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In- line derivatization of L- and D-amino acids by capillary electrophoresis

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List of abbreviations

AA: amino acid
ACN: acetonitrile
aCSF: artificial cerebrospinal fluid
ALS: amyotrophic lateral sclerosis
AQC: 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate

BGE: background electrolyte
CBQCA: 3-(4-carboxybenzoyl)-2-quinolinecarboxaldehyde
CD: cyclodextrin
CM-β-CD: carboxymethyl-β-CD
CE: capillary electrophoresis
CSF: cerebrospinal fluid
CZE: capillary zone electrophoresis
DAO: D-amino acid oxidase
DBD-PyNCS: R(−)-4-(3-isothiocyanatopyrrolidin-1-yl)-7-(N,N-dimethylaminosulfonyl)-2,1,3-benzoxadiazole
DM-β-CD: heptakis(2,6-di-*O*-methyl)-β-CD
Dns-Cl: Dansyl chloride
EOF: electroosmotic flow
FCCD: face-centered central composite design
FDAA: 1-fluoro-2,4-dinitrophenyl-5-l-alanine amide
FITC: fluorescein isothiocyanate
FLEC: 1-(9-fluorenyl)ethyl chloroformate
FMOC: 1-(9-fluorenyl)methyl chloroformate
GC: gaz chromatography
GITC: 2,3,4,6-Tetra-*O*-acetyl-β-D-glucopyranosyl isothiocyanate
HDAS-β-CD: heptakis(2,3-di-*O*-acetyl-6-*O*-sulfo)-β-CD
HPLC: high performance liquid chromatography
IPA: propan-2-ol
IS: internal standard
LIF: laser induced fluorescence
MEKC: micellar electrokinetic chromatography
NAR: Normalized Area Ratio
NBD-F : 7-nitro-2,1,3-benzoxadiazole
NDA: Naphthalene-2,3-dicarboxaldehyde
NMDAr: N-methyl-D-aspartate recptor
OPA: *o*-phtaldialdehyde
OPA/NAC: *o*-phtaldialdehyde and N-acetyl-L-Cys
OPA/IBC: *o*-phtaldialdehyde and N-isobutyryl-L-Cys
OS-γ-CD: octakis(2,3-dihydroxy-6-*O*-sulfo)-γ-CD
RP-HPLC: reversed-phase high-performance liquid chromatographic
R_s: resolution

RSD: relative standard deviation
SDS: sodium dodecyl sulfate
(S)-NIFE: (S)-N-(4-nitrophenoxycarbonyl)-Phe methoxyethyl ester
TFA: trifluoroacetic acid
TM-β-CD: heptakis(2,3,6-tri-*O*-methyl)-β-CD

Abstract

Over the past few years there has been a growing interest in chiral analysis of amino acids (AAs) since several D-forms are thought to play a major role in a number of neurodegenerative diseases. Research in this field is strongly connected to progress in analytical methodology with regard to speed of analysis, sensitivity, high separation performance and simplicity of sample preparation. In this research work, chiral analysis of AAs by MEKC has been studied using both direct and indirect approaches coupled with the full automation of in-capillary derivatization procedure.

In the first part of this work, a dual CD-CZE method was developed for the separation of 9-fluoroenylmethyl chloroformate (FMOC) labeled AAs. Firstly, a background electrolyte (BGE) composed of 30 mM β-CD, 30 mM octakis(2,3-dihydroxy-6-*O*-sulfo)-γ-CD (OS-γ-CD), 40 mM tetraborate and 15% isopropanol (IPA) was selected and led to 17 baseline resolved pairs and 2 partially resolved pairs. Experimental conditions for in-capillary derivatization were then optimized. The best labelling conditions were obtained using successive hydrodynamic injections (30 mbar) of AAs for 2 s, borate buffer for 4 s and 10 mM FMOC solution for 6 s, followed by a mixing at 3 kV for 72 s and wait time of 1 min. The use of MEKC, by adding SDS as surfactant led to the best separation of a standard mixture of ten AA racemates using a BGE containing 30 mM β-CD, 30 mM OS-γ-CD, 25 mM SDS, 40 mM sodium tetraborate and 17% IPA.

In the second part of this work, an original MEKC method using in-capillary derivatization with (-)-9-fluoroenylethyl chloroformate (FLEC) as chiral labelling reagent was developed for the separation of AA derivatives. Several parameters for in-capillary derivatization and MEKC separation were investigated using experimental designs. Efficient labelling was achieved by sequential injection of AAs and FLEC labelling solution followed by the application of a voltage of 0.2 kV for 570 s. Compared to off-line derivatization, in-capillary relative derivatization rates were higher than 82.3 % and better reproducibility was observed. The BGE composition was then optimized in order to achieve selectivity. Separation of 29 in-capillary labeled FLEC-AA derivatives was achieved in a single run using a buffer made up of 40 mM sodium tetraborate, 21 mM SDS and 8.5% IPA. Finally, the quantitative performance of the optimized in-capillary MEKC method was evaluated showing appropriate linearity, accuracy and precision. The developed method was successfully applied to the analysis of spiked artificial cerebrospinal fluid sample.

Résumé

Au cours des dernières années, il y a eu un intérêt croissant pour l'analyse chirale d'acides aminés (AAs) depuis que plusieurs D-AAs sont supposés jouer un rôle majeur au niveau de certaines maladies neurodégénératives. La recherche dans ce domaine est fortement liée aux progrès dans les méthodes d'analyse, notamment en termes de vitesse d'analyse, de sensibilité, de haute performance de séparation et de simplicité de préparation des échantillons. Dans ce travail de recherche, l'analyse chirale d'AAs par chromatographie électrocinétique micellaire (MEKC) a été étudiée en utilisant des approches à la fois directes et indirectes associées à l'automatisation complète de la procédure de dérivatisation à l'intérieur du capillaire.

Dans la première partie de ce travail, une méthode par électrophorèse capillaire de zone, associant deux cyclodextrines (CDs), a été développée pour la séparation d'AAs dérivatisés avec le chloroformiate de 9-fluoroénylméthyl (FMOC). Une solution d'électrolytes, composée de 30 mM de β-CD, 30 mM d'octakis (2,3-dihydroxy-6-O-sulfo)-γ-CD (OS-γ-CD), 40 mM de tétraborate de sodium et 15% d'isopropanol (IPA) a été sélectionnée et a conduit à la résolution complète de 17 paires et la résolution partielle de 2 paires de FMOC-AA. Les conditions expérimentales pour la dérivatisation dans le capillaire ont ensuite été optimisées. Les meilleures conditions de dérivatisation ont été obtenues en utilisant des injections hydrodynamiques successives (30 mbar) de la solution d'AA pendant 2 s, du tampon borate pendant 4 s et de la solution de 10 mM de FMOC pendant 6 s, suivies par un mélange en appliquant un voltage de 3 kV pendant 72 s et un temps d'attente de 1 min. L'utilisation de la MEKC, en ajoutant du dodécylsulfate de sodium (SDS) comme agent tensioactif, a conduit à la meilleure séparation d'un mélange de dix racémates d'AAs au moyen d'une solution d'électrolytes contenant 30 mM de β-CD, 30 mM d'OS-γ-CD, 25 mM de SDS, 40 mM de tétraborate de sodium et 17% d'IPA.

Dans la deuxième partie de ce travail, une méthode originale utilisant la MEKC couplée à la dérivatisation dans le capillaire, avec le chloroformiate de (-)-9-fluoroényléthyl (FLEC) comme réactif de dérivatisation chiral, a été développée pour la séparation des dérivés d'AAs. Plusieurs paramètres de dérivatisation dans le capillaire et la séparation par MEKC ont été étudiés en utilisant la méthodologie des plans d'expériences. Une dérivatisation efficace a été obtenue par l'injection séquentielle de la solution d'AA et la solution de FLEC suivies par l'application d'un voltage de 0,2 kV pendant 570 s. Les taux de dérivatisation relatifs calculés pour la dérivatisation dans le capillaire, par rapport à la dérivatisation hors ligne, étaient

supérieurs à 82,3% avec une meilleure reproductibilité. La composition de la solution d'électrolytes a ensuite été optimisée afin d'obtenir une bonne sélectivité. La séparation de 29 dérivés de FLEC-AA, dérivatisés dans le capillaire, a été atteinte en une seule analyse en utilisant un tampon constitué de 40 mM de tétraborate de sodium, 21 mM de SDS et 8,5% d'IPA. Enfin, les performances quantitatives de la procédure de dérivatisation dans le capillaire et de la méthode de séparation optimisée ont été évaluées montrant une linéarité, une exactitude et une précision appropriées. La méthode développée a été appliquée avec succès à l'analyse d'un échantillon de liquide céphalo-rachidien artificiel enrichi avec des AAs.

Contents

General introduction

Chapter 1

Capillary Electrophoresis

Section 1.1

Separation technologies are in the heart of all aspects of pharmaceutical and biomedical analysis. Capillary electrophoresis (CE) is a separation microanalytical technique where the analytes migrate under an electric field according to their charge-to-size ratio. Based on different separation mechanism compared to that in chromatography, CE is now recognized as complementary, orthogonal technique to High Performance Liquid Chromatography (HPLC), each with its advantages and disadvantages with regard to a given set of analytes.

The advantages of CE over chromatographic techniques are the extremely high peak efficiency and its applicability for the separation of widely different compounds, inorganic ions, organic molecules and larges biomolecules, using the same instrument while changing only the running buffer composition [1]. In addition, CE may offer simpler method development, minimal requirement of sample and chemical amounts, lower costs associated with analysis. A major feature of CE is the availability of various modes, which expands its application range. The most common modes are capillary zone electrophoresis (CZE, based on analytes charge to-mass- ratio) and micellar electrokinetic chromatography (MEKC, based on chromatographic partition of analytes between micelles and the background electrolyte). The advent of MEKC made possible the separation of neutral analytes and improved the selectivity of CE.

Over the last decade, several innovations enabled the development of CE as a versatile platform integrating in-capillary sample pretreatement such as the in-capillary derivatization and the in-capillary sample preconcentration which further improved sensitivity and applicability of CE.

Initially considered for the analysis of biological macromolecules, CE demonstrated its usefulness for the separations of compounds such as amino acids, chiral drugs, vitamins, pesticides, inorganic ions, organic acids, peptides and proteins, carbohydrates, oligonucleotides and DNA restriction fragments, and even whole cells and virus particles [2]-[3][4].

1. History

The first electrophoresis in open tube of protein, nucleic acids and inorganic was demonstrated by Hjerten in 1967, using a 300 µm inner diameter (i. d.) capillary and UV detection [5]. In 1974, Virtanen described a potentiometric detection method for electrophoresis of the alkali cations in approximately 200-µm inner diameter (i.d.) capillaries [6]. The influence of electroosmotic flow (EOF) on electrophoretic behaviour of an analyte was also mentioned by Virtanen [6]. Although these authors showed the advantages of the technique, they were unable to demonstrate the high separation efficiency due to poor detection sensitivity and large injection volumes. In early 1980's, Jorgenson and Lukacs published the invention of a simple CE instrument [7][8].

In 1984, Terabe and co-workers introduced the MEKC which expanded the application range of capillary electrophoretic techniques to neutral analytes. Separation of uncharged compounds was achieved using sodium dodecyl sulphate (SDS) micelles added to the electrophoretic buffer as pseudostationnary phase [9].

The first commercial CE instrument was available in 1987. Since then, the number of publications on CE has been rapidly increasing.

2. Principles

The principle of separation of CE is based on the migration, inside a capillary, of charged analytes dissolved in an electrolyte solution, under the influence of an electric field [10]. The electrophoretic migration of charged analytes takes place in a buffer which is called background electrolyte (BGE).

Upon application of an electric field of intensity *E*, the migration velocity of an analyte is determined by the electrophoretic mobility of the analyte (μ_{ep}) and the electro-osmotic mobility (μ_{EOF}) of the buffer inside the capillary.

The electrophoretic mobility of a solute depends on the characteristics of the solute (electric charge, molecular size and shape) and those of the BGE (type and ionic strength of the electrolyte, pH, viscosity and additives) [10]. The electrophoretic velocity (v_{ep}) of an analyte is given by the equation:

$$v_{ep} = \mu_{ep} \times E = \left(\frac{q}{6\pi\eta r}\right) \times \left(\frac{V}{L}\right) \qquad 1.1.$$

where q = effective charge of the solute,

η = viscosity of the electrolyte solution,

r = Stoke's radius of the solute,

V = applied voltage,

L = total length of the capillary.

When an electric field is applied through the capillary filled with buffer, a flow of solvent is generated inside the capillary, called electro-osmotic flow (EOF). This phenomenon is related to the ionization of the silanol groups of the inner surface of uncoated fused silica capillaries above pH 3. Positively charged molecules present in the solvent will be electrostatically attracted to the negatively charged surface and an electric double layer is formed. This potential difference, which is created very close to the wall, is known as zeta potential (ζ). The movement of solvated cations drags the solvent toward the cathode. The magnitude of the EOF can be expressed in terms of velocity (v_{EOF}) or mobility (μ_{EOF}) as follows:

$$v_{EOF} = \mu_{EOF} \times E = \left(\frac{\varepsilon\zeta}{\eta}\right) \times \left(\frac{V}{L}\right) \qquad 1.2.$$

where ε = dielectric constant of the buffer and ζ = zeta potential of the capillary surface.

The velocity of the EOF depends on μ_{EOF} which in turn depends on the charge density on the capillary internal wall and the buffer characteristics including pH, concentration/ionic strength of the BGE, additives, temperature.

The velocity of the solute (v) is given by:

$$v = v_{ep} + v_{EOF} \qquad 1.3.$$

The electrophoretic mobility of the analyte and the electro-osmotic mobility may act in the same direction or in opposite directions, depending on the charge of the solute. Under classical conditions, analytes are introduced at the anode and are detected at the cathode. Cations will migrate first with the highest velocities as they have the same direction as the EOF and their velocities will be greater than the electro-osmotic velocity. All neutral compounds migrate with the same velocity of EOF and are not separated. Lastly, anions will migrate in the opposite direction to the electro-osmotic flow and their velocities will be smaller than the electro-osmotic velocity. Therefore, the presence of the EOF enables the detection of cations, neutral compounds, and anions at the same end of the capillary (cf. Fig1).

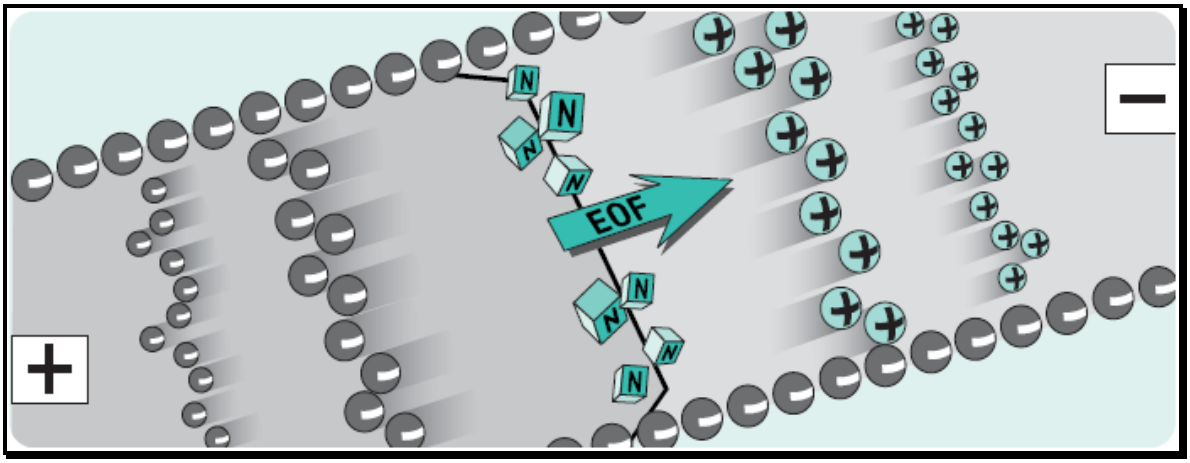

Figure 1. Migration of charged and neutral species [11].

The EOF must remain constant from run to run to obtain good reproducibility of the migration velocity of the solutes. For some applications, it may be necessary to reduce or suppress the electro-osmotic flow by changing the concentration, composition and/or pH of the buffer solution or by modifying the inner wall of the capillary using coatings. These coatings can decrease, neutralize or reverse the surface charge and thus the EOF [12][13].

3. Instrumentation

One key feature of CE is the simplicity of the instrumentation. A typical CE instrument consists of sample introduction system, a capillary, a high-voltage power supply, detection system connected to a data acquisition device and an autosampler (cf. Fig. 2.).

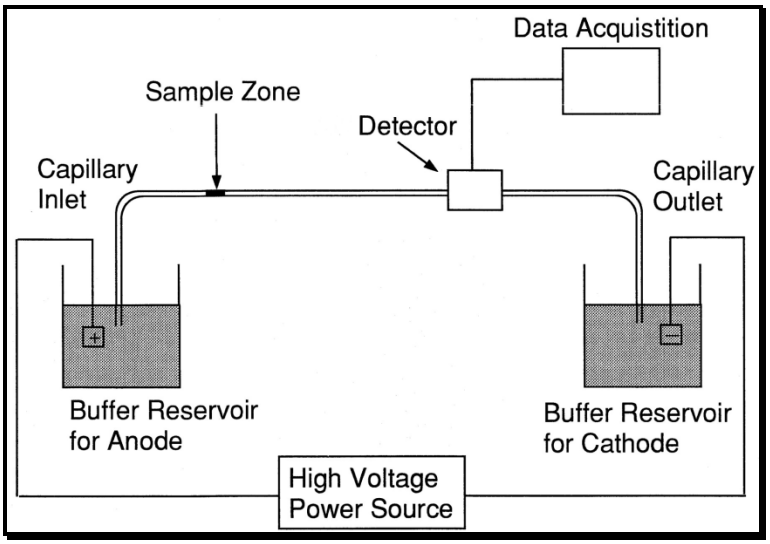

Figure 2. Schematic representation of a capillary electrophoresis system.

The capillary is first filled with the electrolyte. Two electrodes are immersed in the buffer reservoirs to make electrical contact between the high voltage power supply and the capillary. Fused silica capillaries externally coated with a protective layer of polyimide are the primary material employed today. Capillaries with a length of 20 cm to 1 m and internal diameters ranging from 25 to 100 µm id are typically employed in CE [3]. Capillaries should be thermostated to maintain a constant temperature throughout an analysis.

A rigorous pre-conditioning/washing of the capillary should be developed for each analytical method to remove residual absorbates from the capillary wall and to achieve reproducible migration times of the solutes [10].

Sample introduction can be achieved by applying either a pressure difference (hydrodynamic injection) or an electric field (electrokinetic injection) between the two opposite sides of the capillary. To avoid overloading of the capillary with the sample, which can lead to decrease in peak efficiency, the injected volume of sample injected should not exceed 1 % of the total volume of capillary. This corresponds to a few nanoliters (1 – 50 nl), depending on the length and inner diameter of the capillary [14][15].

Hydrodynamic sample injection is the most commonly applied mode. The volume injected by hydrodynamic mode is not dependent on the operating conditions, as there is only an influence of the viscosity of the solutions and the density of the electrolyte in this case. Whereas, with the electrokinetic injection mode, the amount of each ion introduced depends on its effective mobility. Therefore, the amount of analytes introduced differs with the conductivity of sample solutions [16][17]. Nevertheless, this injection can improve the sensitivity by increasing the quantity of analyte injected in some cases [18].

The high-voltage power supply allows voltages up to 30 kV in most CE systems [15]. A range of 15 – 30 kV is generally used in either a positive or negative polarity.

Detection in CE can be performed directly through the capillary wall, thus sample zone broadening is minimum. Several detection modes are applicable in CE including UV-Vis - diode array spectrophotometer, spectrofluorometer, and electrochemical detector. The most commonly used mode is UV spectrophotometry and to a lesser extent fluorimetry (with or without laser source) [19].

Detection limit in UV spectrophotometry depends on the molar absorptivity of the analyte and the optical path length. The latter is limited by the inner diameter of the capillary. Therefore the detection limits are generally between 10^{-4} to 10^{-6} mole/l (10^{-12} to 10^{-14} mole for an injected volume of 10 nl) [19][21].

CE can be directly coupled to mass spectrometry to provide both sensitivity and molecular weight or structural information [20].

4. Modes of operation in CE

The most commonly used modes in CE are CZE and MEKC.

