

**"IULIU HATIEGANU" UNIVERSITY OF MEDICINE AND PHARMACY CLUJ-NAPOCA**

**UNIVERSITY OF LIEGE**

**DOCTORAL SCHOOL**



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PhD THESIS

# Development and validation of electrophoretic and chromatographic methods coupled with mass spectrometry for metabolomic analyses

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*“Whatever it is you're seeking won't come in the form you're expecting.”*

*Haruki Murakami*

*To my family  
(familie i mele)*



## **PUBLICATIONS LIST**

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2. Moldovan R-C, Bodoki E, Servais A-C, Chankvetadze B, Crommen J, Oprean R, et al. Capillary electrophoresis-mass spectrometry of derivatized amino acids for targeted neurometabolomics – pH mediated reversal of diastereomer migration order. *J Chromatogr A.* 2018 Aug;1564:199-206. (ISI 3.716) (study included in chapter 4)
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## ABBREVIATIONS

$\mu_{\text{EFF}}$	- effective electrophoretic mobility
ACN	- acetonitrile
aCSF	- artificial cerebrospinal fluid
Ala	- alanine
ALS	- amyotrophic lateral sclerosis
ADAM	- 1-aminoadamantane
APFO	- ammonium perfluorooctanoate
Arg	- arginine
Asn	- asparagine
Asp	- aspartic acid
BGE	- background electrolyte
CD	- cyclodextrin
CDA	- chiral derivatizing agent
CE	- capillary electrophoresis
CNS	- central nervous system
CSF	- cerebrospinal fluid
Cys	- cysteine
CZE	- capillary zone electrophoresis
DAAO	- D-amino acid oxidase
DNPA	- 2,4-dinitrophenyl-5-L-alanine amide
FAD	- flavin adenine nucleotide
FL	- fluorescence detection
(±)-FLEC	- (±)-1-(9-fluorenyl)ethyl chloroformate
FMOC	- 9-fluorenylmethyl chloroformate
GITC	- 2,3,4,6-tetra-O-acetyl- $\beta$ -D-glucopyranosyl isothiocyanate
Gln	- glutamine
Glu	- glutamic acid
Gly	- glycine
HEPES	- 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
His	- histidine
Ile	- isoleucine
IPA	- isopropyl alcohol
I.S.	- internal standard
Leu	- leucine
LC	- liquid chromatography
LIF	- laser induced fluorescence
LOD	- limit of detection

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LOQ	- limit of quantification
Lys	- lysine
Marfey's reagent	- 1-fluoro-2,4-dinitro-phenyl-5-D-alanineamide
MEKC	- micellar electrikinetic chromatography
MeOH	- methanol
MDA	- 3,4-methylenedioxymphetamine
MDMA	- 3,4- methylenedioxymethamphetamine
MDE	- 3,4-methylenedioxymethylamphetamine
MeOH	- methanol
Met	- methionine
MS	- mass spectrometry
NaTB	- sodium tetraborate buffer
NEC	- (+)-l-(l-naphthyl)ethyl carbamoyl
NMDA	- <i>N</i> -methyl-D-aspartate
NMDAR	- <i>N</i> -methyl-D-aspartate receptor
NMLA	- <i>N</i> -methyl-L-aspartate
OPA	- <i>o</i> -phthalaldehyde
OPA-NAC	- <i>o</i> -phthalaldehyde/ <i>N</i> -acetyl-L-cysteine
OPA-NAP	- <i>o</i> -phthalaldehyde/ <i>N</i> -acetyl-D-penicillamine
Phe	- phenylalanine
PLP	- pyridoxal 5'-phosphate
Pro	- proline
RP	- reversed phase
SDS	- sodium dodecylsulphate
Ser	- serine
SPE	- solid phase extraction
SR	- serine racemase
TBABr	- tetrabutylammonium bromide
TBA <sup>+</sup> OH <sup>-</sup>	- tetrabutylammonium hydroxide
THF	- tetrahydrofuran
Thr	- threonine
Trp	- tryptophan
Tyr	- tyrosine
Val	- valine

