	0.99	4.5
	Phe	1	11.68	1.025	1.29	3.1
	Thr	1	13.75	1.019	0.97	3.9
	7		11–14[f]	1.018–1.031[f] 1.023[b]	0.94–1.53[f] 1.18[b]	2.8–4.5 3.6[b]
FMOC-	15	2	18–32[f]	1.024–1.074[f] 1.044[b,e]	1.06–4.12[f] 2.35[b]	1.8–5.8[f] 2.3[b]
FLEC-	16	3	5–11[f]	1.012–1.159[f] 1.064[b,c,e]	0.97–6.66[f] 3.25[b,c]	0.7–3.6[f] 1.0[b,c]

[a] Methods: 1 = EKC-HP-β-CD; 2 = MEKC-β-CD; 3 = MEKC-indirect.
[b] Mean values for separated amino acids.
[c] For FLEC-AAs with ACN as in Table 1.
[d] Conditions: Buffer, 100 m*M* phosphate (pH = 6.0) containing 2.5 m*M* HP-β-CD; separation column, 62 cm (effective length 45 cm) × 25 μm I.D.; applied voltage, 30 kV; current 28.4 μA.
[e] α larger than given data.
[f] Range.

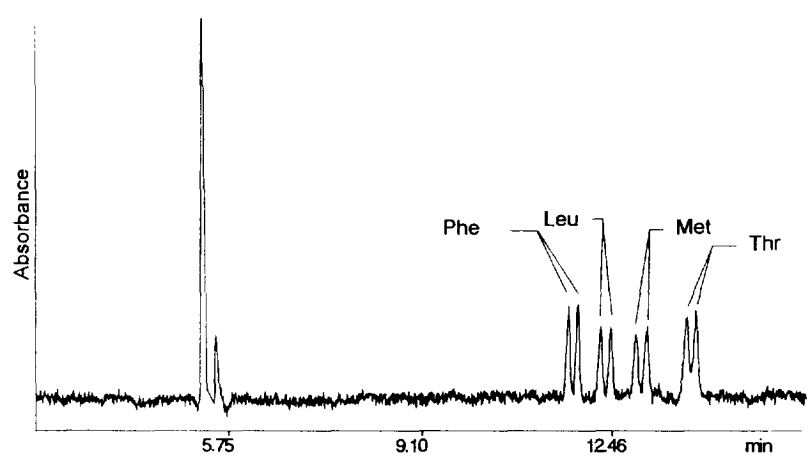


Fig. 3. Electropherogram, UV detection, of 4 amino acids derivatized with FMOC. Separation capillary, fused silica, 360 μm O.D., 25 μm I.D., total length 62 cm, effective length 45 cm; background electrolyte, 100 m*M* phosphate (pH = 6.02), 2.5 m*M* HP-β-CD; applied voltage, 30 kV; current, 28.4 μA; temperature 25°C.

IPA in the buffer was examined with negative results.

When β-CD was used as chiral selector, chiral separation was observed only with one type of organic modifier, IPA, in the CZE-mode as well as in the MEKC-mode. In the CZE-mode, 6 FMOC-AAs, Phe, Leu, Met, Thr, Asp and Glu, were examined, all of these being separated at pH 5.5 with 10 m*M* β-CD and 10% IPA. Migration times of Phe, Leu, Met and Thr were in the same range. For Glu and Asp, which were not resolved by diastereomeric separation, baseline separation was obtained with resolutions of 2.71 and 1.69.

It was noted that the resolutions of the FMOC-AAs were largely unchanged when the pH was raised from 5.5 to 7.0. This indicated that the pH had less effect on enantioselectivity in this case and that the pH could be kept unchanged during further optimization.