4. 1. Capillary Zone Electrophoresis

Capillary zone electrophoresis is the simplest and most versatile CE mode, in which different solutes in the sample migrate as discrete bands with different velocities. The velocity of each analyte depends on the electrophoretic mobility of the solute which in turn depends on his charge and size as well as the electro-osmotic flow in the capillary [21].

With this mode, the analysis of both small (M_r < 2000) and large molecules (2000 < M_r < 100 000) can be accomplished. Owing to the high efficiency obtained in CZE, separation of molecules having only small differences in their charge-to-mass ratio can be achieved. This separation mode also allows the separation of chiral compounds by addition of chiral selectors to the separation buffer [10].

4. 2. Micellar Electrokinetic Chromatography

MEKC is the most flexible CE mode which can be used for the separation of both neutral and charged solutes, maintaining the efficiency, speed and instrumental suitability of capillary electrophoresis [22][23]. In MEKC, surfactants above their critical micellar concentration (*cmc*) are added into the electrolyte solution to form micelles [24][25]. Separation takes place by different partitioning of the analytes between the aqueous buffer and the pseudo-stationary phase composed of micelles. The technique can therefore be considered as a hybrid of electrophoresis and chromatography. One of the most widely used surfactants in MEKC is the anionic surfactant SDS, although other surfactants, for example cationic surfactants cetyltrimethylammonium salts, are also used [10].

A schematic principle of separation in MEKC is shown in Fig. 3 [11], where anionic surfactant is added to the running buffer. At neutral and alkaline pH, the EOF is stronger (toward the cathode) than the electrophoretic migration of the anionic micelles (toward the anode) and hence, the micelles migrate toward the cathode at a retarded overall migration velocity [35]. In the case of charged solutes, the migration velocity depends on both the electrophoretic mobility of the solute in the absence of micelles, and on the partition coefficient of the solute between the micelle and the aqueous buffer. For neutral solutes, since the analyte has no electrophoretic mobility, the analyte migration velocity will depend only on

the partition coefficient between the micelles and the aqueous buffer. The stronger the solute interacts with the micelles the longer is its migration time. When the solute is not partitioned into the micelles it is simply carried by the EOF. In the electropherogram, the peaks corresponding to each uncharged solute are always between that of the EOF marker and that of the micelles (the time elapsed between these two peaks is called the separation window).

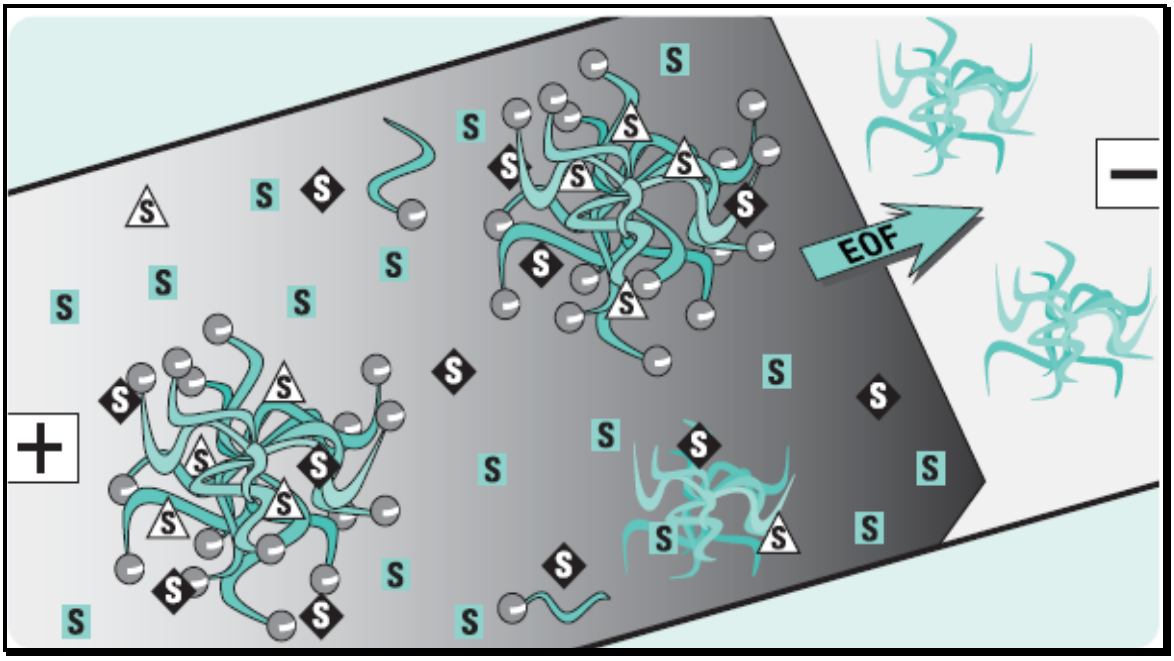

Figure 3: Schematic illustration of the separation principle in MEKC (S = solute).

5. Capillary as preconcentrator and microreactor

CE shows high sensitivity in terms of absolute amounts. However, the limited light-path length and the small amounts of injected samples leads to poor concentration sensitivity in the mM range (when UV detection is used). An increase of the detectability can be obtained by using a more sensitive detector such as LIF or sample pretreatment such as sample preconcentration and chemical derivatization. In recent years, different in-capillary techniques have been developed for either sample preconcentration or derivatization for CE.

5.1. In-capillary sample preconcentration

Numerous strategies have been developed to improve the sensitivity of CE, through the use of on-line sample preconcentration techniques [27][31].

These methods are based on large volume of the sample injection by pressurized or electrokinetic methods and selective concentration of the analyte inside the capillary into a short and sharp zone (stack) before separation.

The principle of most techniques is based on the change in electrophoretic velocity of analyte between two zones, sample solution and BGE caused by different electrical field strengths (field enhanced sample stacking), pH titration (pH dynamic junction) or additive complexation (sweenping) [32][33].

These techniques can be performed individually or in combination and up to 5000 fold-improvement of sensitivity relative to conventional injection method can be achieved.

5.2. In-capillary derivatization

Derivatization is a modification intended to give the analytes of interest more suitable characteristics. Derivatization is mainly used for enhancement of the detection sensitivity in CE. Moreover derivatization can be used to enhance chiral separation, to change the hydrophobicity to enable the use of MEKC separations or to improve mass spectrometric detection.

In-capillary derivatization has several advantages such as low consumption of reagents and samples, minimal sample dilution, high derivatization yield, and full automation of the derivatization step without additional equipment requirement.

In this approach, capillary is not only used as a separation chamber, but also as a microreactor in which derivatization reaction is performed during electrophoresis of the reagents and analytes. The reaction may take place throughout the whole capillary (throughout-capillary), in the middle (zone-passing) of the capillary or at the inlet end (at-inlet) of the capillary [26].

In throughout-capillary derivatization, the sample solution is introduced at the inlet of the capillary previously filled with BGE which contains the reagent. When the separation voltage is applied, the analyte migrates and reacts with the reagent. A relatively large volume of reagent is required compared with the two other modes.

The zone-passing technique is based on passing either the sample or a reagent zone through the other by applying the voltage just after the introduction of the sample solution and the labeling reagent solution into the capillary. The order and site of sample/reagent introduction must be determined based on the magnitude and sign of the mobility and peak intensity varies with passing period of time. This technique is useful for fast kinetic processes of rapid reactions. It is advantageous since only nanoliter volumes of reagent are required without modifying bulk properties of the BGE. Moreover, the zone passing mode is ideal for labile reagents that undergo rapid hydrolysis or side-product formation over extended time scales since reaction times are fast and excess label is electrophoretically removed from the sample due to differential mobility of analyte and reagent zones.

In the at-inlet mode, sample and reagent solutions are introduced to the inlet of a capillary either by tandem mode (*i.e.*, reagent solution–sample solution) or sandwich mode (*i.e.*, reagent solution–sample solution–reagent solution). The reactants in the successive plugs are then mixed by diffusion and allowed to react by standing for a specified period of time. The stability of the reagent in the BGE is highly required in these two modes. Otherwise some byproduct peaks, which would disturb the separation of analyte, can be observed. Using this technique, in-capillary derivatization of amino acids with orthophtaldehyde was achieved in high yields by Taga and Honda [34].

6. Some crucial parameters in CE

6. 1. Migration time

In the presence of an EOF, the migration time of a charged ion is given by the equation [33]:

$$t = \frac{l}{v} = \frac{l \cdot L}{(\mu_{ep} + \mu_{EOF}) \cdot V} \qquad 1.4.$$

where t = the observed migration time (s)

v = the ion migration velocity (cm/s)

l = the effective capillary length (to the detector) (cm)

L = the total capillary length (cm)

A neutral analyte migrates with the EOF and its migration time (t_{EOF}) is determined by:

$$t_{EOF} = \frac{l}{v_{EOF}} = \frac{l \cdot L}{\mu_{EOF} \cdot V} \qquad 1.5.$$

6. 2. Efficiency

The efficiency is generally employed to characterize the zone broadening as in HPLC. Assuming that the only contribution to peak broadening is longitudinal diffusion, it is expressed in number of theoretical plates (N) by:

$$N = \frac{l}{H} = \frac{(\mu_{ep} + \mu_{EOF}) \cdot V \cdot l}{2 \cdot D \cdot L} = 5.54 \cdot \left(\frac{t}{w_{1/2}}\right)^2 \qquad 1.6.$$

$$H = \frac{\sigma^2}{l} = \frac{2 \cdot D \cdot t}{l} \qquad 1.7.$$

where σ^2 = the variance of the migrating zone width,

D = the diffusion coefficient of the analyte,

$w_{1/2}$ = the peak width at half height.

From equation 1.6., efficiency increases with the applied voltage. However, high voltages are responsible of production of heat by the passage of the electrical current (Joule heating) which can give rise to zone broadening. Therefore, it is extremely important to control the capillary temperature [36][37].

Other parameters such as large injection volumes, sample adsorption onto the capillary wall and sample overloading can cause zone broadening and thus loss of efficiency [38].

On the other hand, stacking effects allowing a significant improvement of the efficiency could be obtained by dissolving the sample in water or diluted BGE [39].

6.3. Selectivity

Selectivity ($\propto$) is commonly defined as the ratio of the effective electrophoretic mobilities. This selectivity can be qualified as effective selectivity and only depends on the electrophoretic migration:

$$\propto = \frac{\mu_{ep1}}{\mu_{ep2}}$$

This definition includes the influence of all separation parameters with the exception of the EOF [40][43].

6.4. Resolution

Resolution (*Rs*) between 2 adjacent peaks is most simply defined as in liquid chromatography:

$$R_s = \frac{t_2 - t_1}{4 \times \sigma_t}$$

where t_1 and t_2 = migration times of first and second peak

σ_t = the average standard deviation expressed in time units

In CE, resolution between two peaks can be obtained by modifying the electrophoretic mobility of the analytes, the electro-osmotic mobility induced in the capillary and by increasing the efficiency for the band of each analyte, according to the equation [10]:

$$R_s = \frac{\sqrt{N}\left(\mu_{epb} - \mu_{epa}\right)}{4\left(\bar{\mu}_{ep} + \mu_{EOF}\right)}$$

where μ_{epa} and μ_{epb} = electrophoretic mobilities of the 2 analytes separated,

$\bar{\mu}_{ep}$ = mean electrophoretic mobility of the 2 analytes [10].

6.5. Quantification

Since separation is dependent on analytes migrating at different velocities, the separated analytes will pass the detector at different rates. Therefore, for quantitative CE, corrected areas must be calculated by dividing peak areas by the corresponding migration time in order to:

- compensate for the shift in migration time from run to run, thus reducing the variation of the response,
- compensate for the different responses of sample constituents with different migration times [10].

In order to achieve optimal precision, the use of an internal standard (IS) is usually required to compensate for injection volume variability [2].

Amino acids

Section 1.2

1. Biological interest of D-amino acids

A few decades ago, it was believed that only L-amino acids are present in Mammals. However, with the recent advances in analytical technologies, significant levels of free D-amino acids were found in mammals including humans.

D-Serine was found to play an important role as a neurotransmitter in the human central nervous system by binding of the N-methyl-D-aspartate recptor (NMDAr), which mediates excitatory neurotransmission and cognitive function [44]. D-serine, as a co-agonist of NMDAr, mediates synaptic plasticity, cell migration and neurotoxicity via glycine-binding site [45].

Considering the elevated levels of D-serine in diseased brain and the involvement of excessive activation of NMDAr in a variety of neurodegenerative diseases, such as stroke, amyotrophic lateral sclerosis, Parkinson's disease, Alzheimer's disease, and Huntington's disease, it is probable that D-serine contribute to the pathogeneses of many neurodegenerative disorders. Therefore, D-serine represents a potential and novel target for the development of therapeutic strategies in improving NMDAr-mediated neural injuries, especially those caused by activation of NMDAR via glycine-binding site [44][49].

D-serine may also be involved in the pathogenesis of HIV associated neurocognitive disorders, but the mechanisms for regulating D-serine release and resultant neurotoxicity remain to be determined [50].

The role of D-amino acids in amyotrophic lateral sclerosis pathogenesis was also suggested since D-Serine is elevated both in spinal cord from sporadic cases of amyotrophic lateral sclerosis (ALS) and in an animal model of ALS. Besides, it was shown that a mutation in D-amino acid oxidase (DAO), an enzyme strongly localized to spinal cord motor neurons and brain stem motor nuclei, is associated with familial ALS. In sporadic ALS cases, elevated D-serine may arise from induction of serine synthetic enzyme (serine racemase), caused by cell stress and inflammatory processes thought to contribute to disease progression [51].

As well as D-Ser, D-aspartate acts as an endogenous agonist on NMDAr. Based on this and considering the abundance of D-Asp during prenatal and early life, future studies will be crucial to address the effect of this D-amino acid in the developmental processes of the brain controlled by the activation of NMDArs. In addition, administration of D-Asp to old mice is able to rescue the physiological age-related decay of hippocampal long-term potentiation [52], [53].

Recently, relatively high levels of D-alanine (D-Ala) have been stored in islets of Langerhans isolated from rat pancreas. Additionnally, glucose stimulation of the islets resulted in D-Ala release, suggesting a hormonal role D-Ala [54][55].

Small amounts of D-Ala, D-Proline, D-threonine and D-*allo*-Threonine has been demonstrated in rats and mice by K. Hamase and *col*. Based on these findings they suggest that each D-AA has some physiological role in mammals [56].

There is no doubt that knowledge regarding the interactions of D-amino acids with brain function have markedly increased during the last few decades. However, it is also clear that much still remains to be determined. Research in this field largely depends on progress of the selective and sensitive analytical methodologies.

2. Amino acids derivatization

The amino acids analysis by CE, liquid chromatography or gas chromatography is generally achieved using chemical derivatization. Amino acids are highly polar compounds and, therefore, derivatization can be effectively employed to improve selectivity of conventional reversed-phase high-performance liquid chromatographic (RP-HPLC) or gas chromatographic (GC) analysis and to overcome the lack of a chromophore on many AAs. Since the AAs are chargeable compounds, for their analysis by CE, derivatization step is mainly necessary to improve the sensitivity when the optical detection is used.

Therefore, the choice of a derivatization reagent is crucial, and great demands are placed on its properties. The majority of reagents used react with the amino group. Some reagents react only with primary amines, but ideally secondary amines, such as proline and hydroxyproline, are also covered. Another option is to derivatize the carboxy function [57].

The most common derivatization reagents are listed in Table 1 and their use will be discussed in the following sections.

Table 1: Most usual derivatizing agents for the various separation techniques described throughout this section.

Derivatizing agent	**Abreviation**	**Ref.**
Dinitrophenyl	DNP	[59]
Dansyl chloride	Dns-Cl	[60][63]
6-aminoquinolyl-N-hydroxysuccinimidyl carbamate	AQC	[64]
Naphthalene-2,3-dicarboxaldehyde	NDA	[65], [67]
1,2-naphthoquinone-4-sulphonate	NQS	[70]
7-nitro-2,1,3-benzoxadiazole	NBD-F	[75]
3-(4-carboxybenzoyl)-2-quinolinecarboxaldehyde	CBQCA	[76]
fluorescein isothiocyanate	FITC	[77][79][81]
o-phtaldialdehyde	OPA	[92][93][99]
1-(9-fluorenyl)methyl chloroformate	FMOC	[92], [93]
Chiral reagents		
1-(9-fluorenyl)ethyl chloroformate	FLEC	[71]
R(−)-4-(3-isothiocyanatopyrrolidin-1-yl)-7-(N,N-dimethylaminosulfonyl)-2,1,3-benzoxadiazole	DBD-PyNCS	[72]
o-phtaldialdehyde and N-acetyl-L-Cys	OPA/NAC	[73][74][89]
2,3,4,6-Tetra-*O*-acetyl-β-D-glucopyranosyl isothiocyanate	GITC	[84]
1-fluoro-2,4-dinitrophenyl-5-l-alanine amide	FDAA	[85], [87]
o-phtaldialdehyde and N-isobutyryl-L-Cys	OPA/IBC	[88][90]
(S)-N-(4-nitrophenoxycarbonyl)-Phe methoxyethyl ester	(S)-NIFE	[91][106]

The OPA-thiol and FMOC reagents are the most frequently used derivatization reagents prior to analysis of AAs. With both of these reagents, fluorescent derivatives of high selectivity and sensitivity can be rapidly obtained in aqueous solutions and at ambient temperature [92]. Nevertheless, the FMOC has the advantage of reacting with both primary and secondary amines unlike OPA which reacts only with primary amines [93].

Alternative reagents for AAs derivatization are Dns-Cl, NBD-F, AQC and CBQCA [61][63][75], [76].

A widely used derivatizing agent is FITC. Compared with the previously mentioned agents FITC presents the advantages of higher quantum efficiency and good electrophoretic properties. However, FITC requires longer times of derivatization than the other previously

mentioned dyes. At least 4–18 h of incubation are necessary to obtain stable and well detected FITC derivatives [77][81].

A variety of chiral derivatisation reargents have been also employed to form diastereomeric AA-derivatives, and therefore, allow enantioselective analysis by means of nonchiral reversed phase HPLC or BGE. The most widely used chiral derivatizing agents are compiled in table 1 [82][197].

One group of common chiral labelling reagents comprises aryl chloroformates, which show strong fluorescence, and in most cases ensure a very low detection level of amino compounds. The most important chloroformate is FLEC, developed by Einarsson et al. [83]. The FLEC reagent equally labels primary and secondary amino groups under mild conditions at room temperature in basic solution within 4 min, giving rise to fluorescent and stable diastereomers. An advantage for applications to real samples is the possibility to perform the reaction either in organic solvents or in an aqueous medium.

Isothiocyanates represent another class of optically active reagents for resolution of amino acid enantiomers. The well-known representative of this group is the GITC. This reagent reacts with both primary and secondary amino groups to form the corresponding thiourea derivatives. Compared with FLEC, GITC reacts slowly with AAs (reaction time of 2h at 45°C) [84].

Marfey's reagent or FDAA, is one of the most widely used reagents for indirect enantioselective analysis of amino acids [85][86]. The nucleophilic attack of the α- or β-amino group of the amino acids on the C–F bond results in diastereomeric aniline derivatives with good UV detectability. Harada et al. were the first to analyse several types of FDAA-derivatized proteinogenic AAs and to examine the applicability and limitations of Marfey's method [87]. The neutral amino acids displayed good enantioselectivity, while the separation for hydroxy and acidic amino acid derivatives was insufficiant. Basic amino acids yielded three different derivatives and indicated that an α-amino group is essential for the resolution of both diastereomers. The Marfey's method proved to have wide applicability for standard α-amino acids, except for a few basic amino acids.

Another general reaction for the chiral derivatization of amino acids is their reaction with OPA in combination with chiral thiols to form highly fluorescent isoindole derivatives. The reaction takes place at room temperature in 2 minutes. OPA and chiral thiol system is widely

applied for the determination of the amino acids enantiomers by applying IBC or NAC as chiral thiols [73], [74][88]-[90]. However, the main drawbacks of the method are the inability of OPA reagent to react with secondary amino groups and the derivative instability, which necessitates immediate analysis of prepared samples.

A new "active ester-type" chiral reagent, namely, (S)-NIFE was introduced as chiral derivatizing agent by Peter et al. [91]. (*S*)-NIFE was found to combine excellent sensitivity, good stereoselective properties, and a mild, fast derivatization procedure [106].