# INTRODUCTION

In the last 20 years many important roles in the human organism have been attributed to D-amino acids. A summary of the relevant literature is described in **Chapter 1**. For instance, some AAs (D-Ser, D-Ala, Gly) have been identified to be as co-agonists of the *N*-methyl-D-aspartate excitatory receptor of glutamate<sup>1-4</sup>. Their misregulation has been linked to numerous neurological and neurodegenerative diseases, such as schizophrenia<sup>5-9</sup>, depression<sup>10</sup>, epilepsy<sup>11</sup>, amyotrophic lateral sclerosis<sup>12-14</sup>, Parkinson's disease<sup>15</sup> etc. Some D-AAs have also been introduced in therapy, such as D-Ser (together with antipsychotics) in the treatment of positive, negative and cognitive symptoms of schizophrenia<sup>16</sup>.

The chiral analysis of amino acids usually proves to be challenging. Considering the small molecular mass of the amino acids and their lack of chromophore or fluorophore moieties in the structure, their separation and detection proved to be difficult. In order to overcome this, chiral derivatization is a common practice, increasing the detectability of the amino acids and, at the same time, offering the opportunity to tune the selectivity.

Over the last 30 years, ( $\pm$ )-1-(9-fluorenyl) ethyl chloroformate (( $\pm$ )-FLEC) was used as a chiral derivatizing agent (CDA) in various analytical applications involving a wide range of endogenous, pharmaceutical and environmentally relevant molecules. A comprehensive literature review on the use of FLEC as a CDA is presented in **Chapter 2**. The aim was to present all the significant aspects related to the state of the art in FLEC labeling and subsequent chiral separation of the resulting diastereomers using LC, SFC and CE techniques.

In the context of bioanalytical method development, process automatization is nowadays a necessity in order to save time, improve method reliability and reduce costs. The **3<sup>rd</sup> Chapter** presents, for the first time, the development of a fully automatized MEKC-MS method with in-capillary derivatization for the chiral analysis of D- and L-amino acids using FLEC as labeling reagent. The derivatization procedure was optimized using an experimental design approach leading to the following conditions: sample and FLEC plugs in a 2:1 ratio (15s, 30mbar: 7.5s, 30mbar) followed by 15 min of mixing using a voltage of 0.1 kV. The formed diastereomers were then separated using a background electrolyte (BGE) consisting of 150 mM ammonium perfluorooctanoate (APFO) (pH=9.5) and detected by mass spectrometry (MS). Complete chiral resolution was obtained for 8 amino acids, while partial separation was achieved for 6 other amino acid pairs. The method showed good reproducibility and linearity in the low micromolar

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concentration range. The applicability of the method to biological samples was tested by analyzing artificial cerebrospinal fluid (aCSF) samples.

Given the fact that until now only some D-AAs have been found to be biologically relevant, the development of a targeted CE-MS approach is presented in **Chapter 4**. The method is intended to be used for the chiral analysis of five biologically relevant amino acids (Ser, Asn, Asp, Gln and Glu) in CSF. In order to achieve chiral resolution, the amino acids were derivatized with (+)-FLEC and the chiral selectivity was found to be highly dependent on pH for all analytes and the optimized BGE consisted of 150 mM acetic acid, adjusted to pH 3.7 with NH<sub>4</sub>OH. Furthermore, a reversal of the migration order of Asp derivatives was observed. This phenomenon seems to be caused by intra-molecular interactions affecting the pK<sub>a</sub> of the second ionizable group (the side chain carboxyl).

The applicability of this method was evaluated using aCSF. A solid phase extraction (SPE) protocol was developed for the selective extraction of the FLEC derivatives. A full evaluation of the matrix effect and extraction yield was performed concluding that the matrix effect is marginal and the recoveries are between 46 and 92%. The method offers adequate sensitivity (limits of detection below 1 µM).