In order to obtain optimized conditions, and to separate as many AAs as possible by MEKC, an optimization by means of a full factorial design which contained 3 factors. The low factors employed were, SDS concentration 8 m*M*, CD concentration 3 m*M* and 5% isopropanol. The high factors were, SDS concentration 15 m*M*, CD concentration 10 m*M* and 15% isopropanol. For the factorial design 16 experiments including two centre points, were performed at pH 7.0 with a mixture of 6 FMOC-AAs. The optimization showed that all 6 FMOC-AAs had similar optimal conditions, which was completely different from the result obtained from the optimization of FLEC-AAs. This result suggests that more than one analyte should be considered in optimization so as to obtain optimal conditions that are suitable for many analogous analytes. In Figs. 4 A–C are shown an example of optimization for Phe-FMOC. It can be seen from Fig. 4, that the concentrations of β-CD and IPA were the most important parameters, and that optimal SDS and β-CD concentrations were dependent on the IPA concentration used. Additionally, the model prediction indicated maximum resolution for 18% IPA, 12 m*M* β-CD and 18 m*M* SDS. Considering that analysis time is increased when high IPA and SDS concentrations are employed, 15% IPA, 12 m*M* β-CD and 15 m*M* SDS were adopted as optimal conditions for testing all FMOC-AAs. Under these conditions, 15 of 19 FMOC-AAs were separated, Table 3. Only Ala, Cys, Tyr and Lys could not be separated. The system showed a good selectivity for the separation of FMOC-derivatized proline isomers. These analytes were, however, eluted as relatively broad bands, Table 3, baseline separation could therefore not be achieved in this case. In Fig. 5 is shown a typical chromatogram of the separation of a 7 FMOC-AAs mixture in the MEKC-mode. The D-form was first eluted, thus indicating that the L-form is more strongly bound to β-CD than the D-form. This elution order is beneficial in the determination of large enantiomeric excess for natural L-AAs, since the D-enantiomer is usually considered as an impurity and its elution prior to the large peak favours quantitation.

3.5. Effect of organic modifiers on chiral recognition

Addition of organic solvents could change the inclusion complex formation constant, thus affecting the optimum concentration of CD; separation may thereby be improved or worsened [59,60]. As mentioned above, chiral recognition for FMOC-AAs was found with β-CD only in the presence of IPA. The presence of isopropanol resulted in greatly decreased electroosmotic flow (EOF) as compared with methanol and ACN, as has been reported earlier [61–64]. Only low amounts of IPA, 5%, were required for enantioselectivity. The benefit of small amounts of a modifier, strongly binding to the β-CD, in the buffer for the moderation of the analyte–CD interaction was recently reported [65]. Armstrong et al. [66,67] demonstrated that chiral recognition could not be obtained by HPLC when the mobile phase contained water, but separation could be achieved in water-free systems. It was concluded that the presence of water forced the FMOC-part of the derivative into the cavity of the CD, when the interaction of the chiral part of the derivative, the amino acid, with the CD would thereby be insufficient