R(-)-4-(3-isothiocyanatopyrrolidin-1-yl)-7-(N,N-dimethylaminosulfonyl)-2,1,3-benzoxadiazole DBD-PyNCS was used as a chiral fluorescence tagged reagent for separation of Tyr enantiomers. The proposed method has been successfully applied to the determination of Tyr in a commercial amino acid oral solution [72].

3. Chiral separation

The chiral analysis of amino acids enantiomers is a widely investigated topic in analytical chemistry. Until now, numerous analytical methods using GC, HPLC and CE have been reported [94], [97].

For the separation of chiral compounds, including amino acids, two main strategies have evolved:

- an indirect approach, based on the formation of diastereomers by the reactions of AAs with chiral derivatizing agent and separation of the diastereomeric derivatives in achiral environment, and

- a direct approach performed in chiral environment by the interaction of enantiomers with a chiral selector on a chiral stationary phase or added to the running buffer.

3.1. Enantiomeric separation of amino acids by HPLC method

The majority of the investigations were perfomed using one-dimensional HPLC methods by applying the chiral derivatization reagents or chiral stationary phases.

Since the efficiency of a reversed phase column is usually higher than that of enantioselective column, the separation of diastereomers derivatives by a reverse phase column following the chiral derivatization of AAs is widely utilized.

The OPA reagent in combination with chiral thiol compounds was usually employed for the determination of D-amino acids in biological samples. Initially, the OPA chiral derivatization method was established using N-acetyl-l-cysteine (NAC) as a chiral thiol [98][100]. The OPA-NAC method was applied to mammalian samples for the determination of D-Asp in the testis and pituitary gland of the rat [101] and D-Asp and D-Glu in the liver, kidney and brain of mature rats [102].

The widely used Marfey's reagent FDAA is a very interesting derivatization reagent as it allows successful resolution of DL-amino acids from a mixture. Application of Marfey's reagent and analogs for chiral amino acid analysis was reviewed by Bhushan and Brückner [86].

The use of FLEC was also reported for the enantioselective determination of amino acids. Simultaneous determination of D- and L-amino acids using precolumn derivatization with FLEC and reversed-phase ion-pair high-performance liquid chromatography was achieved in the nervous tissues of crustaceans [103].

Since the determination of trace amounts of D-AAs in real biological matrices has always been interfered with thousands of substances, highly selective analytical method is needed. Therefore, 1D-HPLC-MS/MS combining diastereomer separation using reversed-phase HPLC or enantiomer separation using chiral stationary phase and the highly selective determination using MS or MS/MS detectors have been established.

A pre-column derivatization using OPA in combination with the chiral thiol isobuteryl-L-cysteine (IBLC) has been applied in UHPLC-MS for the enantioseparation and selective detection of D-AAs in complex biological samples (serum, plasma, urine and gut) [105].

(S)-N- (4-Nitrophenoxycarbonyl)-l-phenylalanine-2-methoxyethyl ester ((S)-NIFE) has also been reported, using a common UHPLC-MS/MS instrument equipped with a C-18 reverse-phase column [106]. By using this method, the D-enantiomers of all chiral proteinogenic amino acids can be quantified in human biological fluid.

Several chiral stationary phases have been also used for the determination of D-amino acids. A Pirkle-type chiral stationary phase, Sumichiral OA-2500S, was used for the enantioseparation of 4-fluoro-7-nitro-2,1,3-benzoxadiazole (NBD-F) derivatized aspartate [104].

Enantiomers of 19 succinimidyl ferrocenyl propionate derivatized amino acids were separated by a cinchona alkaloid-based chiral stationary phase, Chiralpak QD-AX, and selectively determined by MS/MS detection [107].

A sensitive and selective one-dimensional chiral HPLC-MS/MS method for the chiral separation of all proteinogenic amino acids derivatized with the succinimide ester of 6-methoxyquinoline-4-carboxylic acid has been reported [115].

Numerous two-dimensional systems combining reversed-phase separation and an enantioselective separation have been established for the selective determination of small amounts of specific D-amino acids.

Phe, Tyr, Trp and Leu were isolated by using an ODS column in their native forms, and the fraction was introduced to a chiral crown ether column (Crownpack Cr (+)) to separate the enantiomers. Fluorescence detection of amino acids post-column derivatized with OPA plus mercaptoethanol was then performed [108], [109].

A 2D-HPLC system combining an ODS column and a β-cyclodextrin (CD) bonded enantioselective column was reported for the determination of precolumn derivatized FMOC-Pro enantiomers [110].

Coupled column HPLC system combining a reversed-phase and a pirkle-type enantioselective column following the precolumn labeling with NBD-F have also been reported for the determination of Ser [111], [112].

For the simultaneous determination of multiple D-AAs in biological samples, the multiloop two-dimensional column switching strategy was developed. This system consists of a nonenantioselective narrow-bore RP column for AA preseparation followed by a multiloop valve device for trapping, retaining and transferring particular peak fractions onto chiral column.

A multi-loop 2D-HPLC method was employed for the simultaneous determination of 10 hydrophilic D-AAs (His, Asn, Ser, Gln, Arg, Asp, Gly, Allo-Thr, Glu and Thr) precolumn derivatized with NBD-F. A long microbore-monolithic ODS column (0.53 mm ID x 1000 mm) was used for the first dimension. The automatically collected fractions of the target peaks were then transferred to the second dimension consisting of a narrow-bore-pirkle type (Sumichiral OA 2500 S 1.5 mm ID x 250mm) enantioselective column [114]. This method was also employed to the simultaneous determination of N-methyl-D-aspartic acid analogues in mammals and bivalves [113].

3.2. Enantiomeric separation of amino acids by CE method

CE represents a very attractive tool for chiral separation and it is currently the most extensively applied technique to the enantioselective analysis of AAs [116]. Various ways, including the use of cyclodextrins and their derivatives, ligand exchange, chiral surfactant, and chiral derivatization have been described to separate AA enantiomers by CE and MEKC.

Cyclodextrins

The first successful capillary electrophoresis (CE) separations of AA enantiomers were accomplished using with cyclodextrins (CDs) as enantioselective discriminators in the running electrolyte. CDs are cyclic oligosaccharides composed of α-1,4-linked glucose units. Many ring sizes are available: α-CDs, ß-CDs, γ-CDs and δ-CDs containing respectively six, seven, eight and nine α-D-glucopyranoside units. CDs have many desirable features such as good enantioselctivity, optical transparency in the short wavelength UV-light and have good water solubility. CDs have a three-dimensional structure usually described as open conical cylindrical molecules with relatively hydrophobic cavity in the middle and a relatively hydrophilic surface on the outside. This structure facilitates enantioselectivity by formation of inclusion host-guest complexes, with one enantiomer being preferred over the other owing to better steric fitting. In addition to the hydrophobic inclusion of the analytes, hydrogen bonding with the groups of the CD rim affects separation.

Native CDs and their many derivatives have been extensively used for the enantioseparation of a wide range of chiral analytes and are the most frequently used selectors for enantiomeric separation of AAs. Since the size of AA derivatives correlate with the size of CD cavity, derivatization influences the enantiomeric separation. In addition to CD type and concentration, enantioselectivity is also influenced by the buffer, pH, capillary temperature, applied current, and velocity of EOF [117].

While α-CDs are suitable for the enantioseparation of native Trp [118][119], AAs labeled with Dns-Cl, FITC, OPA and FMOC could be resolved by β-CD [120][121] and γ-CD [122][123]. In addition, δ-CD has been applied to separate dansyl and FMOC derivatives of several AAs. Since the ring size of δ-CD is different from γ-CDs, provided similar or quite different enantiomeric resolution depending on the structure of the analytes [124]. Derivatized neutral CDs such as hydroxypropyl-β-CD (HP-α-CD), HP-β-CD, methyl-β-CD (Me-β-CD)

and dimethyl-β-CD (DM-β-CD) have been also employed and gave higher selectivity for amino acid racemates than the natural CDs [125][128].

The charged CDs were widely employed for the enantioseparation of AAs. As ionized CDs work as a pseudostationary phase, their interaction with AA racemates results in a different velocity of the analytes from a surrounding phase due to the electrophoretic migration of the ionic CDs. Among various anionic [68][69][129][133][134][152][153] and cationic CDs [59], [154][174] employed for chiral separation of AAs, sulfated CDs [129][130] and highly sulfated CDs (HS-CDs) [68][69][131][134] are still the major applied chiral selectors.

For resolving AA enantiomers in complicated sample matrices, CD-MEKC mode was largely used. In many applications of CD-MEKC, a neutral or basic buffer (pH 7–10) containing both SDS and β-CD is used as BGE [135][148]. CDs derivatives such as HP-β-CD [148][150], DM-β-CD [150] and HP-γ-CD [151] have also been employed in the CD-MEKC mode.

Ligand-exchange

Ligand-exchange CE was also applied to several enantioseparations of AAs. Since the stability constants of the diastereomeric mixed metal complexes between the analytes and the chiral selector ligand are different, the enantioseparation is due to the difference in migration velocities between the target racemates. In general, chiral metal ion complexes are formed using Cu(II) and Zn(II) cations as the central metal; l-AAs and l-hydroxy-Pro as the chiral selector ligand. The simplest method consists of adding both metal ions and chiral selector ligand into the BGE.

In addition to l-amino acids and l-hydroxy-Pro, (S)-3-aminopyrrolydine [175], l-amino acid amides [176], d-sugar acids [177] and1-alkyl-3-methylimidazolium l-Pro [178] have been used as the chiral selector ligands for the separation of native, DNS-, FMOC-labeled amino acid racemates.

Chiral surfactants

Enantioseparation by CE may be improved by means of dual chiral selector system that contains a chiral surfactant in addition to the CD selector. Chiral surfactants such as sodium taurocholate (STC) and sodium taurodeoxycholate (STDC) bile salts have been utilized [80][179][186].

As an example, the use of a mixture of 20 mM β-CD and 30 mM STC with 80 mM borate buffer (pH 9.3) gave baseline resolutions of 20 pairs of FITC-labeled AAs enantiomers. A simultaneous separation of only 6 pairs of the enantiomers could be obtained [80].

Enantioselective MEKC separation of amino acid enantiomers derivatized with AQC was realized by Swartz *et al.* using a buffer consisting of 25 mM $Na_2HPO_4/Na_2B_4O_7$, at pH 9.0, with 100 mM (*R*)-*N*-dodecoxycarbonylvaline (a synthetic surfactant). Twelve pairs of AQC-derivatized AAs were enantioresolved and, interestingly, both chiral and achiral separations could be achieved simultaneously [64].

Chiral derivatization agents

MEKC separations of AA enantiomers after their derivatization with chiral reagents, such as FDAA, OPA/chiral thiol and FLEC, were also reported [71][187][188].

Baseline resolution of seven pre-column labeled L-FDAA-AA racemates (Ala, Val, Leu, Asp, Glu, Phe, and Trp) was achieved within 23 min [187]. Indirect enantiomeric separations were also achieved by using MEKC after precapillary derivatization with FLEC. Either a 10 mM sodium phosphate (pH 6.8), or a 5 mM buffer (pH 9.2), each containing 25 mM SDS and 10–15% ACN were used. All FLEC-derivatized AAs (Ser, Ala, Val, Met, Leu, Trp, and Phe) and their diastereomeric pairs were resolved. The FLEC derivatives of Glu, Asp, and Pro were separated using a 10 mM sodium citrate buffer, pH 4.4 [71].

In-capillary derivatization applications

Several new studies report the use of in-capillary derivatization, applying various reagents, to simplify the sample preparation work.

As a successful application of in-capillary derivatization, AQC was used and successive injections of ACN (2 s), derivatizing agent (10 mM AQC, 3 s), and sample in borate (1:1 v/v, 6 s) in tandem (at 50 mbar) was realized. A washing step with DMSO between injections was necessary to minimize the initial current problems due to the formation of precipitates into the capillary. A dual CD system of 5% highly sulfated-β-CD and 2% acetylated-γ-CD was employed to the enantioseparation of 18 protein amino acids and Orn labeled with AQC. The developed method showed appropriate analytical characteristics (linearity, LOD and LOQ, precision, absence of matrix interferences, and accuracy). The enantiomers of Arg, Lys, and Orn were determined in dietary supplements and wines [68][69].

On column labeling technique and chiral ligand-exchange CE with Zn(II)-L-Arg complex as a chiral selector was described for analysis of dansylated AA enantiomers. It was achieved using a sequential injection of buffer (100 mM boric acid, 5 mM ammonium acetate, 3 mM $ZnSO_4$, and 6 mM L-Arg, pH 8.4, 10s), Dns-Cl solution (1.5 mM) 3s, AA solution (24s), and buffer (10s) at 0.2 psi. After injection, 35 min reaction time was allowed in the capillary. Using the above mentioned buffer, nine pairs of AA enantiomers were baseline-resolved, with LODs reported to be approximately 9 µM using UV detection (214 nm) [60].

A binary chiral selector system with a mixture of ß-CD and STDC was also used to separate 19 pairs of in-capillary FMOC-labeled AAs. By using running buffer composed of 150 mM borate, 30 mM ß-CD, 30 mM STDC, and 15 % isopropanol (pH 9.0) 17 pairs that were fully enantioresolved and two pairs that were partially resolved [178].

In-capillary chemical derivatization using OPA/NAC with on-line sample preconcentration was also developed. Using this method, direct CE analysis of D-AAs in a 95% enantiomeric excess mixture with submicromolar detection limits (using UV detection) was achieved. The method was then applied to study the enantioselective AA flux in *E. coli* bacteria cultures which demonstrated a unique L-Ala efflux into the extracellular medium [188].

Aim and scope

Chapter 2

During the last decade, there has been a growing interest in chiral analysis of amino acids (AAs) since several D-forms are thought to play a major role in a number of neurodegenerative diseases. To promote the progress of research on the detection of D-AAs in biological matrix and better understanding of their pathophysiological roles, easy to use and highly selective chiral methods have still to be developed.

Capillary electrophoresis is considered as an appropriate analytical technique for this type of separation. As most AAs do not contain chromophore groups, in-capillary derivatization seems to be an interesting new approach for their detection.

The aim of this research work is to develop a CE method for AA chiral analysis, which combines high chemo- and enantioselectivity with the full automation of in-capillary derivatisation procedure.

The first part of the present thesis aims to develop a new effective method to further improve the chiral separation performance of mixtures of L- and D-AAs using in-capillary derivatization with FMOC. Firstly, the selection of the basic experimental conditions to separate efficiently the FMOC-AAs enantiomers will be performed using pre-capillary derivatisation. Experimental conditions for in-capillary derivatization will then be optimized. A special focus will be addressed on improving separation chemoselectivity. Moreover, the BGE composition will be optimized in order to maximize resolution between both enantiomers and AAs in mixture.

In the second part of this work, FLEC will be used as chiral labeling reagent. In-capillary derivatization conditions will be optimized by experimental design aiming to provide the best derivatization performance. A comparison of pre- and in-capillary derivatization performances will be realized. Optimal background electrolyte composition for the separation of in-capillary formed FLEC-AAs will then be determined using a multivariate approach.

Finally, the applicability of the method to cerebrospinal fluid (CSF) will be evaluated through the analysis of spiked artificial CSF.

Results

Chapter 3

Chemo-and enantio-selective method for the analysis of amino acids by capillary electrophoresis with in-capillary derivatization

Section 3.1

Preface

CE has become one of the most powerful analytical tools for the chiral determination of AAs because it offers the advantages of rapidity, very high peak efficiency while requiring very small sample sizes. In addition, CE represents a versatile platform for integrating sample pretreatement, namely in-capillary derivatization, which has several advantages, such as low consumption of reagents, sample, short reaction time, minimal sample dilution and the possibility of automation. This last feature is particularly interesting for analysis of AAs that lack intrinsic chromophores (except aromatic AAs). Among the various derivatization agents, 9-fluoroenylmethyl chloroformate (FMOC) has attracted our attention as it has good and rapid reactivity with both primary and secondary amines.

In this work, the in-capillary derivatization of AAs with FMOC and the subsequent chiral separation of AAs were investigated by CE. Special attention was paid to the optimization of the factors having a significant influence on the enantioselectivity but also the chemoselectivity of AAs including the use of cyclodextrins, organic modifier and surfactant.

Chemo-and enantio-selective method for the analysis of amino acids by capillary electrophoresis with in-capillary derivatization

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Running title: Enantioseparation of amino acids by CE with in-capillary derivatization

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Abstract

A novel dual chiral CE method was developed for the separation of L- and D- amino acids (AAs), using in-capillary derivatization with 9-fluoroenylmethyl chloroformate (FMOC). Firstly, using pre-column derivatisation, the enantioseparation of FMOC-AAs was optimized according to the nature of cyclodextrins (CD). A background electrolyte (BGE) composed of 30 mM β-CD, 30 mM octakis(2,3-dihydroxy-6-*O*-sulfo)-γ-CD (OS-γ-CD), 40 mM tetraborate and 15% isopropanol (IPA) was selected and led to 17 baseline resolved pairs (Rs = 1.7–5.8) and two partially resolved pairs (Lys, Rs = 0.5 and Arg, Rs = 1.2). Experimental conditions for in-capillary derivatization were then optimized. Several parameters, such as mixing voltage and time, concentration of labelling solution and the length of the spacer plug were studied. The optimal conditions for in-capillary derivatization procedure were obtained using successive hydrodynamic injections (30 mbar) of AAs for 2 s, borate buffer for 4 s and 10 mM FMOC solution for 6 s, followed by a mixing at 3 kV for 72 s and wait time of 1 min. Moreover, a particular attention was paid to improve separation chemoselectivity. The effect on stereoselectivity and chemoselectivity of different factors, such as decrease of pH and tetraborate concentration and the addition of sodium dodecyl sulfate (SDS), was investigated using the in-capillary derivatization procedure. The best separation of a standard mixture of ten AA racemates was observed using a BGE containing 30 mM β-CD, 30 mM OS-γ-CD, 25 mM SDS, 40 mM sodium tetraborate and 17% IPA.

Keywords: Amino acids; chiral capillary electrophoresis; in-capillary derivatization.

1. Introduction

Increasing attention is currently being paid to the chiral analysis of amino acids (AAs) since D-forms are believed to represent an important class of low abundance chiral metabolites with biological significance [46][48][189][192]. As D-AAs are expected to be potential drug candidates and biomarkers, their separation from L-enantiomers has been extensively studied and many biological and food applications of AAs enantioseparations have been published , [96], [106][107][120][150][166][174][183][193][200]. Analysis of D-AAs may be used as a diagnostic tool and these chiral metabolites might also be administrated to treat diseases [46], [189][191]. For example, D-Serine (D-Ser) has been recently identified as neurotransmitter and an endogenous ligand for N-methyl-D-aspartate (NMDA) receptors in the brain [47], [48]. It has also been shown on *in vitro* assays that aggregation of amyloid beta is stereospecific, suggesting the role of D-AAs in neurodegeneration [192].

Despite the progress made, chiral analysis of AAs remains an analytical challenge. Many liquid chromatographic methods using chiral stationary phases or chiral derivatization reagents were applied to the separation of AA enantiomers [94][106][195], [199]. These methods often show rather good enantioselectivity but limited chemoselectivity due to low peak efficiencies. That is why a second chromatographic dimension or mass spectrometry (MS) was sometimes necessary to increase selectivity for the analysis of AAs mixtures [94][106]. 2D-gas chromatography-MS has also been successfully used for enantioseparation and quantification of 8 D-AAs in serum [199]. Beside this, chiral capillary electrophoresis (CE) can be a valuable alternative, based on high peak efficiency, enormous resolving power and small sample and reagents volume consumption. Nevertheless, the low injected amount has to be compensated for by the use of a sensitive detector such as laser induced fluorescence (LIF) or MS. Progress in CE and micellar electrokinetic chromatography (MEKC) analysis of AAs has been recently reviewed by Kitagawa and Viglio, respectively [96][200].

Except for the three aromatic AAs (i.e. tryptophan, phenylalanine and tyrosine), the remaining AAs need to be derivatized when UV detection is applied due to their lack of chromophore groups. Recently, in-capillary derivatization has received increasing attention due to its remarkable advantages, i.e. low consumption of reagents and samples, high derivatization efficiency, short reaction time, minimal sample dilution and the possibility of automation. Up to now, many derivatization agents, such as dansyl chloride (Dns-Cl) [60], 3-(2-

furoyl)quinoline-2-carboxaldehyde (FQ) [203][204], 6-aminoquinolyl-*N*-hydrosuccinimidyl carbamate [69], naphthalene-2,3-dicarboxaldehyde (NDA) [149][205], *ortho*-phthalaldehyde/N-acetyl L-cysteine (OPA/NAC) [188], 1,2-naphtoquinone-4-sulfonate (NQS) [206] and 9-fluoroenylmethyl chloroformate (FMOC) have already been used for in-capillary derivatization of AAs. FMOC is a cheap chemical that can rapidly react with very dilute amino compounds to yield stable derivatives. Under alkaline conditions, it can react with primary and secondary amines and so it can be applied to all common AAs [207][210].