The study comprised in **Chapter 5** is meant to progress the knowledge in the liquid chromatographic separations of FLEC-DL-AAs. All the methods described before for their diastereomeric separation had some important drawbacks. For example, all studies employed C4, C8 or C18 stationary phases in combination with THF in various ratios as mobile phases. It is well known that THF use is detrimental to the liquid chromatographic instruments, which cause damage and/or shorten the life of different plastic or rubber components. The aim of this study was to evaluate the selectivity of two phenyl stationary phases for the separation of FLEC-DL-AAs. For this, gradient elution of ammonium acetate in combination with different organic solvents was employed. The diastereoresolution was observed to be closely influenced by the pH of the mobile phase; at the same time, the resolution was less influenced by other variables such as the nature of organic solvent, length of the gradient or the starting percentage of organic solvent. For a better understanding of these phenomena, an experimental design was employed, several correlations being established explaining the chromatographic behavior.

A general discussion is comprised in **Chapter 6** comparing the different analysis methods that were developed and their potential use.

## **LITERATURE REVIEW**



## Chapter 1:

# D-amino acids' implications in neurological and neurodegenerative diseases

Proteins are the largest group of macromolecules found in all living cells. All proteins, starting from bacteria up to the most complex living organisms, are formed by 20 amino acids. These amino acids are covalently bound in countless combinations to form unique sequences corresponding to all different proteins. All these 20 proteinogenic amino acids are  $\alpha$ -amino acids, therefore the general structure includes an amino group and a carboxyl group linked to the same carbon atom.

A common feature of almost all  $\alpha$ -amino acids (except Gly) is that they have at least one chiral center. Stereochemistry is the study of the 3D structure of chemical compounds, stereoisomers being isomers of the same substance that only differ in their 3D arrangement. Moreover, if they only differ in the capacity of rotating the plane of the polarized light passing through them, then they are called enantiomers. Amino acids' optical activity was first reported by Pasteur in 1851, their stereochemistry being extensively studied ever since. The vast majority of amino acids occurring in nature are L-amino acids. But even if the D-forms are less prevalent, they seem to play significant roles, endorsing the efforts to develop new tools for their analysis.

Over the years, numerous studies dealt with the significance of D-amino acids in various samples. The research areas ranged from food and environmental analysis to medicine and pharmaceuticals, being lately reviewed in several publications<sup>1,17–25</sup>.

D-AAs were detected in numerous tissues of vertebrates and invertebrates, either in free form or peptide-bound<sup>26,27</sup>. Among them, D-Ser, D-Asp, D-Ala, D-Glu and D-Gln are the free D-AAs occurring in significant levels in mammalian tissues. With few exceptions, the roles of D-AAs in the human organism are generally undesirable. For instance, in diet they affect the digestibility of the proteins, peptide bonds with D-AAs being more resistant to proteases<sup>28</sup>.

## 1.1 Origins of D-amino acids

The D-amino acids can be found in minute quantities in many living organisms, their origin being both exogenic and endogenic. D-AAs can be acquired by human beings

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by food ingestion, release from metabolically unstable polypeptides and biosynthesis of L-AAs.

The exogenic amino acids usually have a dietary origin, either occurring naturally in food, or as a result of food processing (D-AAs produced under certain conditions: high temperatures, strong acidic or basic conditions, fermentation etc.). Csapo et al.<sup>29</sup> reported considerable quantities of D-AAs obtained by racemization in different common prepared foods (fruit juices, cereals, milk, baked potatoes etc.). Racemization was found to be induced by extreme pH values and heat treatment, which are some of the most common food processing procedures. For example, the presence of D-AAs in dairy products can be used as a marker of thermal and alkaline treatments. Oancea et al.<sup>30</sup> found that a concentration above 4% of D-Ala in milk can represent microbial contamination, while the presence of D-Pro in wine can be an indicator of its age.

D-AAs can also be the result of fermentation processes; therefore, they are commonly found in dairy products and fermented beverages (wine, beer etc.). Different D-AAs (D-Ala, D-Asp, D-Glu, D-Ser, D-Lys) have been reported by Albert et al.<sup>31</sup> in different sorts of milk (cow, sheep and goat milk), but not in human milk. Their presence in the milk of these animals is associated with the enzymatic digestion of D-AA containing peptides and proteins derived from peptidoglycan in the ruminants' rumen.