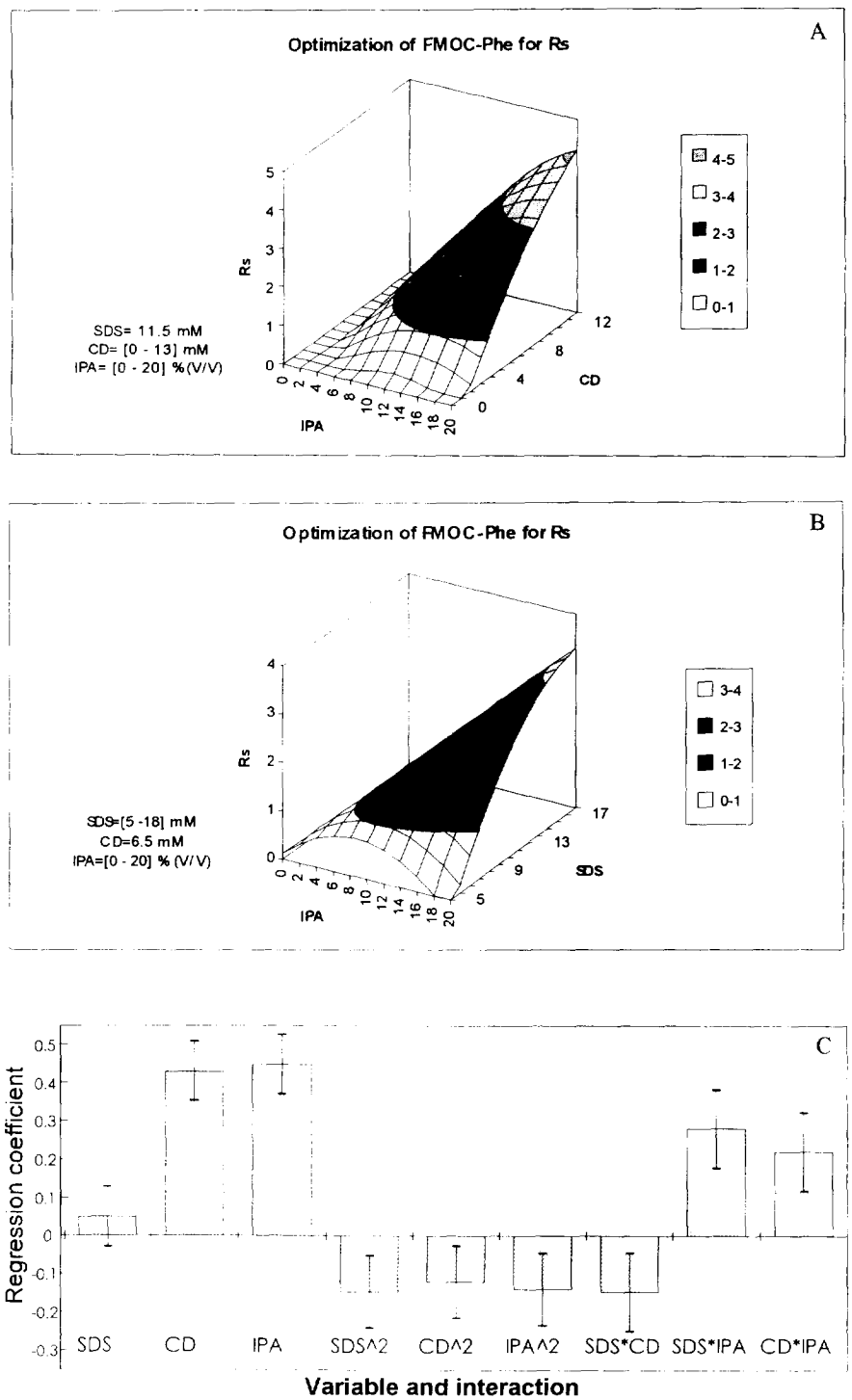


Fig. 4. Three-dimensional peak resolution surface phenylalanine derivatized with FMOC as a function of IPA and β-CD concentrations (A) and IPA and SDS concentrations (B). C is a bar chart for the regression coefficient and interaction effects of the three main variables for the resolution of phenylalanine derivatized with FMOC.

Table 3
Enantiomeric separation of FMOC-AAs by CD-MEKC

Amino acid	t (D-isomer) (min)	Selectivity $\alpha >$	R_s	N/m ($\times 10^5$)
Arg	21.99	1.040	1.10	2.26
Asp	32.64	1.026	1.68	3.24
Glu	30.30	1.051	3.20	3.36
His	18.60	1.040	1.80	4.80
Ile	20.85	1.074	4.14	5.20
Leu	22.07	1.022	2.72	5.28
Met	20.47	1.034	1.82	5.40
Nleu	23.34	1.039	2.38	4.40
Nval	20.88	1.037	1.83	3.70
Phe	23.68	1.062	4.12	5.52
Pro	21.75	1.033	1.08	1.72
Ser	19.07	1.024	1.06	3.64
Thr	19.14	1.027	1.41	4.94
Trp	25.13	1.048	3.31	4.92
Val	19.73	1.076	3.52	4.16

Conditions: buffer, 45 mM phosphate (pH = 7.0) containing 15 mM SDS, 12 mM β-CD and 15% IPA; separation column, 70 cm × 25 μm I.D. (effective length 50 cm); applied voltage, 25 kV; current, 6.8 μA.

for enantioselectivity. In water-free systems, with acetonitrile as the main mobile phase component, it was anticipated that ACN occupied the internal part of the CD, the AAs derivatives thus having to interact chirally with the external parts of the CD. It may be speculated that isopropanol, being less polar than methanol and ACN, has a similar role in the present system. For the 7 investigated FMOC-AAs, the effect of IPA concentration on the resolution of different