However, a simultaneous separation of extended mixture of L- and D- AAs has been found in few papers [69]. The enantiomeric separation of ornithine in complex mixtures of AAs, using a dual cyclodextrine (CD) system containing highly sulphated-β-CD (HS-β-CD) and acetylated-γ-CD in phosphate buffer pH 2, was reported by Marínez-Girón et al. [69]. Wan et al. achieved the enantioseparation of a mixture of 13 DL-FMOC-AAs, off-line derivatized, using CD-MEKC containing 50 mM sodium dodecyl sulfate (SDS) and 10 mM β-CD in 50 mM phosphate buffer (pH 7.5) and 15% isopropanol (IPA) [211]. Baseline chiral separation of a mixture of 8 pairs of FMOC-AAs was achieved in one run, with application of in-capillary derivatization, using dual chiral selector system consisting of β-CD and sodium taurodeoxycholate in sodium tetraborate buffer (pH 9) [189].

The main purpose of this work is the development of an effective approach to further improve the chiral separation performance of mixtures of L- and D-AAs, using a dual chiral selector system in combination with in-capillary derivatization with FMOC, in order to obtain a fully integrated system for the chiral analysis of AAs by CE.

2. Experimental

2.1. Instrumentation

All experiments were carried out on a $HP^{3D}CE$ system (Hewlett-Packard, Waldbronn, Germany) equipped with an autosampler, an on-column diode-array detector and a temperature control system (15-60 °C ± 0.1 °C). A CE Chemstation (Hewlett-Packard) was used for instrument control, data acquisition and data handling. Fused silica capillaries were provided by Thermo Separation Products (San Jose, CA, USA).

2.2. Chemicals and reagents

The D,L-AAs (alanine, valine, leucine, isoleucine, proline, phenylalanine, methionine, tryptophan, serine, threonine, asparagine, glutamine, aspartate, glutamate, cysteine, tyrosine, histidine, lysine and arginine) and FMOC were purchased from Sigma-Aldrich (Saint-Louis, MO, USA). Sodium tetraborate was supplied by Merck (Darmstadt, Germany) and SDS by Acros-Organics (Geel, Belgium).

β-CD, γ-CD, heptakis(2,3,6-tri-*O*-methyl)-β-CD (TM-β-CD), heptakis (2,6-di-*O*-methyl)-β-CD (DM-β-CD), carboxymethyl-β-CD (CM-β-CD) and HS- β -CD were from Sigma-Aldrich (Saint-Louis, MO, USA). Heptakis(2,3-di-*O*-acetyl-6-*O*-sulfo)-β-cyclodextrin (HDAS-β-CD) was obtained from Antek Instruments (Houston, TX, USA). Octakis (2,3-dihydroxy-6-*O*-sulfo)-γ-CD (OS-γ-CD) was a gift from G.Vigh (Texas A&M university, College Station, TX). Water used in all experiments was of Milli-Q quality (Millipore, Bedford, MA, USA). Background electrolytes (BGEs) and samples solutions were filtered through a Polypure polypropylene membrane filter (0.2 µm) from Alltech (Laarne, Belgium) before use.

Running buffers were prepared by dissolving sodium tetraborate and required additives in water. The pH was adjusted to 9.2 with 1 M boric acid or 1 M sodium hydroxide.
FMOC solution was prepared in acetonitrile and stored at 4°C.

2.3. Electrophoretic technique

Electrophoretic separations were carried out with uncoated fused silica capillaries having 50 µm internal diameter and 48.5 cm length (40 cm to the detector). A new capillary was conditioned with 1 M sodium hydroxide and water, each for 15 min. At the beginning of each working day, the capillary was washed with 1 M sodium hydroxide, water and the BGE, each for 15 min. At the end of each working day, the capillary was rinsed with 1 M sodium hydroxide and with water each for 15 min. Before each injection, different capillary washing sequences were applied according to the derivatization mode; when precapillary derivatization is performed, the capillary was washed with 1 M sodium hydroxide, water for 2 min and then equilibrated with the BGE for 2 min. For in-capillary derivatization, the capillary was washed successively with water for 1 min, acetonitrile for 2 min (to remove the excess of the derivatization agent), water for 1 min, 1 M sodium hydroxide for 2 min and then equilibrated with the BGE for 2 min. Capillary wash cycles were performed at a pressure of approximately 1 bar. The applied voltage was 20 kV and UV detection was performed at 210, 250, 260 and 280 nm.
Injections were made by applying a pressure of 50 mbar for a period of 3 s (corresponding to 1.9 nL) and the capillary was thermostated at 25 °C.

2.4. Precapillary derivatization

Precapillary derivatization of AAs with FMOC was performed as described in ref [48]. One ml of 10 mM FMOC was mixed with 1 ml of 3 mM AA in 40 mM sodium tetraborate (pH 9.2). After reacting at room temperature for 2 min, the mixture was extracted with 2.5 ml of pentane to remove the excess reagent. The solution was then diluted ten times with water and stored at 4°C for one month.

2.5 In-capillary derivatization

Derivatization of AAs with FMOC was performed directly in the capillary by sequential injection of 3 mM AA in 40 mM tetraborate solution for 2 s, 40 mM tetraborate buffer for 4 s and 20 mM FMOC labeling solution in acetonitrile for 6 s at 30 mbar. After each injection of sample or FMOC, the inlet end of the capillary was washed by immersion in a vial containing milli-Q water. Then, vials containing the separation buffer were placed at both ends of the

capillary and the sandwich sections were mixed by applying a voltage of 3 kV (mixing voltage) for 72 s (mixing time). After mixing, the derivatization reaction was allowed to proceed for 1 minute (reaction time) without voltage. Once the reaction time elapsed, the separation was performed at 20 kV.

2.6 Calculation method

The resolution (R_s) of the enantiomers, calculated according to the standard expression based on the peak width at half height, was obtained from the Chemstation software [208].
To estimate the ratio of derivatization (Y), the following equation was used [209]:

$$Y = \frac{A_{N\ FMOC\text{-}Trp}}{A_{N\ FMOC\text{-}Trp} + A_{N\ Trp}} \times 100 \qquad (1)$$

Where $A_{N\ FMOC\text{-}Trp}$ and $A_{N\ Trp}$ are normalized peak areas of derivatized tryptophan (FMOC-Trp) and tryptophan, respectively.
Fmoc-Trp and Trp having different absorptivities, the equation (1) is only used to provide a rough estimation of the derivatization yield.

3. Results and discussion

3.1. Optimization of the chiral separation

A pre-capillary derivatization step using FMOC was first performed. FMOC was selected as it is a fast labeling agent producing stable AA derivatives. Preliminary experiments concerning BGE composition (ie. sodium tetraborate concentration) were carried out with ten D-, L-AAs, namely Glu, Asp, Leu, Ser, Met, Phe, Trp, Tyr, Ala and His. The selected BGE was made up of 40 mM sodium tetraborate at pH 9.2. The FMOC-derivatization seems to favour chiral recognition by the CD. Indeed, under the conditions leading to a complete separation of FMOC-Trp and FMOC-Phe enantiomers, no enantioseparation of underivatized Trp and Phe was observed. M.C. Waldhier et al. already suggestted that bulkier AAs, obtained by derivatization, give rise to enantioselective interaction with the CD cavity [197]. Furhtermore, L.A. Riddle also showed that the fluorescent tag affected the migration order of the enantiomers, when CDs are used as selectors [214].

Two native CDs, namely β-CD and γ-CD, two neutral derivatives of β-CD; i.e. TM-β-CD and DM-β-CD, as well as charged CD derivatives (CM-β-CD, HDAS-β-CD, HS-β-CD and OS-γ-CD) were tested as chiral selectors for the enantioseparation of FMOC-AAs.
Because of the low solubility of β-CD in water, a concentration of 20 mM was tested. For the sulfated CDs (OS-γ-CD, HDAS-β-CD and HS-β-CD), a concentration of 20 mM was also used in order to avoid high current values (>120 µA). Other CDs were tested at 30 mM.
No enantiomeric resolution was observed with HDAS-β-CD. As can be seen in Table 1, the highest resolution values were obtained with OS-γ-CD for all tested FMOC-AAs, except for tryptophan and phenylalanine which were enantioseparated only with TM-β-CD.

Table 1. Influence of the nature of the CD on FMOC-AA enantiomeric resolution.

Analytes	**Cyclodextrin**					
	β-CD	γ-CD	DM-β-CD	TM-β-CD	HS-β-CD	OS-γ-CD
	20 mM	30 mM	30 mM	30 Mm	20 mM	20 mM
Ser	-	-	-	-	-	**1.2**
Asp	-	-	0.5	-	-	0.5
Glu	-	-	0.5	-	0.5	**1.7**
Leu	0.5	-	-	-	1.0	**3.3**
Ala	0.5	-	-	-	0.9	**1.5**
Tyr	-	0.5	-	-	-	**2.7**
Trp	0.5	-	-	**1.4**	-	-
Phe	-	-	-	**1.7**	-	-
Met	-	-	-	-	-	**2.1**
His	-	-	-	-	-	**2.4**

-, no enantiomeric separation observed (R_s < 0.5)

BGE: 40 mM sodium tetraborate and 20 or 30 mM CD; other conditions as described in Section 2.

Dual CD systems were then tested to enhance the enantioseparation. Combinations of 20 mM OS-γ-CD and TM-β-CD (from 5 to 20 mM) were first applied. Unfortunately, as shown in Fig. 1, no optimum for all AAs could be obtained. For example, the enantioresolution of phenylalanine increases while that of serine decreases with the increase of TM-β-CD concentration. Indeed, the mobility difference between a pair of enantiomers can be enlarged by the addition of the second chiral selector provided the effect of the latter on the mobility of the enantiomers is different from that of the first selector [210][211][212][213][214].

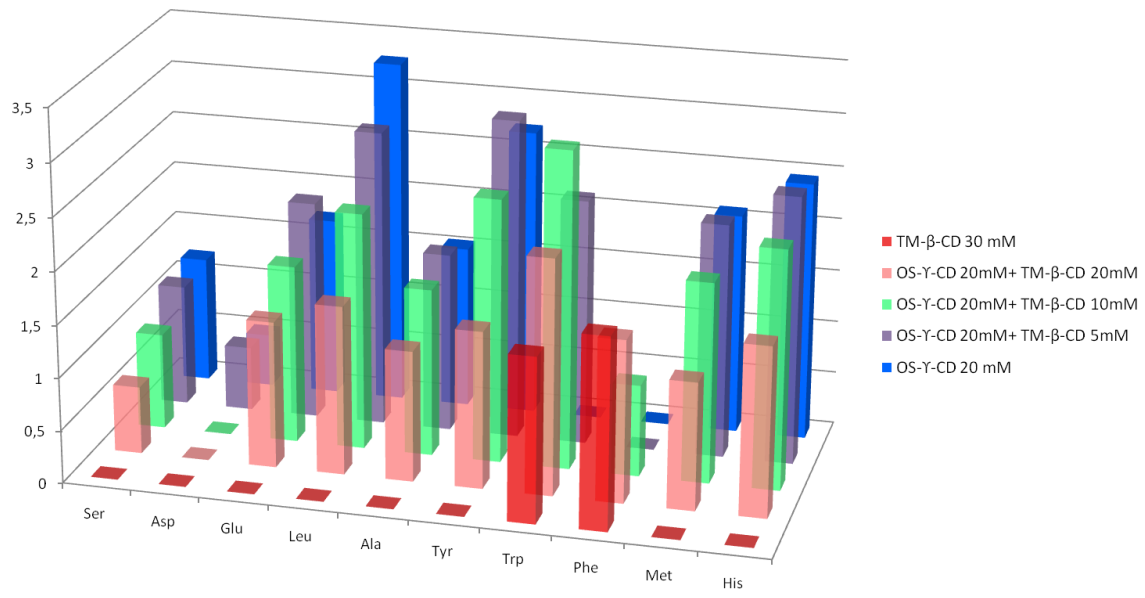

Figure 1. Effect of the association of 20 mM OS-ɣ-CD with TM-β-CD (from 5 to 20 mM) on enantioresolution of FMOC-AAs. BGE: 40 mM sodium tetraborate.

Since a compromise for all AAs could not be found with OS-γ-CD and TM-β-CD, higher concentrations of β-CD and OS-γ-CD were tested in the presence of an organic solvent not only to enhance the β-CD solubility but also to prevent too high current values generated with OS-γ-CD. Among the various organic solvents tested (methanol, acetonitrile, ethanol and IPA) the highest enantioresolution values were observed with a BGE containing 15% IPA. A combination of β-CD and OS-γ-CD (each at 30 mM in the BGE containing 15% IPA) was found to lead to the complete enantioseparation of the ten pairs of AAs (*cf*. Fig. 2). The corresponding electropherograms are shown in Fig. 3.

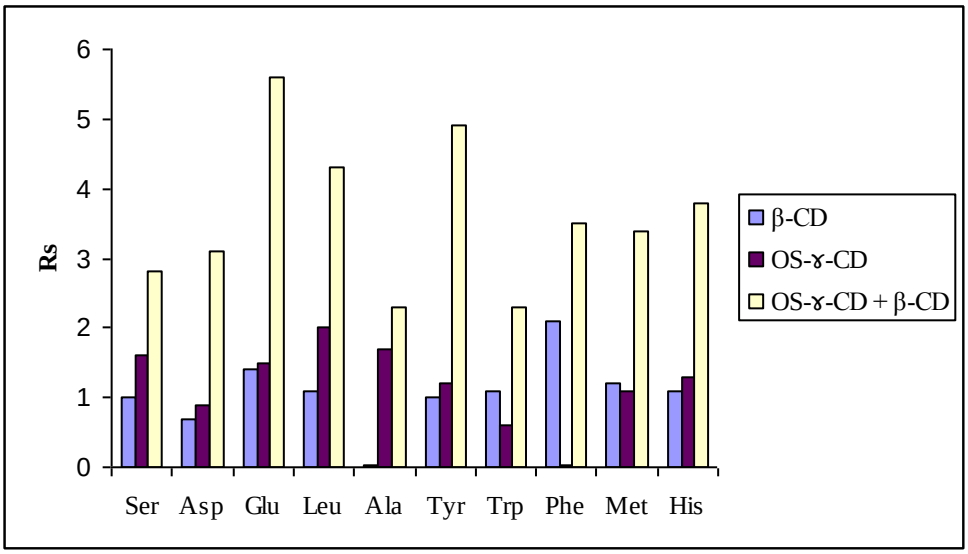

Figure 2. Influence of the association of β-CD and OS-γ-CD on enantiomeric resolution.

BGEs: (A) 30 mM β-CD (B) 30 mM OS-ɣ-CD and (C) 30 mM β-CD and 30 mM OS-ɣ-CD in 40 mM sodium tetraborate containing 15% IPA. Other conditions as described in Section 2.

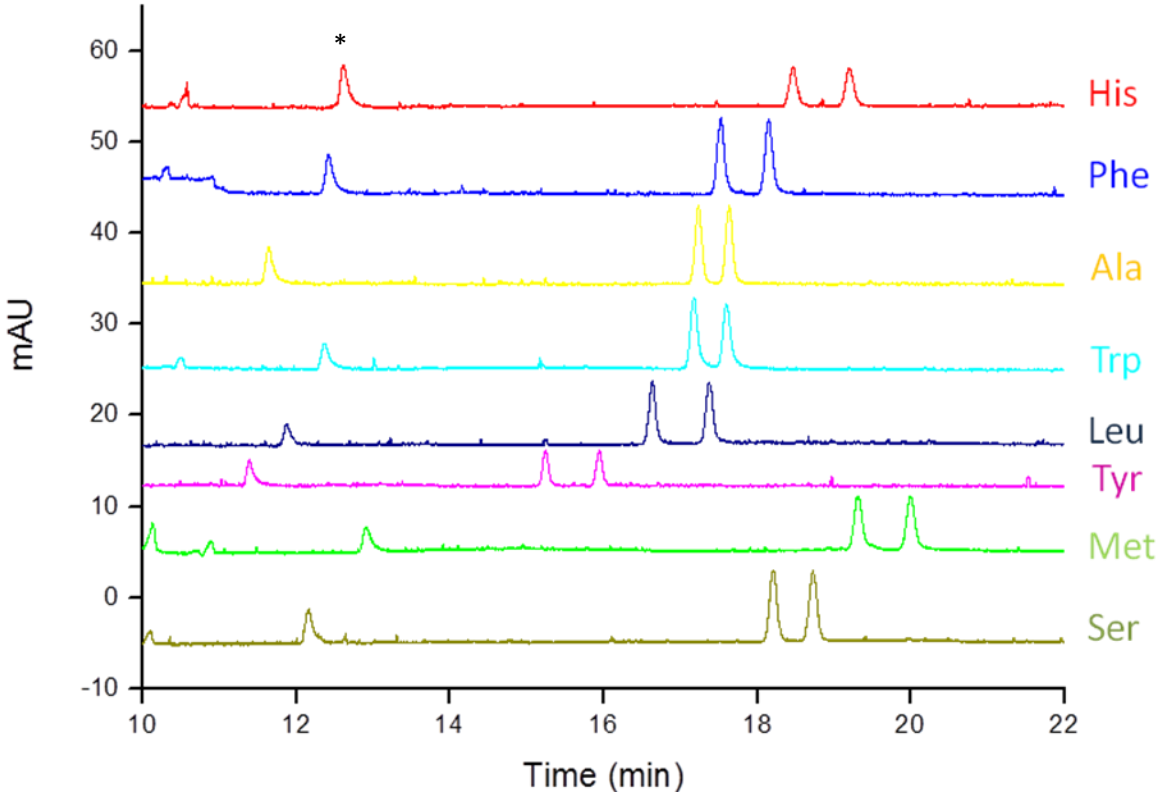

Figure 3. Electropherograms showing enantioresolution of FMOC-AA.

BGE: 30 mM β-CD and 30 mM OS-ɣ-CD in 40 mM sodium tetraborate containing 15% IPA.

*: peak corresponding to the FMOC excess remaining in the FMOC-AA solution.

Detection at 210 nm ; Other conditions as described in Section 2.

The selected conditions were then applied to all proteinogenic chiral AAs. As can be seen in Table 2, except for Lys (Rs = 0.5) and Arg (Rs = 1.2), all pairs of FMOC-AAs were baseline resolved (Rs = 1.7-5.8). Under these conditions the D-enantiomers migrated first.

3.2. Optimization of the in-capillary derivatization conditions

In-capillary derivatization of AAs with FMOC can be achieved by successive introductions of AA, borate buffer and FMOC labeling solution followed by application of voltage, as illustrated in Fig. 4. The following injection sequences were examined: FMOC-sample, sample-FMOC, FMOC-sample-FMOC and sample-buffer-FMOC. It was found that AAs have to be injected ahead of FMOC, otherwise no peak could be detected. Moreover, it was shown that introducing a plug of high pH borate buffer between the sample and FMOC plugs increases the derivatization performance, as already demonstrated in [186]. This can be explained by the deprotonation of the AA during their migration in the plug of borate buffer, which is crucial for their labelling with FMOC [207]. In fact, under these conditions, FMOC migrates with the EOF, faster than the anionic AAs. Therefore, it comes in contact with the AAs and then quickly moves away from the AA plug once the voltage is applied.

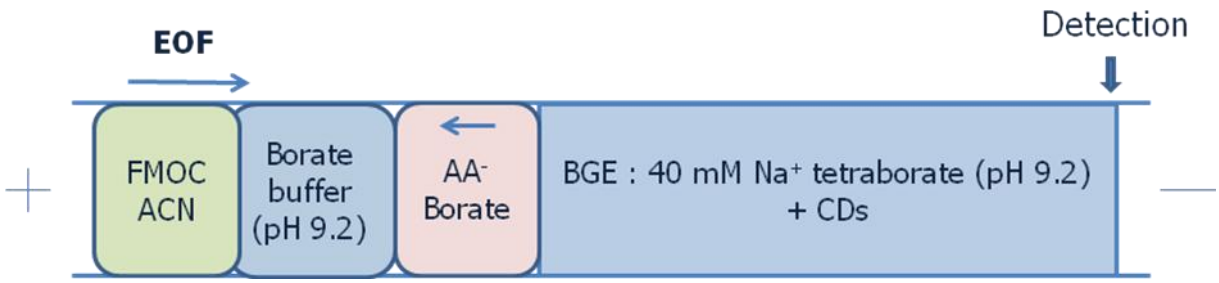

Figure 4. Scheme of in-capillary derivatization.

The influence of various factors, such as the mixing voltage and time, concentration of labelling solution and the length of the spacer plug, on the in-capillary derivatization performance was then investigated.

To optimise these parameters, tryptophan was selected in order to estimate the yield of derivatization (cf. Equation 1) as it possesses a chromophore.

3.2.1. Effect of mixing voltage and time

The mixing time determines the mixing extent of the reactants. Based on an applied voltage of 12 kV, the effect of mixing time (9-24 s) was investigated. As illustrated in Fig. 5, the derivatization yield increases remarkably as the mixing time increases from 9 to 18 s, and then decreases slightly.

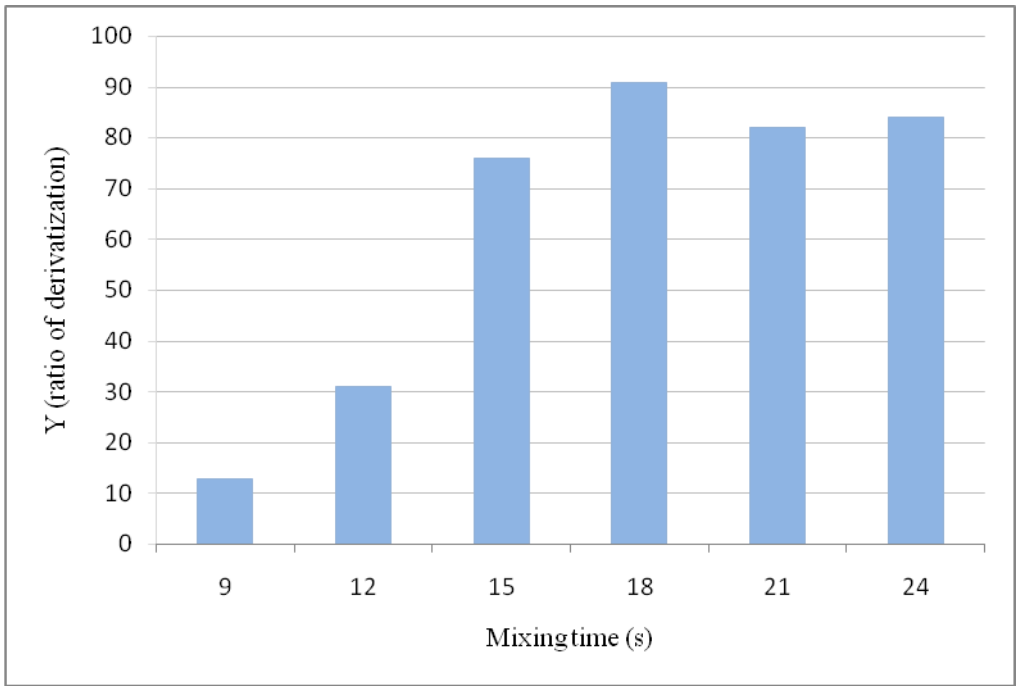

Figure 5. Effect of the electrokinetic mixing time on in-capillary derivatization yield. Derivatization conditions: successive hydrodynamic injections of sample, borate buffer, 20 mM FMOC solution (30 mbar for 2 s for each); mixing voltage (12 kV). BGE: 40 mM sodium tetraborate. Other conditions as described in Section 2.

The optimization of the mixing voltage was then performed, keeping the same global electrokinetic mixing equivalent to 12 kV for 18 s. Different couples of voltage/time were used. The maximum derivatization yield was reached when a voltage of 3 kV was applied for 72 s (data not shown).

3.2.2. Effect of the concentration of FMOC solution and plug length

The target analyte has to be fully labelled using an excess of FMOC. Nevertheless, in the case of in-capillary derivatization, the amount has to be well controlled in order to maintain the CE

resolution and obtain reproducible results. For the first experiments, solutions of 3 mM AA and 10 mM FMOC were injected for 2 s.

When the concentration of the FMOC solution increases (from 5 to 20 mM), the labelling rate increases from 83% to 99%. However, it is worth noting that 20 mM FMOC solution for 6 s gives rise to a noisy baseline. Moreover, for the same amount of FMOC introduced (10 mM FMOC solution for 6 s or 20 mM FMOC solution for 3 s), the labelling yield was enhanced (96% instead of 85%), using a longer plug of FMOC. Plug length longer than 6 s could not be tested because of current break. Accordingly, the FMOC plug consisting of a solution of 10 mM FMOC injected for 6 s was selected.

3.2.3. Effect of the length of the borate spacer plug

The influence of the plug length of borate buffer introduced between the sample and the FMOC was then examined. The injection time of borate buffer was varied from 2 to 6 s while those of AA and FMOC remained constant.

The ratio of derivatized tryptophan increased remarkably as the spacer plug time increased from 2 to 4 s, and then decreased slightly (data not shown). Therefore, the optimum spacer plug length was obtained by injecting the borate buffer for 4 s.

3.2.4. Effect of the reaction time

The impact of the reaction time (from 0 to 2 min) after mixing on the labeling yield was then studied. The reaction time had no significant influence on the in-capillary derivatization performance (data not shown). It was concluded that the labeling reaction occurred immediately after mixing. The reaction time was finally set to one minute.

Finally, the optimal in-capillary derivatization procedure was obtained using the following conditions: successive hydrodynamic injections (30 mbar) of AA for 2 s, borate buffer for 4 s and 10 mM FMOC solution for 6 s, mixing at 3 kV for 72 s and wait time of 1 min. Under these conditions, a limit of detection of 0.3 mM of FMOC-Trp (corresponding to 0.57 10^{-12} moles injected), estimated as the minimum concentration yielding an S/N ratio equal to 3, was obtained.

A typical electropherogram of tryptophan obtained under the optimal conditions for in-capillary derivatization is shown in Fig. 6. It should be noticed that no chiral selector was added to the BGE and hence Trp enantiomers were not separated.

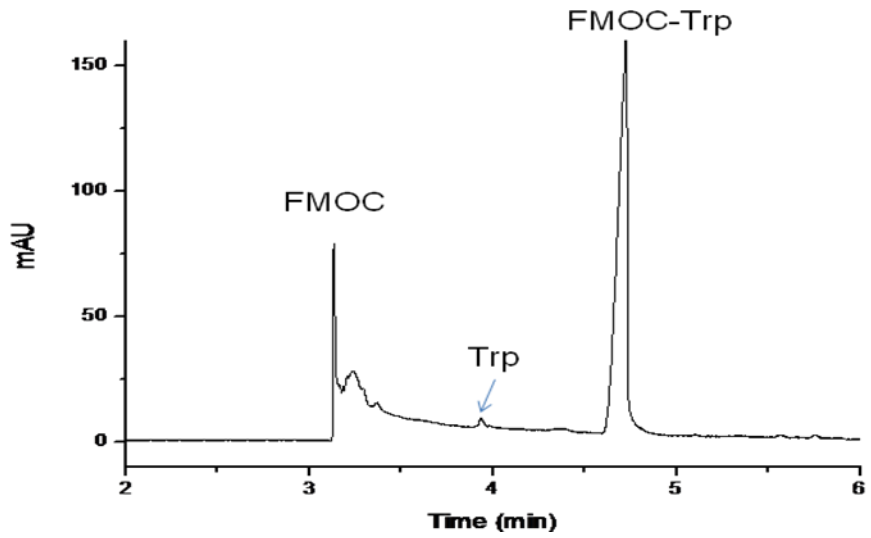

Figure 6. Typical electropherogram of tryptophan obtained under the optimal conditions. Derivatization conditions: successive hydrodynamic injections of sample (30 mbar for 2 s), borate buffer (30 mbar for 4 s) and 10 mM FMOC solution (30 mbar for 6 s); mixing voltage 3 kV for 72 s and wait time 1 minute. BGE: 40 mM sodium tetraborate. Detection at 210 nm ; Other conditions as described in Section 2.

Moreover, it is worth noting that in-capillary derivatization did not have a significant impact on the enantiomeric resolution (Table 2).

Table 2. Influence of the in-capillary derivatization on enantiomeric resolution.

	CZE[a]		MEKC[b]
FMOC-AA	Precapillary Derivatization	In-capillary derivatization	In-capillary derivatization
Ala	2.3	2.0	2.6
Val	5.8	5.3	8.1
Leu	4.3	3.8	7.6
Ileu	4.6	4.7	7.4
Pro	1.7	0.9	1.8
Phe	3.5	3.1	7.8
Met	3.4	3.0	5.5
Trp	2.3	2.5	6.9
Ser	2.8	2.2	3.7
Thre	2.3	2.3	7.0
Asn	2.9	2.0	3.3
Gln	2.9	2.8	3.4
Asp	3.1	3.1	4.5
Glu	5.6	5.6	9.1
Cys	2.8	1.8	-*
Tyr	4.9	4.9	7.3
His	3.8	3.2	4.4
Lys	0.5	1.0	-*
Arg	1.2	-	2.7

-, no enantiomeric separation observed (R_s < 0.5)

*, no peak detected within 140 min

BGE: a) 30 mM β-CD, 30 mM OS-γ-CD, 15% IPA in 40 mM sodium tetraborate ;

b) 30 mM β-CD, 30 mM OS-γ-CD, 25 mM SDS, 17% IPA in 40 mM sodium tetraborate; other conditions as described in Section 2.

3.3. Optimization of chemoselectivity

The selected BGE leads to the enantiomeric separation of 16 pairs of FMOC-AAs. However, under these conditions, FMOC-AAs were not all separated from each other. The BGE was then optimized in order to improve separation chemoselectivity. Therefore the effect on the stereo selectivity and chemoselectivity of different factors, such as decrease of pH and tetraborate concentration and the addition of SDS, was investigated.

To achieve this goal, 19 AA racemates were tested individually, using in-capillary derivatization. In addition, a standard mixture of 10 AAs was derivatized inside the capillary.

The use of 50 mM sodium phosphate buffer at pH 7 slightly improved enantiomeric resolution. Nevertheless, the separation among AAs did not increase. When a lower sodium tetraborate concentration (20 mM) was used, the separation between the enantiomers and among the FMOC-AAs did not improve.

The application of MEKC, by adding SDS as surfactant to the BGE was then investigated. Different SDS concentrations were tested (20-25-30 mM) and 25 mM SDS was found to give rise to the best separation of the standard mixture. SDS seems to play a role not only in improving the separation among the FMOC-AAs but also in increasing the enantiomeric resolution (Table 2).

Since the addition of organic solvents usually extends the migration window, higher percentages of IPA (15-20%) were investigated. The best separation of the standard mixture was observed using a BGE containing 30 mM β-CD, 30 mM OS-γ-CD, 25 mM SDS and 17% IPA in 40 mM sodium tetraborate. Under these conditions, all FMOC-AAs of the standard mixture were resolved. The enantioresolution was also increased for all AAs, except for Lys and Cys which have such a high affinity for the micelles that they were detected in the negative polarity mode (Table 2).

Fig. 7 shows the electrophoretic separation of the standard mixture of AAs under the optimum conditions. The identification of each AA in this sample was assessed by spiking the sample with solution of each individual AA.

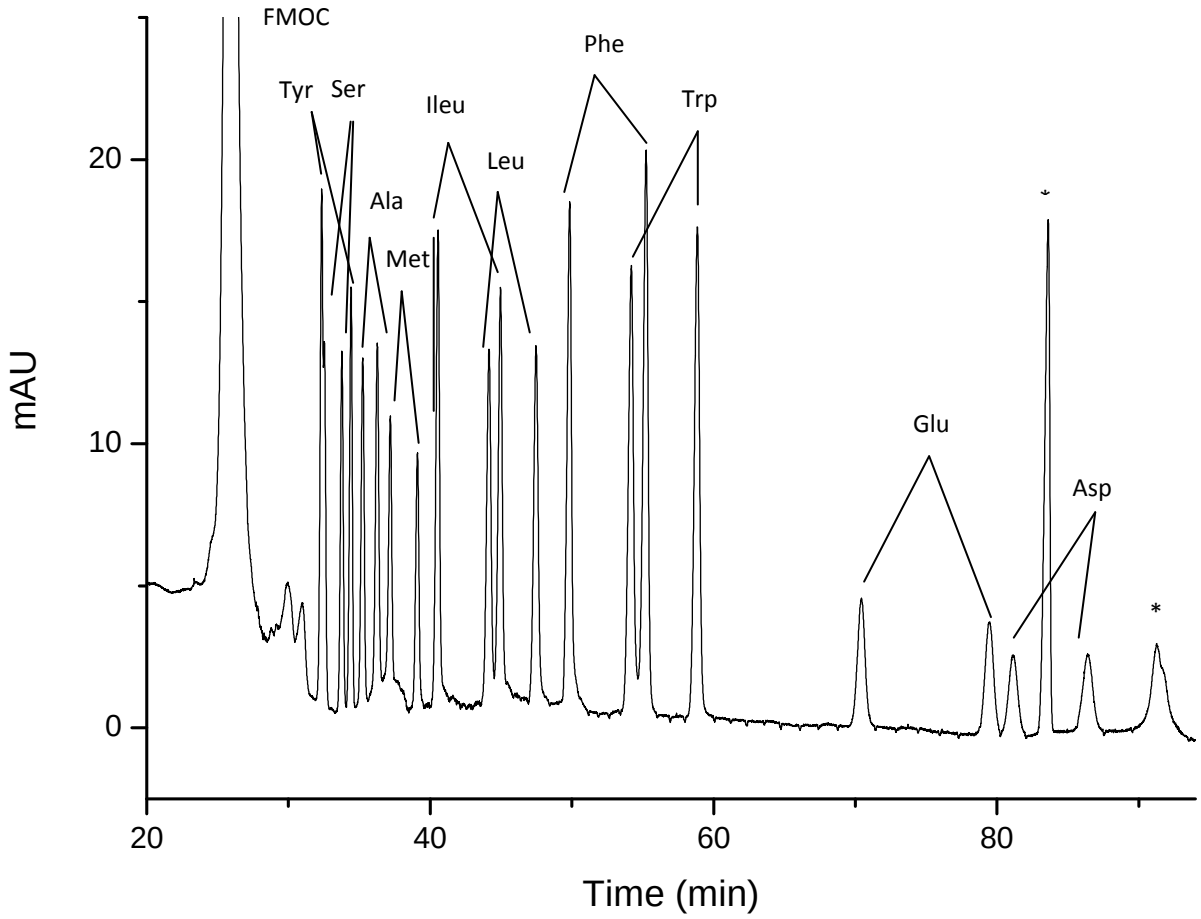

Figure 7. Electropherogram showing the separation of standard AA mixture under the optimum conditions.

Derivatization conditions: successive hydrodynamic injections of sample (30 mbar for 2 s); borate buffer (30 mbar for 4 s) and 10 mM FMOC solution (30 mbar for 6 s); mixing voltage 3 kV for 72 s and wait time 1 minute.

BGE: 30 mM β-CD, 30 mM OS-γ-CD, 25 mM SDS 17% IPA and 40 mM sodium tetraborate; *: unknown peak. Detection at 210 nm; other conditions as described in Section 2.

4. Conclusion

A new chiral MEKC analytical method was developed for the analysis of mixtures of AAs derivatized inside the capillary with FMOC, using β-CD and OS-γ-CD as chiral selectors, SDS as surfactant and IPA as organic modifier. This proposed method represents a selective and easy to use approach for fully automated analysis of FMOC-AAs. The best enantiomeric separations were obtained with dual CD system composed of 30 mM β-CD and 30 mM OS-γ-CD. For efficient in-capillary derivatization, it is important to apply the appropriate voltage and time to mix the plugs and to use a rather long plug length of diluted FMOC solution. The use of MEKC, by adding SDS as surfactant, improves not only enantioselectivity, but also chemoselectivity, leading to the simultaneous separation of ten pairs of FMOC-AAs. The UV detection was used to optimize the CE integrated analytical system. In a second step, his method will be applied to biological samples with LIF detection in order to improve sensitivity.

Acknowledgements

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In-capillary derivatization with (-)-1-(9-fluorenyl)ethyl chloroformate (FLEC) as chiral labelling agent for the electrophoretic separation of amino acids

Section 3.2

Preface

In the first part of this work, we developed a dual chiral CE method, using β-CD and OS-γ-CD as chiral selectors, for the separation of in-capillary derivatized FMOC-AA enantiomers.

The proposed method provides (i) an excellent chiral separation for 17 FMOC-AAs and (ii) efficient in-capillary derivatization with FMOC.

The use of MEKC improves not only enantioselectivity, but also chemoselectivity, leading to the enantiomeric separation of a mixture of 10 pairs of FMOC-AAs in a single run. However, during prevalidation studies, poor reproducibility of the method was noticed. In order to simplify the composition of the BGE and to improve the separation repeatability, we considered the use of a chiral derivatization agent.

In this second part of the work, we undertook to investigate the potentiality of a chiral derivatization reagent, namely (-)-1-(9-fluorenyl)-ethyl chloroformate (FLEC), for in-capillary derivatization as well as chiral separation of AAs in mixture.

In-capillary derivatization with (-)-1-(9-fluorenyl)ethyl chloroformate as chiral labelling agent for the electrophoretic separation of amino acids

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Abstract

An original micellar electrokinetic chromatography (MEKC) method using in-capillary derivatization with a chiral labelling reagent was developed for the separation of amino acid (AA) derivatives. The potential of (-)-1-(9-fluorenyl)-ethyl chloroformate (FLEC) as in-capillary derivatization agent is described for the first time. Several parameters for in-capillary derivatization and subsequent MEKC separation were systematically investigated using experimental designs. Firstly experimental conditions for in-capillary derivatization were optimized using face-centered central composite design (FCCD). Mixing voltage and time as well as concentration of the labelling solution were investigated. Efficient labelling was achieved by sequential injection of AAs and FLEC labelling solution followed by the application of a voltage of 0.2 kV for 570 s. The background electrolyte (BGE) composition was then optimized in order to achieve selectivity. A FCCD was performed with two factors, namely the sodium dodecyl sulfate (SDS) concentration and the percentage of propan-2-ol (IPA). The separation of 12 pairs of derivatized AA (FLEC-AA) diastereomers was achieved with resolution values comprised between 3 and 20. Furthermore, an efficient derivatization and separation of 29 FLEC-AA derivatives were achieved in a single run using a buffer made up of 40 mM sodium tetraborate, 21 mM SDS and 8.5% IPA. The method was successfully applied to the analysis of spiked artificial cerebrospinal fluid (aCSF) sample.

1 Introduction

Over the past few years, much attention has been focused on the biological functions played by D-AAs [46][47]. For example, D-Ser and D-Asp were recently identified as endogenous components involved in the modulation of neurotransmission and functional plasticity at glutamatergic synapses of the central nervous system [45][48]. These AAs are thought to play a role in aging schizophrenia as well as in acute and chronic neurodegeneration [45]. It has also been shown that synaptic D-Ser may play a major role in motor neuron degeneration in amyotrophic lateral sclerosis pathogenesis [215]. Concerning D-Ala, it was reported that this AA is localized in insulin producing beta-cells in mammalian pancreas and would be involved in the regulation of blood glucose level [54]. Therefore, studying D-AAs may have clinical and diagnostic significances [189]. Moreover, these chiral metabolites might also lead to the development of new treatment approaches. The involvement of other D-AAs in physiopathological processes is not yet well investigated and research in this field is strongly connected to progress in analytical methodology with regard to speed of analysis, sensitivity, high separation performance and simplicity of sample preparation [190]. In this context, various chemoselective and enantioselective separations of AAs have been published [197]. Some methods include multidimensional approaches like 2-D-GC and 2-D-LC to increase selectivity. Applications based on hyphenation to mass spectrometry also appeared to be particularly promising [106][115]. For example, Reischl et al. developed a 1-dimensional LC-MS/MS method for the enantioseparation of all proteinogenic AAs derivatized with the non chiral reagent 6-methoxyquinoline-4-carboxylic acid using a chiral anion exchanger column [115]. Another approach is to employ a chiral derivatization agent. This enables the separation of the formed diastereomers on a non-chiral column [216]. For all the above mentioned methods, AA derivatization prior to chromatography is necessary in order to enable detection and/or enantioseparation, which can be time and reagent consuming and requires skilful handling.