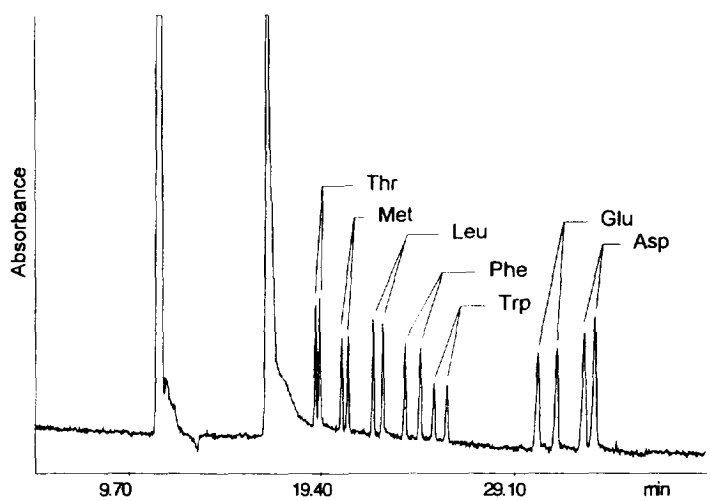


Fig. 5. Chromatogram, UV detection, of 7 amino acids derivatized with FMOC. Separation capillary, fused silica 360 μm O.D., 25 μm I.D., total length 70 cm, effective length 50 cm; background electrolyte, 45 mM phosphate (pH = 7.0), 15 mM SDS, 12 mM β-CD, 15% (v/v) IPA; applied voltage, 25 kV; current, 6.8 μA; temperature, 25°C.

FMOC-AAs is shown in Fig. 6 (top). It is evident that the best resolution for most of these FMOC-AAs was found at 15% IPA. This is also in good agreement with the conditions suggested above. On the other hand, the best efficiency was observed with 10% IPA, Fig. 6 (bottom). Further increasing the IPA concentration resulted in decreased efficiencies. This is because slow EOF led to a slow overall migration rate, which in turn resulted in increased band broadening by means of longitudinal diffusion [68]. Furthermore, the separation efficiencies of FMOC-AAs in the presence of either β-CD or HP-β-CD, were, in general, lower than the efficiencies obtained with the diastereomers, Table 2. This suggests that slow kinetics in the