Besides, capillary electrophoresis (CE) is proved to be a powerful separation technique for the chiral analysis of AAs since it has the advantages of high peak efficiency and resolution, fast separation, as well as small amount of sample and reagent consumption. The usefulness and practical applications of capillary electro-migration methods for AA analysis have been reviewed recently [216][217]. Over the past few decades, in-capillary derivatization has emerged as a powerful labelling technique due to its remarkable advantages, such as low

consumption of reagents and samples, high derivatization efficiency, minimal sample dilution, and full automation of the derivatization step without additional equipment requirement [26][60][69][188][203][206][218][221]. The derivatization of AAs using in-capillary techniques has been mainly reported using dansyl chloride (Dns-Cl) [60], 3-(2-furoyl)-quinoline-2-carboxaldehyde (FQ) [203], [204], 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate (AQC) [69], naphthalene-2,3-dicarboxaldehyde (NDA)/CN^- [205], *o*-phthaldialdehyde (OPA) [218], *o*-phthaldialdehyde/N-acetyl L-cysteine (OPA/NAC) [188][219], 1,2-naphtoquinone-4-sulfonate (NQS) [206], 4-fluoro-7-nitro-2,1,3-benzoxadiazole (NBD-F) [220] and 9-fluorenylmethyl chloroformate (FMOC) [186][221]. Although these reagents have their own merits, it should be emphasized that, except OPA/NAC, they all require the use of a chiral selector in the separation buffer to enable the enantioseparation of labelled AAs.

In order to develop a new and easy-to-use in-line method, we focused our attention on the chiral reagent FLEC which has been introduced as a fluorogenic chiral reagent in 1987 by Einarsson et al [222]. FLEC reacts quickly and at room temperature with primary and secondary amines to form highly stable and fluorescent derivatives. Chiral separation of FLEC-AA has already been achieved by high performance liquid chromatography [223][224] or CE [71][207] using precolumn derivatization. However, to the best of our knowledge, the use of this reagent for the in-capillary derivatization of amines has not yet been reported.

In the present study, the potential of FLEC to perform in-capillary derivatization of AAs by CE is examined. Several parameters that influence the in-capillary derivatization of AAs, namely FLEC concentration, mixing voltage and time were optimized using a multivariate approach. Moreover, we explored the chemo and chiral separation power of the FLEC labelling reagent in a classical MEKC buffer. To achieve this goal, a FCCD was used to deduce optimal BGE composition for the separation of in-capillary formed AA derivatives.

2 Experimental

2.1 Instrumentation

All experiments were performed on a $HP^{3D}CE$ system (Agilent Technologies, Waldbronn, Germany) equipped with an autosampler, an on-column diode-array detector and a temperature control system (15-60 °C ± 0.1 °C). A CE Chemstation (Hewlett-Packard, Palo Alto, CA, USA) was used for instrument control, data acquisition and data handling. Fused silica capillaries were provided by Thermo Separation Products (San Jose, CA, USA).

2.2 Chemicals and reagents

D,L-AAs (alanine, valine, leucine, isoleucine, proline, phenylalanine, methionine, tryptophan, serine, threonine, asparagine, glutamine, aspartate, glutamate, cysteine, tyrosine, histidine, lysine and arginine), glycine, N-acetyl-DL-tryptophan and a 18 mM acetone solution of FLEC (chemical structure shown in Fig. 1) were purchased from Sigma-Aldrich (Saint-Louis, MO, USA). The optical purity of FLEC was > 99.9 % according to the manufacturer. Sodium tetraborate was supplied by Merck (Darmstadt, Germany) and SDS by Acros-Organics (Geel, Belgium). Sodium hydroxide, acetonitrile (ACN), IPA and trifluoroacetic acid (TFA) were from VWR chemicals Prolabo (Leuven, Belgium). Formic acid 98-100 % and ethanol were from Merck (Darmstadt, Germany). Water used in all experiments was of Milli-Q quality (Millipore, Bedford, MA, USA).

2.3 Electrophoretic technique

Electrophoretic separations were carried out with uncoated fused silica capillaries having 50 µm internal diameter and 80.5 cm length (72 cm to the detector). A new capillary was conditioned with 1 M sodium hydroxide and water, each for 15 min. At the beginning of each working day, the capillary was washed with 1 M sodium hydroxide, water and the BGE, each for 10 min. At the end of each working day, the capillary was rinsed with water, water / ACN mixture (50/50, v/v) and water each for 15 min.

In order to obtain a satisfactory run-to-run repeatability, before each analysis the capillary was washed with 0.1 M TFA solution for 1 min, then the inlet and the outlet of the capillary were moved to vials containing 0.05 M TFA solution and a 10 kV potential was applied for 3 min and finally, the capillary was washed with water for 1 min and the running buffer for 3 min.

At the end of the analysis, the capillary was washed with water, water / ACN mixture (50/50, v/v) and water, each for 2 min. Capillary wash cycles were performed at a pressure of 1 bar.

The applied separation voltage was 30 kV and UV detection was performed at 210, 250, 260 and 280 nm.

Injections were made by applying a pressure of 30 mbar for a period of 5 s (corresponding to 1.9 nL) and the capillary was thermostated at 25 °C.

2.4 Buffer and sample preparation

Buffers were made up of 40 mM sodium tetraborate decahydrate pH 9.3. Unless otherwise mentioned, the BGE was prepared by the addition of 10-35 mM SDS and 5-20 % (v/v) IPA.

Stock solutions of the individual AAs (0.5 mM) were diluted in 5 mM sodium tetraborate solution containing 0.5 mM of N-acetyl-DL-tryptophan (internal standard), to obtain the working solutions.

To reduce the number of analyses, during the optimisation, several AAs were mixed at a concentration of 0.5 mM (cf. Table 1).

Table 1. Optimisation of the separation conditions: division of the AAs in six groups.

Group 1	**Group 2**	**Group 3**	**Group 4**	**Group 5**	**Group 6**
Gln	Ser	Asn	Ala	Thr	Pro
Met	Val	Tyr	Ileu	Gly	Glu
Phe	Trp	Leu	Cys	Asp	His
Arg	Lys				

A stock solution of a mixture of 19 AAs (all natural AAs except Pro) was prepared in a 5 mM sodium tetraborate solution containing 0.5 mM of N-acetyl-DL-tryptophan to obtain a concentration of 0.5 mM of each AA.

BGEs and sample solutions were filtered through a syringe filter and stored at 4°C.

aCSF was prepared according to a procedure from DURECT Corporation (Cupertino, CA) [225]. Sodium chloride (8.66 g), potassium chloride (0.224 g), magnesium chloride

hexahydrate (0.163 g), calcium chloride dihydrate (0.206 g) were dissolved in 0.5L water (solution A). The solution B was prepared by dissolving 0.214 g sodium phosphate dibasic heptahydrate and 0.027 g sodium phosphate monobasic monohydrate in 0.5L of water. Solutions A and B were then mixed in equal proportions before spiking with AAs. This solution (ions concentration in mM: Na 150, K 3, Ca 1.4, Mg 0.8, P 1.0 and Cl 155) closely matches the electrolyte concentrations of human CSF [226] The solution was stored at +4 °C until use.

For spiked aCSF sample, a mixture of 1 mM AAs prepared in aCSF was diluted twice with 5 mM sodium tetraborate solution containing 1 mM N-acetyl-DL-tryptophan as IS.

2.5 Pre-capillary derivatization

The following off-line derivatization procedure was applied to the AAs solutions: to 200 µl sample aliquot (containing AA and IS at a concentration of 1 mM), 200 µl of 12 mM FLEC solution (diluted with ACN) were added. This mixture was kept for 2 min at room temperature, then extracted with 0.5 mL hexane to remove excess of FLEC labelling reagent. The ACN resulting sample was ready for injection into the CE apparatus.

2.6 In-capillary derivatization

The derivatization of AAs with FLEC was performed directly in the capillary by hydrodynamic injections (5 s at 30 mbar) of the sample solution (AA and internal standard at a concentration of 0.5 mM each in 5 mM sodium tetraborate buffer) followed by the solution of FLEC diluted in ACN (concentrations ranging from 2 to 6 mM). After each injection, the inlet end of the capillary was washed by immersion in a vial containing ACN, then in a vial containing water to avoid any cross contamination. Vials containing the separation buffer were then placed at both ends of the capillary and the plugs were mixed by applying a specified voltage ranging from 0.1 to 1 kV (mixing voltage) for specified periods of time ranging from 120 to 600 s (mixing time). After mixing, the separation was performed at 30 kV.

2.7 Multivariate analysis

For in-capillary derivatization optimisation, a FCCD was defined with 18 experimental conditions, including four repetitions at the center point. Three levels for each factor were

investigated (for mixing time: 120, 360, 600s; for mixing voltage: 0.1, 0.5, 1 kV and for FLEC concentration: 2, 4 and 6 mM). The 18 conditions were performed in random order. For each condition and for each compound, three injections were made and the mean was considered.

For the optimization of the separation conditions, a FCCD was designed with 11 experiments including three repetitions at the center point. Three levels were considered for each factor (10, 22.5 and 35 mM for SDS; 5, 12.5 and 20 % for IPA) in order to estimate the quadratic effects. The experiments were performed in random order.

2.8 Performance evaluation

The resolution (R_s) of the diastereomers, calculated according to the standard expression based on the peak width at half height, was obtained from the Chemstation software [10].

To assess the in-capillary derivatization performance during optimization, the normalized peak areas of derivatized AAs were expressed relatively to the internal standard. A normalized area ratio (NAR) was calculated for each FLEC-AA according to the equation:

$$NAR_{AA} = \frac{NA_{FLEC-AA}}{NA_{IS}} = \frac{A_{FLEC-AA}/Tm_{FLEC-AA}}{A_{IS}/Tm_{IS}} \quad (1)$$

where $NA_{FLEC\text{-}AA}$ and NA_{IS} are normalized peak areas of FLEC-AA and internal standard (N-acetyl-DL-tryptophan) respectively. $A_{FLEC\text{-}AA}$, $Tm_{FLEC\text{-}AA}$, A_{IS} and Tm_{IS} represent peak areas and migration times of FLEC-AA and internal standard, respectively.

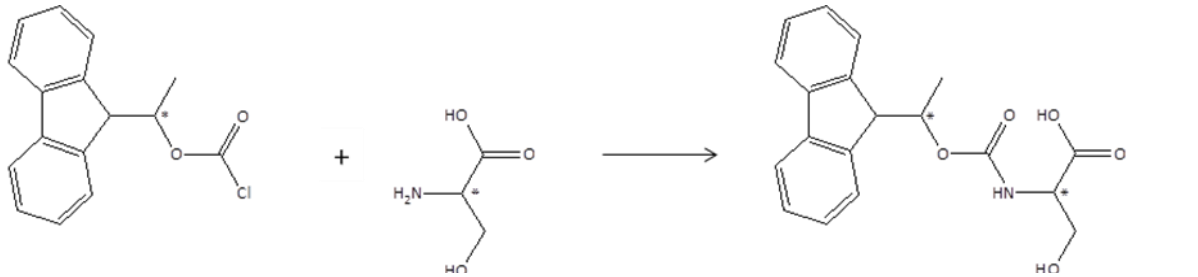
In-capillary relative derivatization rate (Y) was estimated comparatively to the off-line derivatization as follows:

$$Y = \frac{(NAR_{FLEC-AA})_{in-cap}}{(NAR_{FLEC-AA})_{pre-cap}} \times 100 \quad (2)$$

where $(NAR_{FLEC\text{-}AA})_{in\text{-}cap}$ and $(NAR_{FLEC\text{-}AA})_{pre\text{-}cap}$ are normalized area ratio of FLEC-AA obtained using in-capillary and pre-capillary procedure, respectively.

3 Results and discussion

The chiral separation of racemic mixtures of AAs was evaluated after in-capillary derivatization using (-)-FLEC labelling reagent which forms FLEC-AA diastereomeric derivatives potentially separable in non-chiral BGE (cf. Figure 1). It should be mentioned that the reversal of migration order might be simply obtained with (+)-FLEC, as already demonstrated by H. Wan et al. [207].



(-)-FLEC
(-)-1-(9-Fluorenyl)ethyl chloroformate

DL-serine

(-)-FLEC-D-serine and (-)-FLEC-L-serine

Figure 1. Schematic illustration of the derivatization reaction of amino acids with FLEC.

3.1 Preliminary studies

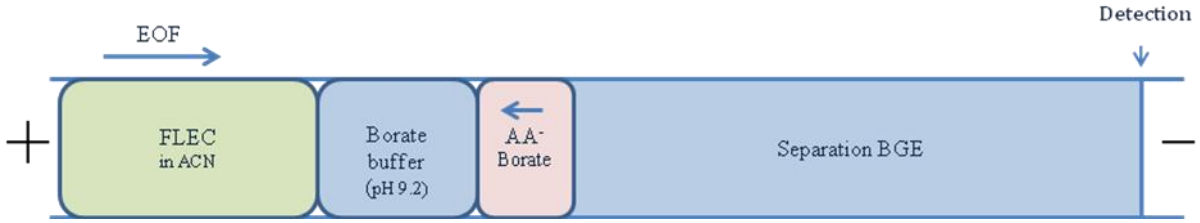
The selection of an appropriate strategy for in-capillary derivatization of AAs with FLEC was first addressed. N-acetyl-DL-tryptophan was used as internal standard as its amino function is blocked and thus it cannot react with FLEC. To perform those preliminary studies, Val, Ser and Glu were selected as model analytes.

According to our previous study of in-capillary derivatization of AAs with FMOC (derivatization reagent chemically similar to FLEC but not chiral), the following conditions were tested: successive hydrodynamic injections (30 mbar) of AA for 2 s, borate buffer for 4 s and 10 mM FLEC solution for 6 s, mixing at 3 kV for 72 s and wait time of 1 min [221] (cf. Fig. 2A). In these preliminary experiments, the same BGE as in [221] was employed: 40 mM sodium tetraborate at pH 9.2, 25 mM SDS and 15 % (v/v) IPA, except the presence of cyclodextrins since FLEC is supposed to bring stereoselectivity. Under these conditions, in-capillary derivatization of the model analytes could be achieved (cf. Fig. 2C (a)). As can be seen in this figure, complete resolution of FLEC-Val and FLEC-Ser diastereomers could be obtained while no separation of FLEC-Glu diastereomers was observed. Nevertheless, the repeatability in terms of NARs was not satisfactory under these conditions (RSD values of

NAR (n=8): 29.2, 26.2 and 30.4 % for FLEC-Ser, FLEC-Glu and FLEC-Val, respectively). Moreover, the peak corresponding to the FLEC excess was large and could potentially interfere with the electrophoretic separation. In order to improve run-to-run repeatability and to limit the adsorption of the FLEC excess on the capillary wall, several capillary washing sequences (between runs) were tested. Formic acid and trifluoroacetic acid solutions were evaluated as first washing step in order to protonate the silanol groups. Different washing sequences at the end of the run using ACN, ACN/water mixture and water were also investigated. Finally, the following washing sequence was used: an applied voltage of 10 kV with 0.1 M TFA solution (3 min), flushes with water (1 min) and BGE (3 min) before the run and water (2 min), ACN/water mixture (50/50, v/v) (2 min) and water (2 min) successively at the end of the run. In parallel, the critical parameters for in-line derivatization including the mixing voltage, the mixing time and the borate spacer plug were also examined. Different mixing voltages (between 0.1 and 3 kV) were tested and in-capillary derivatization of AAs with FLEC was found to increase when the mixing voltage decreased. It is noteworthy that unlike the derivatization with FMOC, for which the borate spacer plug was necessary, derivatization performance was enhanced when the FLEC solution was introduced directly after the AA solution. Moreover, the injection time was set at 5 s (at 30 mbar) for each plug.

Fig. 2B illustrates the scheme of in-capillary derivatization after the preliminary optimization. Under these conditions, the repeatability of the derivatization performance was satisfactory (RSD values of NAR (n=8): 8.3, 6.6 and 11.6 % for FLEC-Ser, FLEC-Glu and FLEC-Val, respectively). The corresponding electropherogram showing the in-capillary derivatization and diastereomeric separation of Ser, Val and Glu is presented in Fig. 2C (b).

A



B

C.

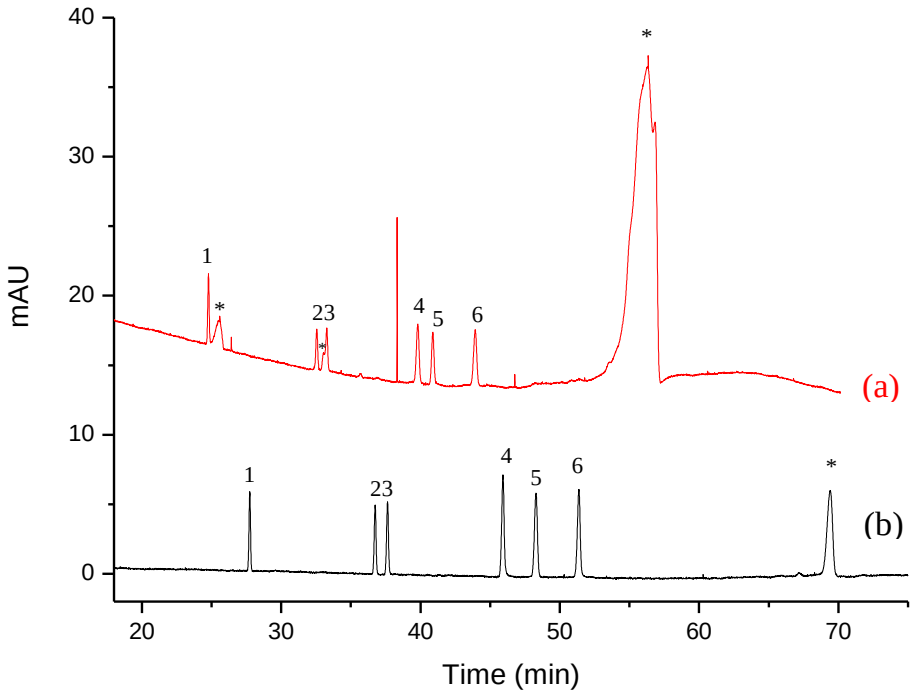


Figure 2. A. Scheme of initial in-capillary derivatization of AA with FLEC. Derivatization conditions as in [221]: successive hydrodynamic injections of sample (30 mbar for 2 s), borate buffer (30 mbar for 4 s) and 10 mM FLEC solution (30 mbar for 6 s); mixing voltage 3 kV for 72 s and wait time 1 min.

Washing sequence before run: water (1 min), ACN (2 min), water (1 min), 1 M NaOH (2 min), water (1 min) and BGE (2 min). Other conditions as described in section 2.

B. Scheme of in-capillary derivatization after preliminary optimization. Derivatization conditions: successive hydrodynamic injections of sample (30 mbar for 5 s) and 10 mM FLEC solution (30 mbar for 5 s); mixing voltage 1 kV for 240 s. Washing sequences before each run: 0.1 M TFA (10 kV for 3 min), water (1 min) and BGE (3 min) and after each run: water (2 min), ACN/water mixture (2 min) and water (2 min). Other conditions as described in section 2.
C. Electropherograms obtained after in-capillary derivatization of Ser, Val and Glu using the same sample solution under the conditions of schemes (A) and (B). Peak identification: (1) internal standard, (2) and (3) FLEC-Ser, (5) FLEC-DL-Glu, (4) and (6) FLEC-Val. Peaks marked with an asterisk correspond to FLEC peaks.
BGE used in A, B and C: 40 mM sodium tetraborate pH 9.2, 25 mM SDS and 15 % (v/v) IPA.

3.2 Optimization of the in-capillary derivatization conditions

An experimental design was first applied to study the influence of the mixing voltage and mixing time as well as the concentration of the FLEC solution (main and quadratic effects) on the NARs of three FLEC-AAs (Ser, Val and Glu selected again as model compounds).

The effect of the mixing voltage was investigated over the 0.1 – 1 kV range, since it was noticed during the preliminary experiments that the application of a low mixing voltage improves derivatization performance. It should be emphasized that, in the absence of application of mixing voltage, the NARs of FLEC-AAs decreased even if a long reaction time was applied.

The mixing voltage time was studied over the 120 – 600 s range. Lower mixing times than 120 s led to small derivatized AA peaks. Longer mixing time (> 600 s) led to current disruption. The lower level of the FLEC solution concentration was settled at 2 mM and the higher one at 6 mM as the introduction of a too large excess of labelling reagent into the capillary should be avoided for the CE separation performance. Indeed, high concentrations of FLEC gave an overloaded FLEC peak shape which was detrimental to FLEC-AA peak separation.

The relationship between the response, namely the NAR of each FLEC-AA (Y) and the factors, i. e. the FLEC concentration (X_1), the mixing voltage (X_2) and the mixing time (X_3) was modeled by the following equation:

$$Y = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_3 X_3 + \beta_{11} X_1^2 + \beta_{22} X_2^2 + \beta_{33} X_3^2 + \beta_{12} X_1 X_2 + \beta_{13} X_1 X_3 + \beta_{23} X_2 X_3 + \varepsilon$$

where β_0 is the intercept, β_1, β_2, and β_3 the main effects, $\beta_{11,}$ β_{22} and β_{33} the quadratic terms, β_{12}, β_{13} and β_{23} the interaction terms, and ε the error term.

The quality of fit of the model was considered as excellent with adjusted R^2 values ranging from 0.81 to 0.87 for the NARs of the tested FLEC-AAs.

The different coefficients of the model and their statistical significance for the five FLEC-AA diastereomers are presented in Table 2.

Table 2. Optimisation of in-capillary derivatization by a FCCD: coefficients of the model and their *p*-values*.

Parameters	NAR									
	FLEC-Ser 1		FLEC-Ser 2		FLEC-Val 1		FLEC-Val 2		FLEC-Glu**	
	Coef.	p-value	Coef.	p-value	Coef.	p-value	Coef.	p-value	Coef.	p-value
β_1 (FLEC concentration□	0.124	**0.0006**	0.132	**0.0009**	0.097	**0.0028**	0.086	**0.0045**	0.162	**0.009**
β_2 (mixing voltage)	-0.154	**0.0001**	-0.167	**0.0002**	-0.125	**0.0006**	-0.121	**0.0006**	-0.398	**< 0.0001**
□3 (mixing time)	0.046	0.078	0.055	0.067	0.060	**0.030**	0.068	**0.0151**	0.079	0.133
β_{11}	-0.074	0.129	-0.066	0.218	0.028	0.537	0.028	0.532	-0.054	0.567
β_{22}	-0.028	0.542	-0.020	0.699	-0.219	**0.0010**	-0.194	**0.0018**	0.266	**0.019**
β_{33}	-0.012	0.786	-0.025	0.629	-0.072	0.138	-0.067	0.150	-0.137	0.171
β_{12}	0.007	0.779	0.002	0.957	0.092	**0.0069**	0.074	**0.018**	-0.032	0.566
β_{13}	0.047	0.104	0.049	0.126	0.017	0.515	0.022	0.398	0.056	0.324
β_{23}	-0.035	0.210	-0.041	0.190	-0.017	0.527	-0.010	0.703	-0.041	0.461

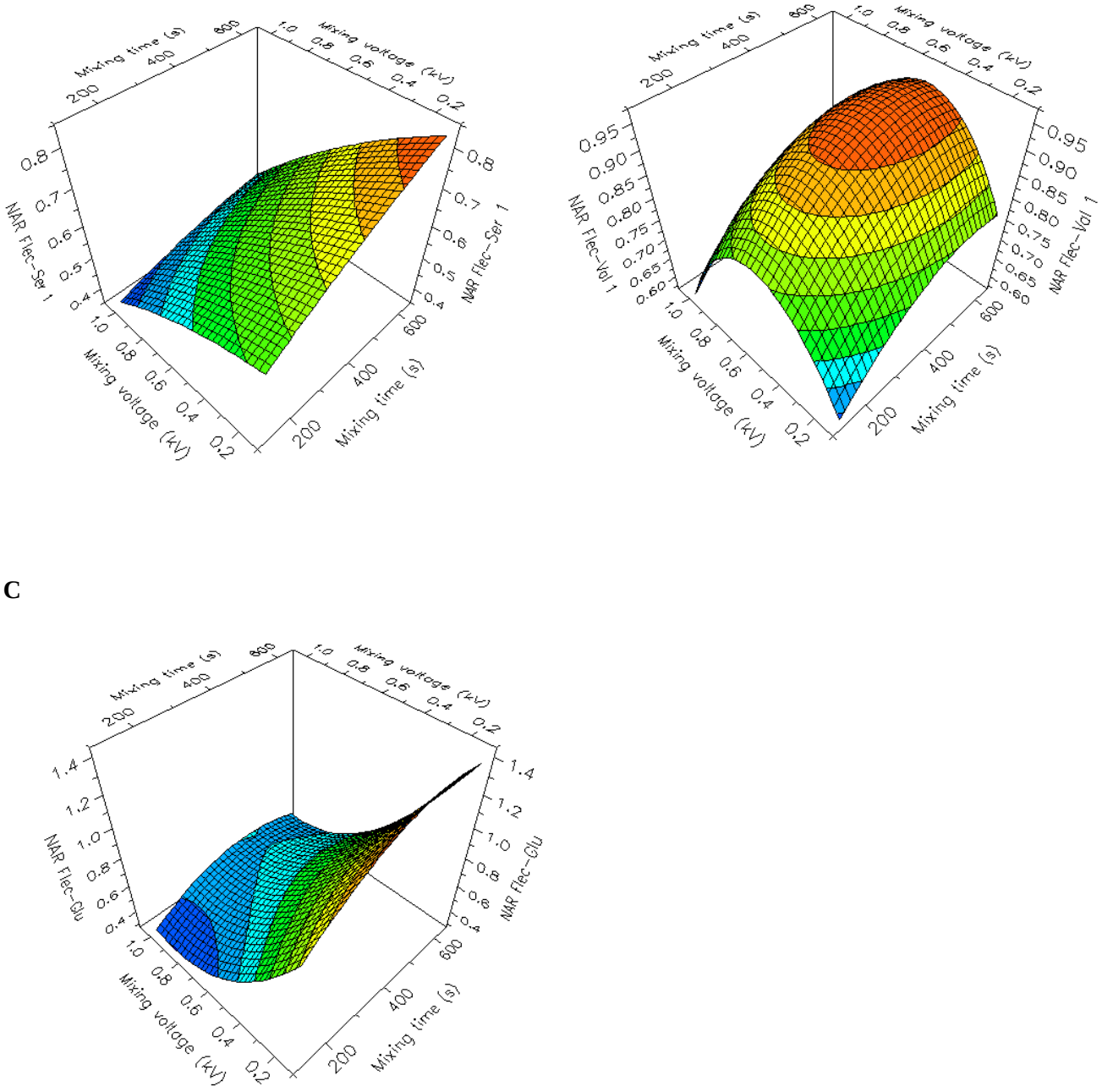
* p-values significant at the 5 % level are printed in bold-face type.

** Diastereomers of FLEC-Glu were not separated under these separation conditions.

The effect is considered as significant if p-value < 0.05. As can be seen in this table, both the concentration of the FLEC solution and the mixing voltage significantly influenced the NARs of derivatized AAs. As expected, the derivatization performance of the studied AAs was found to increase with the FLEC concentration. The mixing time also influenced the derivatization of Val, but to a minor extent. Moreover, the quadratic term of the mixing voltage was significant for FLEC-Val and FLEC-Glu NAR. Interaction terms between X_1 and X_2 were significant for FLEC-Val NARs. Considering the response surfaces obtained with 6 mM FLEC solution (Figure 3), it can be noticed that the influence of the mixing voltage and time differed from an analyte to another. For FLEC-Ser and FLEC-Glu, the highest NARs were obtained when a mixing voltage of 0.1 kV was applied for 600 s whereas a mixing voltage of 0.5 kV for 600 s led to the best NARs for FLEC-Val. It seems that the application of a very low voltage promotes the reaction between FLEC and the AAs.

A

B



C

Figure 3. Response surface plots of normalized area ratio (NAR) of (A) FLEC-Ser 1, (B) FLEC-Val 1 and (C) FLEC-Glu as a function of the mixing voltage and mixing time.

Optimal conditions were predicted as followed: 6 mM FLEC applying a mixing voltage of 0.2 kV during 570 s. To assess the agreement between predicted and observed values, the confidence intervals were calculated (cf. Table 3).

Table 3. Predicted and observed FLEC-AA NAR values, under the optimum derivatization conditions.

Analyte	**NAR**	
	Predicted (confidence interval)	Observed
FLEC-Ser 1	0.809 (0.684-0.936)	0.892
FLEC-Ser 2	0.861 (0.717-1.004)	0.959
FLEC-Val 1	0.869 (0.742-0.996)	0.930
FLEC-Val 2	0.822 (0.699-0.943)	0.872
FLEC-Glu*	1.228 (0.965-1.491)	1.420

* Diastereomers of FLEC-Glu were not separated under these conditions.

Derivatization conditions: successive hydrodynamic injections of sample (30 mbar for 5 s) and 6 mM FLEC solution (30 mbar for 5 s); mixing voltage 0.2 kV for 570 s.

BGE: 40 mM sodium tetraborate, 25 mM SDS and 15 % IPA.

Detection at 210 nm; Other conditions as described in Section 2.

As can be seen in this table, all observed responses were found to be inside the confidence intervals, which demonstrates the suitability of the proposed models.

Finally, using the optimal conditions, the detection limits for Ser, Val, Trp enantiomers as well as DL-Glu, , estimated as the minimum concentration yielding to a S/N ratio equal to 3, were 0.5, 0.6, 1 and 0.75 µg/mL, respectively (which correspond to about 5 µM). It would be of interest to use fluorescence detection in order to achieve the high sensitivity necessary for *in vivo* AAs determination.

In order to estimate the in-capillary derivatization rate under the optimized conditions, a comparison with pre-capillary derivatization performed under the same conditions (concentrations of FLEC solution and AAs, cf. section 2) was made for Ser, Val, Glu and Trp. The relative derivatization rate calculated according to equation 2 (cf. section 2), were 83.4 % for Ser, 85.9 % for Val, 84.9 % for Glu and 82.3 % for Trp, testifying the good performance

of the proposed derivatization conditions. In addition, the RSD values of FLEC-AA NARs obtained using the in-line and off-line procedures (cf. Table 4) demonstrated the better reproducibility of in-capillary derivatization.

Table 4. RSD values (n=3) for pre-capillary and in-capillary derivatized AA NARs.

AA derivatization	**RSD FLEC-AA NAR (%)**			
	Ser	Val	Glu	Trp
Pre-capillary	5.9	8.9	4.0	9.9
In-capillary	2.5	1.6	0.18	11.9

BGE: 40 mM sodium tetraborate, 25 mM SDS and 15 % IPA.
Pre-capillary derivatization: successive hydrodynamic injections of sample (30 mbar for 5 s) and ACN (30 mbar for 5 s); mixing voltage 0.2 kV for 570 s.
In-capillary derivatization: successive hydrodynamic injections of sample (30 mbar for 5 s) and 6 mM FLEC solution (30 mbar for 5 s); mixing voltage 0.2 kV for 570 s.
Detection at 210 nm; Other conditions as described in Section 2.

3.3 Optimization of the separation conditions

After having determined the optimal derivatization conditions, the composition of the BGE was examined in order to further improve the chiral and chemo selectivity as well as to reduce the analysis time of the MEKC separation.

Using the methodology of experimental design, two factors were studied, namely the SDS concentration and the IPA percentage, using a BGE made up of 40 mM sodium tetraborate at pH 9.2.

The effect of SDS concentration was evaluated from 10 to 35 mM since the SDS critical micellar concentration in pure water at 25°C is 8.5 mM and very long analysis times (> 150 minutes in the presence of 20% IPA) were observed at concentrations higher than 35 mM. It is worth noting that the presence of SDS was found necessary to achieve separation of FLEC-diastereomers. Concerning the IPA percentage, the lower value was settled at 5% and the higher one at 20%. Higher percentages resulted in longer analysis time without bringing about significant benefit in terms of chiral separation and/or chemoselectivity. It is noteworthy that

for several FLEC-AAs (Trp, Asp, Glu) very poor diastereomeric resolution was observed in the absence of IPA.

The set of 20 natural AAs was divided into 6 groups according to their migration times (cf. Table 1) in order to study diastereoisomeric resolution. Moreover, a mixture of 19 AAs (all AAs except Pro which gave very poor efficiency) was also analyzed in order to optimize the selectivity by maximizing the number of peaks.

The regression model selected to define the relationship between the response and the factors included six coefficients (the intercept, β_0, the main effects, β_1 and β_2, the quadratic terms, β_{11} and β_{22}, and the interaction term, β_{12}), as indicated in the second order polynomial equation:

$$Y = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_{11} X_1^2 + \beta_{22} X_2^2 + \beta_{12} X_1 X_2 + \varepsilon$$

where Y is the resolution of FLEC-AA diastereomers or the number of peaks from the 19 AA mixture, X_1 the SDS concentration, X_2 the percentage of IPA and ε is the error term.

The resolution of FLEC-diastereomers of acidic AAs (Asp and Glu) and Tyr could not be modeled. Indeed, no complete separation of Asp and Glu diastereomers was observed in all experimental conditions. This is probably due to electrostatic repulsion from the negatively charged SDS micelles. For Tyr, many experiments led to R_s values lower than 0.5 (not detectable resolution). Concerning the basic AAs, only the FLEC-Arg diastereomers could be detected in a reasonable analysis time. His, Lys as well as Cys showed a high affinity for the micelles and could not be detected at the cathode within 180 minutes in most experiments. Finally, Pro gave very bad peak shape.

For the other twelve AAs, the quality of fit of the model was considered as satisfactory with adjusted R^2 values ranging from 0.78 to 0.98 for R_s of FLEC-AAs.

Table 5. Optimisation of FLEC-AA diastereomer separation by a FCCD: coefficients of the model and their *p*-values*.

Parameters	*Rs*											
	FLEC-Gln		FLEC-Asn		FLEC-Ser		FLEC-Threo		FLEC-Ala		FLEC-Val	
	Coef.	p-value	Coef.	p-value	Coef.	p-value	Coef.	p-value	Coef.	p-value	Coef.	p-value
$\beta_{1\ (SDS\ concentration)}$	0.503	**0.0011**	0.498	**<0.0001**	0.967	**0.0001**	1.762	**<0.0001**	1.233	**0.0001**	2.600	**0.0010**
$\beta_{2\ (IPA\ percentage)}$	-1.016	**<0.0001**	-0.422	**0.0001**	-0.525	**0.0011**	-0.990	**0.0005**	-0.276	**0.0458**	-0.518	0.2287
β_{11}	-0.032	0.7337	-0.068	0.2069	-0.041	0.6777	-0.489	**0.0224**	-0.202	0.1660	-0.402	0.4131
β_{22}	-0.089	0.3620	-0.149	**0.0248**	-0.473	**0.0039**	-0.783	**0.0034**	-0.709	**0.0023**	-2.823	**0.0015**
β_{12}	-0.177	0.0539	-0.072	0.1126	-0.063	0.4356	0.129	0.3291	0.042	0.6895	0.653	0.1282

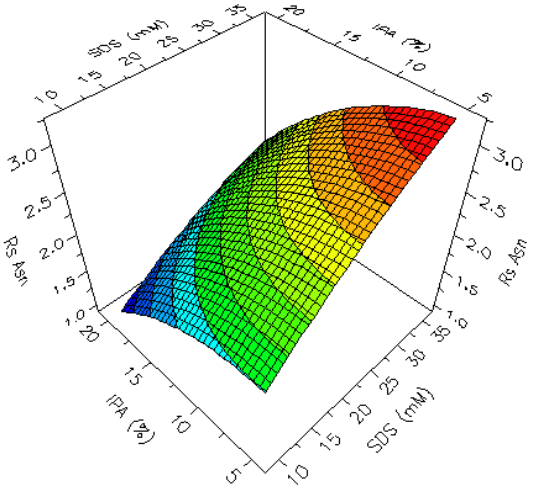
Parameters	*Rs*											
	FLEC-Met		FLEC-Leu		FLEC-Ileu		FLEC-Phe		FLEC-Trp		FLEC-Arg	
	Coef.	p-value	Coef.	p-value	Coef.	p-value	Coef.	p-value	Coef.	p-value	Coef.	p-value
$\beta_{1\ (SDS\ concentration)}$	0.686	**0.0446**	1.186	**0.0143**	2.027	**0.0046**	1.046	0.0988	0.407	**0.0425**	0.063	0.914
$\beta_{2\ (IPA\ percentage)}$	-0.213	0.4454	1.075	**0.0206**	1.330	**0.0242**	0.972	0.1186	1.631	**0.0001**	1.319	0.064
β_{11}	-0.216	0.5130	-0.386	0.3610	-0.459	0.3973	-0.273	0.6762	-0.228	0.2595	-1.044	0.176
β_{22}	-1.026	**0.0204**	-2.894	**0.0007**	-3.327	**0.0011**	-3.876	**0.0015**	-1.302	**0.0008**	-1.569	0.064
β_{12}	0.158	0.5471	1.034	**0.0197**	1.052	**0.0448**	1.658	**0.0196**	0.687	**0.0048**	0.696	0.244

* p-values significant at the 5 % level are printed in bold-face type.

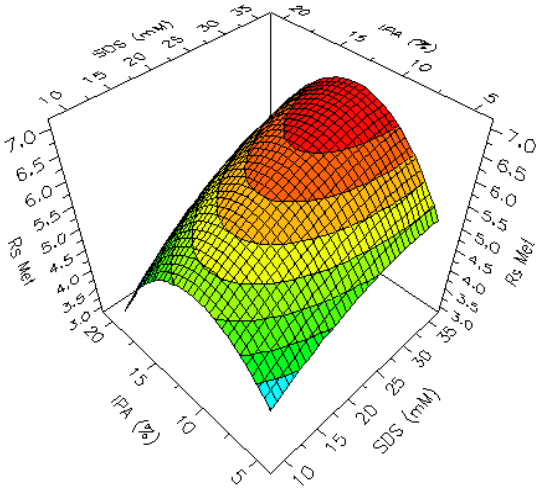
As can be seen in Table 5 and considering the coefficients of the model and their statistical significance for the different AAs, it can be noticed that the SDS concentration increased the separation of all analytes except Phe and Arg for which the influence of SDS concentration was not significant. On the contrary, the influence of the IPA percentage was different from an AA to another. Indeed, the IPA percentage had a negative effect on the diastereomeric resolution of Gln, Asn, Ser, Thr and Ala while an opposite effect was observed for Leu, Ileu and Trp. For Val and Met, it was mainly the quadratic term of the IPA percentage which was found to be also significant. It is worth noting that no factor was significant for the separation of Arg diastereomers and thus no prediction could be made. This is probably related to the important variability in the peak shape.

The response surfaces of the diastereomeric resolution were then plotted in order to obtain a better visualization of the results. Since neutral polar AAs (Gln, Ser, Asn and Thr) exhibited a similar behaviour, only the response surface obtained for Asn is presented in Fig. 4A as an example. Fig. 4B illustrates the response surface of Met, which is comparable to those obtained for the other neutral non polar AAs (Ala, Val, Leu, Ileu and Phe), whereas the response surface for Trp, which had a particular behaviour, is shown in Fig. 4C.

A



B



C

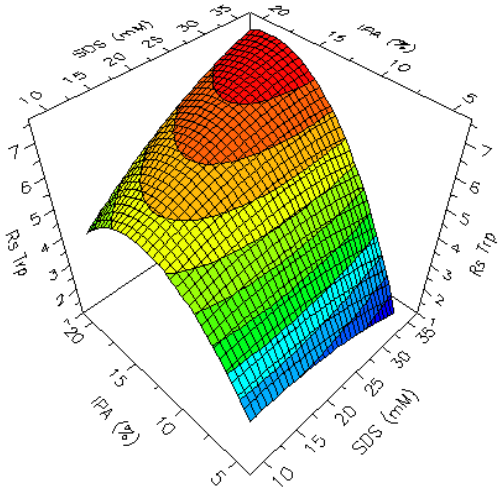


Figure 4. Responses surface plots of chiral resolution as a function of SDS concentration and IPA percentage for (A) Asn, (B) Met, and (C) Trp.