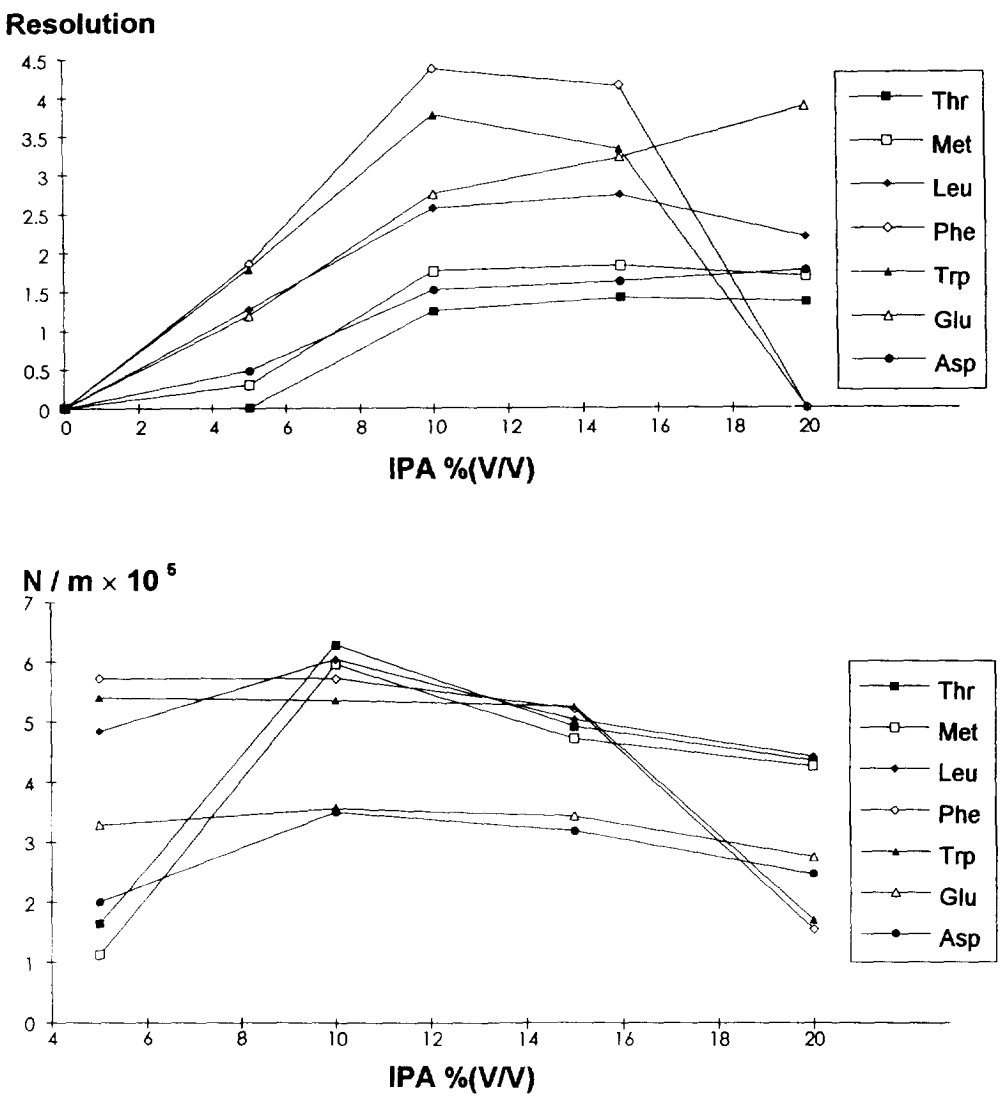


Fig. 6. Graphs of the resolution (top) and separation efficiency (bottom) of 7 FMOC derivatized amino acids as a function of % isopropanol. Separation capillary, applied voltage and temperature as in Fig. 5. Background electrolyte, 45 m*M* phosphate (pH = 7.0), 15 m*M* SDS, 12 m*M* β-CD and different % of isopropanol.

formation of inclusion complexes between analytes and cyclodextrins could also contribute to the band width.

Conclusions

The results are summarized in Table 2. The chiral separation of 19 amino acids has been examined and ca. 80% of these could be resolved by direct as well as by indirect methods. Indirect separation offered higher separation efficiencies than direct separation with or without micelles. The average plate heights were thus, 1.0, 2.3 and 3.6 μm, respectively. Moreover, the average resolution was higher for separation of diastereomers, 3.25, than for chiral separation with micelles, 2.35, or without micelles, 1.18. The speed of separation was higher for indirect separation, the separation time for the direct method using micelles was three times longer.

The number of variables that has to be optimized, depends on the separation task. In the examples given, two variables were optimized in the indirect approach, and three variables in the direct method. However, for the separation of late eluting diastereomers, an organic co-solvent was needed, which led to a three variable optimization also in this case. According to our opinion, the main difference in the method development of the two approaches concerns the scouting that precedes the optimization. As a starting point for the optimization, conditions must be found that result in, at least, some separation. This can be accomplished relatively easily in the indirect method, while the scouting experiments can be quite extensive in connection with the direct separation method. A good knowledge of the function of the significant variables is required for efficient scouting. In conclusion, for the analytical systems investigated, the best performance was achieved with the indirect separation method.

Optimization with different analytes could result in different optimal conditions, especially for diastereomeric separation of FLEC-AAs where the SDS concentration was the most significant factor influencing the separation. In comparison with the diastereomeric separation of amino acids derivatized with Marfey's reagent and GITC by means of MEKC, much lower SDS concentrations were required for the resolution of FLEC-AAs. In the direct separation mode, β-CD showed a wide range of enantioselectivities for FMOC-AAs. The other chiral selector tested, HP-β-CD was less useful.

The FMOC-AAs derivatives are somewhat hydrophobic, and a particular organic modifier, IPA, was found indispensable for the chiral separation of these derivatives. In the presence of IPA, variations in pH had, within limits, only a small effect on the chiral recognition. Due to the low SDS concentrations employed in the chiral separation of FMOC-AAs and FLEC-AAs, relatively high separation efficiencies were obtained with MEKC. Finally, it should be mentioned that the basis for the successful application of the chiral derivatization reagent is its high optical purity. The chiral recognition mechanism for FMOC-AAs with β-CD and its derivatives is being further investigated.

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