As can be seen in Fig. 4A, an increase of IPA proportion appeared to be disadvantageous for the diastereomeric resolution of early migrating FLEC-AAs (neutral polar AAs) while it is favourable to the resolution of last migrating AA diastereomers, namely Trp (Fig. 4C). For non polar AAs (Met, Ala, Val, Leu, Ileu and Phe), an intermediate percentage of IPA gives rise to maximum resolution (cf Fig. 4B). All these results suggest that the partitioning of FLEC-AA diastereomers into the micelles and their interactions with IPA differ strongly from each other.

Table 6 summarizes the maximum diastereomeric resolution values predicted by the model. The use of FLEC as chiral derivatising reagent led to very high resolution values ranging from 3 to 20 for eleven AAs. It is shown that maximum resolution was observed with the highest SDS concentration for all studied AAs.

Table 6. Predicted maximum resolution values for diastereomeric separation of (-)-FLEC-AAs as well as resolution values obtained under the optimal conditions (21 mM SDS and 8.5 % IPA) and their predicted confidence intervals.

AA	Maximum	BGE composition		Optimal conditions
	R_s	SDS (mM)	IPA (%)	Observed R_s (predicted confidence interval)
Gln	4.7	35	5	3.0 (2.7-3.3)
Asn	3.4	35	5	2.3 (2.3-2.6)
Ser	5.0	35	5	3.8 (3.3-3.9)
Thr	9.9	35	6	7.9 (7.6-8.6)
Ala	6.7	35	11.7	4.5 (4.4-5.3)
Val	19.2	35	12.5	15.1 (13.0-16.1)
Met	7.1	35	12.5	6.1 (4.9-7.0)
Ileu	19.6	35	15	15.4 (12.8-16.2)
Leu	15.3	35	15	11.8 (10.5-13.1)
Phe	17.9	35	15	13.9 (11.9-16.1)
Trp	7.8	35	18	4.2 (3.9-5.2)
Arg	Not modeled			4.3
Asp				≤0.5
Glu				≤0.5
Tyr				0.7
His				ND
Lys				ND
Cys				ND

ND: not detected within 80 min.

Special attention was then paid to the method selectivity toward the diastereomers but also towards the different AAs taking into account the analysis time. For this method optimization, the diastereomeric resolution was settled at 4 as target value, since higher resolution is not requested and would be detrimental to the analysis time, while the number of peak was maximized. The optimum BGE composition using this optimization strategy was found to consist of 21 mM of SDS and 8.5% (v/v) IPA. A typical electropherogram of the mixture of 15 chiral AAs and Gly under these conditions is shown in Fig. 5. This figure shows the separation of 29 peaks of FLEC-AAs in a single run. Indeed, the diastereomers of the 12 AAs were completely separated (cf. Table 6); Asp, Glu, Gly appeared as single peaks and Tyr diastereomers started to be separated. It should be noted that under these conditions, His, Lys and Cys migrated after 80 minutes.

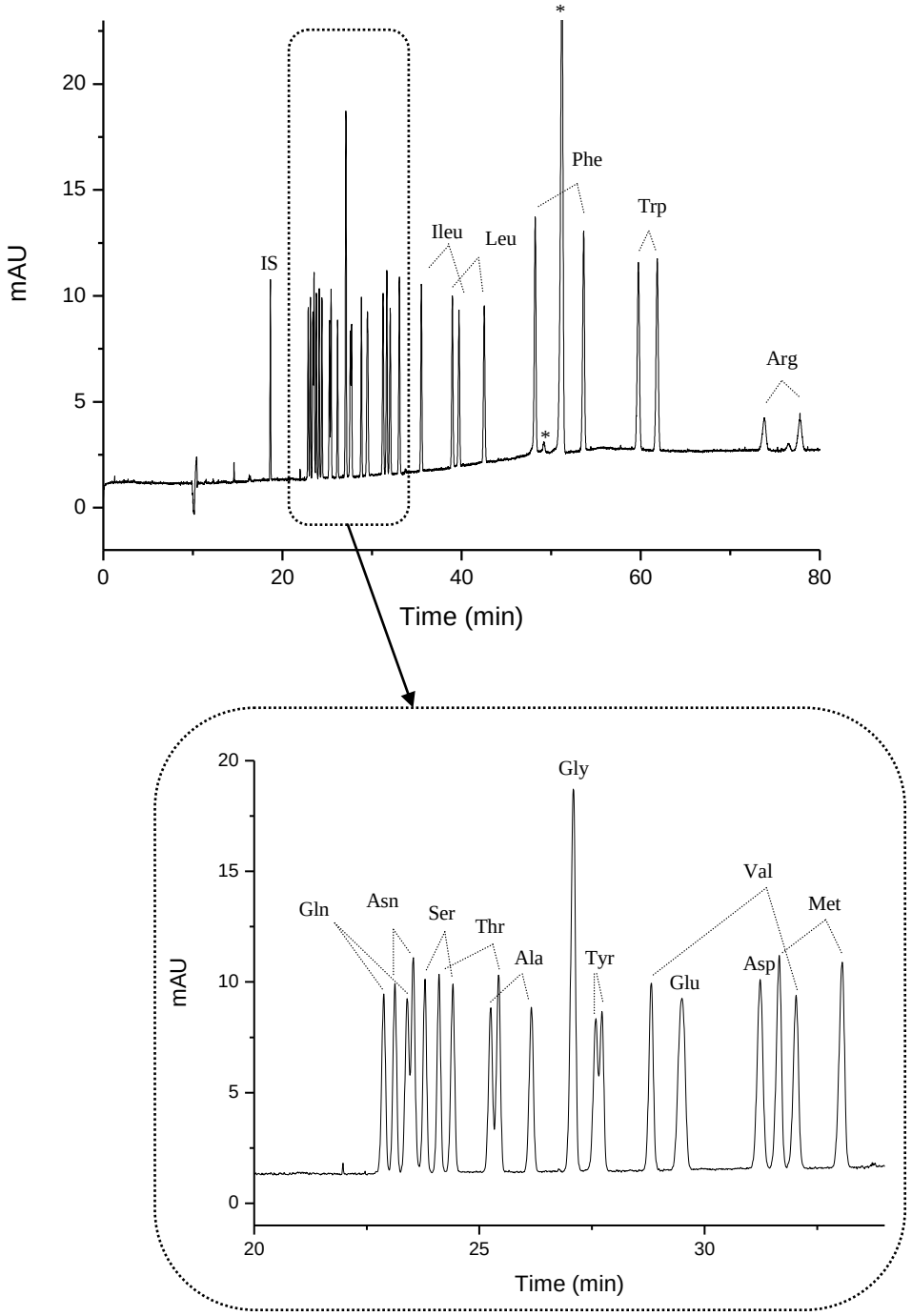


Figure 5. Electropherogram of a mixture of 15 chiral AAs and Gly AAs using in-capillary derivatization with (-)-FLEC. Peaks marked with an asterisk correspond to FLEC peaks. Derivatization conditions: successive hydrodynamic injections of sample (30 mbar for 5 s) and 12 mM FLEC solution (30 mbar for 5 s); mixing voltage 0.2 kV for 570 s.
BGE: 40 mM sodium tetraborate pH 9.2, 21 mM SDS and 8.5 % (v/v) IPA. Other conditions as described in section 2.
*excess of FLEC

Other conditions for optimizing the separation of acidic AAs (Asp and Glu) as well as His, Lys and Cys diastereomers by CE using in-capillary derivatization with (-)-FLEC are currently under study in our laboratory.

After having optimized the derivatization and separation conditions, the applicability of the method for quantitative purposes, including linearity, mean recovery and repeatability, was evaluated using a representative group of AAs (Ser, Val and Trp) (cf. Table 7). The linearity between FLEC-AAs NARs and the AA concentration was investigated in the range of 0.025 – 0.5 mM (per enantiomer). For this purpose, six concentration levels were considered and three replicates were performed at each level. As can be seen in Table 7, the determination coefficients (r^2) obtained for all regression lines demonstrate the excellent relationship between FLEC-AAs NAR and analyte concentration. The mean recoveries for the studied analytes ranged from 101.4 to 102.8 %. As can be seen in Table 7, the RSD values of migration times and FLEC-AAs NARs illustrate the good repeatability of the proposed method considering the in-capillary derivatization procedure.

Table 7: Quantitative performance of the optimized MEKC method from in-capillary derivatization of Ser, Val and Trp.

AA	Linear regression		Determination coefficient (r^2)	Mean recovery (%)[(a)]	Repeatability RSD % (n = 8)[(b)]	
	Slope	Intercept			t_m	*NAR*
D-Ser	2.971	-0.004	0.9961	102.1	2.5	7.7
L-Ser	2.971	-0.009	0.9964	101.4	2.5	7.6
D-Val	3.277	-0.009	0.9995	101.4	2.4	2.8
L-Val	3.236	-0.012	0.9993	101.8	2.5	2.5
D-Trp	4.273	-0.034	0.9994	102.8	2.9	4.4
L-Trp	4.615	-0.033	0.9994	102.8	3.1	4.1

[(a)] The recoveries were determined in triplicate at the concentration levels of 0.025, 0.05, 0.125, 0.25, 0.375 and 0.5 mM.

[(b)] The RSD values were determined at the concentration of 0.25 mM.

Derivatization conditions: successive hydrodynamic injections of sample (30 mbar for 5 s) and 12 mM FLEC solution (30 mbar for 5 s); mixing voltage 0.2 kV for 570 s.

BGE: 40 mM sodium tetraborate pH 9.2, 21 mM SDS and 8.5 % (v/v) IPA. Other conditions as described in section 2.

The applicability of the optimized in-capillary MEKC method for the analysis of CSF was finally evaluated. For this purpose, aCSF spiked with Ser, Val and Trp was analyzed. The electropherogram of spiked aCSF sample is shown in Figure 6. As shown in this figure, the migration order of diastereomers can be different from one AA to another.

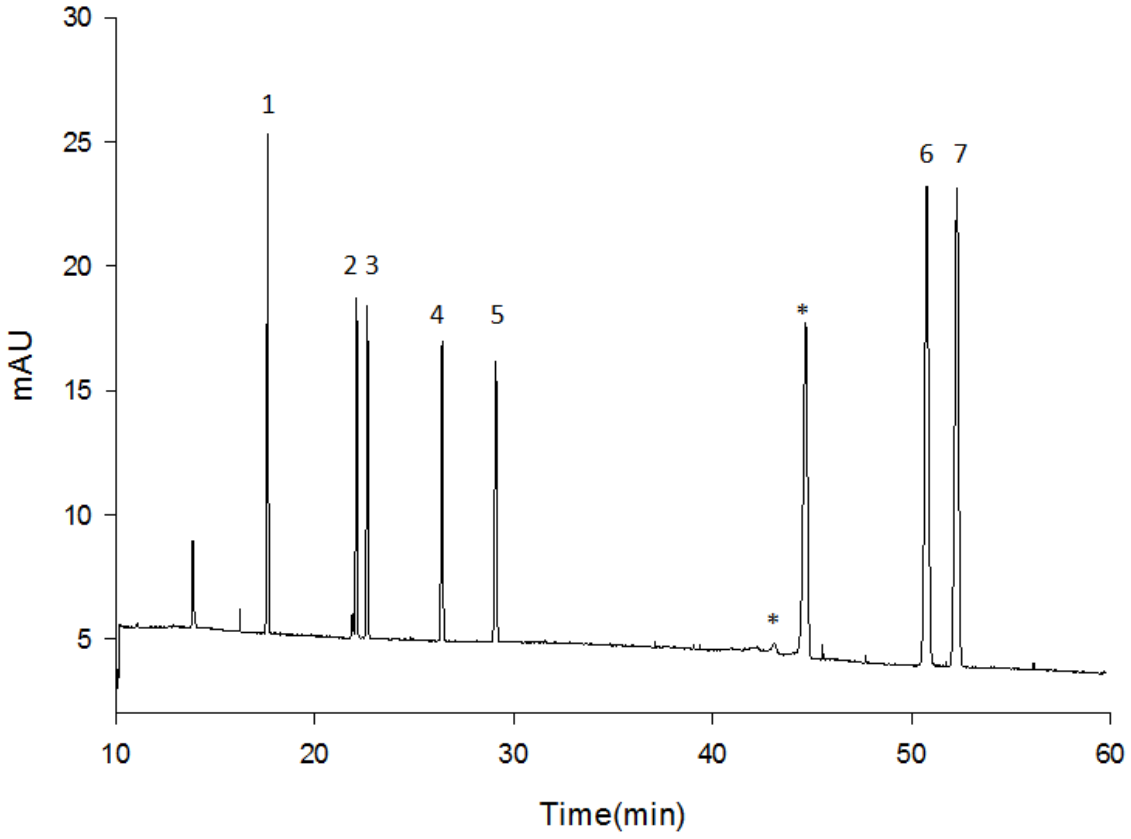


Figure 6

Typical electropherogram of aCSF spiked with a mixture of Ser, Val and Trp under optimized conditions. Peak identification: (1) internal standard, (2) FLEC-L-Ser (3) FLEC-D-Ser, (4) FLEC-D-Val and (5) FLEC-L-Val, (6) FLEC-D-Trp and (7) FLEC-L-Trp. Peaks marked with an asterisk correspond to FLEC peaks.

Derivatization conditions: successive hydrodynamic injections of sample (30 mbar for 5 s) and 6 mM FLEC solution (30 mbar for 5 s); mixing voltage 0.2 kV for 570 s.

BGE: 40 mM sodium tetraborate pH 9.2, 21 mM SDS and 8.5 % (v/v) IPA. Other conditions as described in section 2.

NAR values in aCSF, compared to those of standard sample solution using the same AAs mixture prepared in sodium tetraborate 5 mM, are given in Table 8. As can be seen in this table, despite the high salt concentration of such kind of matrix, high peak efficiencies (N > 300.000) as well as high analyte recoveries (from 75% to 80%) are observed. These data support the suitability of the proposed method for its application to real CSF samples.

Table 8: Comparison of NAR values of AAs mixture in aCSF with the standard sample solution using the optimized in-capillary MEKC method.

AA	NAR		Recovery (%)
	A	B	
D-Ser	0.77	0.61	79.2
L-Ser	0.78	0.61	78.2
D-Val	0.71	0.53	74.6
L-Val	0.70	0.53	75.7
D-Trp	1.22	0.97	79.5
L-Trp	1.18	0.92	78.0

A: AAs solubilised in sodium tetraborate (5 mM); B: AAs spiked in aCSF

Data are means of four analyses.

Derivatization conditions: successive hydrodynamic injections of sample (30 mbar for 5 s) and 12 mM FLEC solution (30 mbar for 5 s); mixing voltage 0.2 kV for 570 s.

BGE: 40 mM sodium tetraborate pH 9.2, 21 mM SDS and 8.5 % (v/v) IPA. Other conditions as described in section 2.

4. Conclusion

In this study, the first in-capillary derivatization using FLEC as labelling reagent is described and the subsequent MEKC separation of formed FLEC-AA diastereomers is optimized. Appropriate conditions for in-capillary derivatization procedure were found by the methodology of experimental design. The results highlight the key role of a very low mixing voltage promoting the reaction between AAs and FLEC inside the capillary. Compared to pre-capillary derivatization, the optimized in-capillary procedure exhibits some relevant advantages such as full automation of derivatization, improvement of reproducibility of normalized peak area ratios and low consumption of sample and reagent.

The BGE composition was then optimised using FCCD in order to allow the resolution of a maximum of FLEC-D/L-AAs pairs as well as the separation of a mixture of 19 AAs. Interestingly, it was found that IPA did not have the same influence on the separation of the diastereomers, while the presence of SDS was found to be necessary in all cases.

After optimization, a chemo and stereoselective separation of 29 derivatives of FLEC-AAs was obtained with a BGE composed of sodium teraborate 40 mM, 21 mM of SDS and 8.5% of IPA using a fully automated method.

Quantitative performance of the developed method was evaluated showing appropriate linearity, accuracy and repeatability.

Finally, the applicability of the in-capillary MEKC method to the analysis of aCSF sample has been demonstrated.

Further increase in sensitivity might be achieved through pre-concentration approaches and the use of more sensitive detectors, such as fluorescence detectors, making the method even more attractive, particularly for *in vivo* applications.

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General conclusion and perspectives

Chapter 4

GENERAL CONCLUSION AND PERSPECTIVES

In this research work, chiral analysis of AAs by MEKC has been investigated using both direct and indirect approaches coupled to in-capillary derivatization.

In the first part of this work (section 3.1), a dual chiral CE method has been optimized for the separation of mixtures of AAs following in-capillary derivatization with FMOC. In order to achieve enantioseparation of FMOC-AAs, BGE composition was optimized. Dual CD system composed of 30 mM β-CD, 30 mM OS-γ-CD, 40 mM tetraborate and 15% IPA was selected and led to 17 baseline resolved pairs and two partially resolved pairs (Lys, Rs = 0.5 and Arg, Rs = 1.2). Experimental conditions for in-capillary derivatization were then optimized. Among the studied parameters, the applied voltage and time to mix the plugs seriously impacted the in-capillary derivatization performance. Meanwhile, the in-capillary derivatization did not decrease the peak efficiency and enantiomeric resolution.

Moreover, the effect on stereoselectivity and chemoselectivity of different factors, such as decrease of pH and tetraborate concentration and the addition of SDS, was investigated using the in-capillary derivatization procedure. The use of MEKC, by adding SDS as surfactant, improved not only enantioselectivity, but also chemoselectivity of FMOC-AAs separation. A separation buffer composed of 30 mM β-CD and 30 mM OS-γ-CD as chiral selectors, 25 mM SDS as surfactant and 17 % IPA as organic modifier led to the simultaneous separation of ten pairs of FMOC-AAs.

The second part of this work was dedicated to the development of a method coupling in-capillary derivatization to form diastereoisomers using (-)-FLEC as chiral labeling agent (section 3.2). Design of experiment methodology was first used to determine the derivatization conditions. FLEC concentration, the mixing voltage as well as mixing time were studied as factors. Using the optimized conditions, in-capillary relative derivatization rates, estimated comparatively to the pre-capillary derivatization for several AAs, were higher than 82.3 %. Moreover, compared to off-line derivatization, in-capillary derivatization exhibited better reproducibility.

After having optimized the derivatization procedure, BGE composition was studied in order to further improve the chiral and chemo selectivity. The influence of SDS concentration and IPA percentage was studied using experimental design. The AAs presented different behaviors according to their chemical properties (basic, acidic, polar, hydrophilic, and

hydrophobic). By optimizing the separation conditions, 13 pairs of FLEC-AAs (Asn, Gln, Ser, Ala, Thr, Tyr, Val, Met, Leu, Ileu, Phe, Trp and Arg) as well as FLEC-Asp, FLEC-Glu (with no diastereomeric resolution) and FLEC-Gly were separated in a single run.

Analytical performance of the developed method was evaluated showing appropriate results (linearity, accuracy and acceptable precision). The applicability of the method to CSF was demonstrated through the analysis of spiked aCSF.

The method was simple, effective, economic and applicable to the quantitative simultaneous chiral analysis of neutral chiral AAs in the presence of acidic and basic AAs.

It was demonstrated that MEKC coupled with in-capillary derivatization is a suitable technique for quantification of amino acids enantiomers even in high salt concentration sample matrices. Most importantly, the in-capillary derivatization procedure appears to have large promise as simple, rapid and automated sample preparation method.

Since FLEC derivatives are highly fluorescent, alternative detection schemes such as LIF, are expected to improve sensitivity enabling the application of the developed method to real biological samples.

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Chapter 5

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List of publications

Chapter 6

1. Scientific papers

1.1. First author publications

I. Fradi, E. Farcas, A. Ben Saïd, M. L. Yans, C. Lamalle, G. W. Somsen, A. Prior, G. J. de Jong, M. Kallel, J. Crommen, A. C. Servais, M. Fillet, In-capillary derivatization with (-)-1-(9-fluorenyl)ethyl chloroformate as chiral labelling agent for the electrophoretic separation of amino acids. Accepted in J. Chromatogr. A, July 2014.

I. Fradi, A. C. Servais, C. Lamalle, M. Kallel, M. Abidi, J. Crommen, M. Fillet, Chemo- and enantio-selective method for the analysis of amino acids by capillary electrophoresis with in-capillary derivatization. J. Chromatogr. A 1267 (2012) 121.

I. Fradi, A. C. Servais, M. Pedrini, P. Chiap, R. Iványi, J. Crommen, M. Fillet, Enantiomeric separation of acidic compounds using single-isomer amino cyclodextrin derivatives in nonaqueous capillary electrophoresis. Electrophoresis 27 (2006) 3434.

1.2. Co-author publication

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2. Oral communication

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