## 1.2 D-amino acids' metabolism

Most of D-AAs' synthesis and degradation is regulated by two enzymes: amino acid racemase and D-amino acid oxidase (DAAO). The racemase catalyzes the conversion between L- and D-AAs. Since L-AAs are most abundant in nature, they act as substrate for generating D-AAs. Yoshimura et al.<sup>32</sup> found that the racemase can be both pyridoxal 5'-phosphate (PLP) dependent and independent. For example, alanine racemase and serine racemase (SR) are PLP-dependent, while glutamate racemase and aspartate racemase are PLP-independent enzymes.

SR, besides being PLP-dependent, also needs Mg<sup>+2</sup>, Ca<sup>+2</sup>, Mn<sup>+2</sup> and ATP for converting L-Ser to D-Ser<sup>33,34</sup>. Chelators, such as EDTA, were found to completely inhibit its enzymatic activity<sup>35</sup>. Enzymatic inhibition occurs also in the presence of L-Asp and L-Asn (by competitive inhibition) and by reagents which react with sulphhydryl groups (glutathione). Besides catalyzing the conversion of L-Ser to D-Ser, SR seems to be also accountable for the racemization of Asp<sup>36</sup>.

In mammals, D-Asp is an amino acid found mainly in the central nervous system and reproductive systems. Even if evidence suggest that its origin is endogenic, no specific enzyme responsible for the biosynthesis of D-Asp was identified.

The enzyme responsible for the degradation of D-AAs is DAAO, degrading them through oxidative deamination. First described by Krebs<sup>37</sup>, this enzyme is flavin adenine nucleotide (FAD) dependent, being found in many organisms, from microbes to mammals. The typical degradation products are hydrogen peroxide, an α-ketoacid and

ammonium. It has a high affinity for neutral D-AAs and a marginal activity for the basic ones, the metabolism of acidic D-AAs being realized by D-aspartate oxidase.

Recent research<sup>38</sup> on the levels of D-AAs on the perfused brain of mice suggests that there are also other processes controlled by other enzymes than DAAO and racemases that control broad levels of D-AAs in brain tissue.

## 1.3 Roles of D-AAs in the nervous and endocrine systems

### 1.3.1 D-Ser

D-Ser was the first D-AA found in mammal tissues, the discovery being made by Hashimoto et al.<sup>39</sup> by analyzing rat brain. Since then, D-Ser has also been identified in peripheral tissues and physiological fluids.

Besides Gly, D-Ser is the only proteinogenic amino acid that is synthesized in the human body for its own use<sup>40</sup>. The synthesis takes place from L-Ser, being mediated by SR. In brain, D-Ser has an enantiospecific binding to *N*-methyl-D-aspartate receptor (NMDAR). This excitatory receptor is one of the ionotropic receptors of glutamate, requiring a co-agonist in addition to glutamate for its activation, therefore the positive modulatory roles of NMDAR co-agonists are of significant importance<sup>1</sup>. Even though both D-Ser and Gly have high affinity for the receptor, D-Ser affinity to NR1/NR2 subunits of the NMDAR was found to be three times more potent than that of Gly<sup>2</sup>. Moreover, D-Ser binding seems to be critical to synaptic NMDAR activity<sup>3,4</sup>. In particular, D-Ser binds primarily to the synaptic NMDARs, but not the extrasynaptic ones<sup>41</sup>. The binding to these glutamate receptors is required for several related processes, such as normal neurotransmission, but also in pathophysiological ones. The regulation mechanisms and their roles in the NMDAR activity are still to be completely elucidated.

Compared to rodents, the regulation of D-Ser in humans is less understood. SR seems to be expressed both in excitatory and inhibitory neurons in human primary cortex<sup>42</sup> and in GFAP-positive astrocytes in human hippocampus<sup>43</sup>. In cerebral cortices of postmortem human brain D-Ser levels have been reported to be between 80 to 150 nmol/g<sup>44</sup>. A recent study<sup>45</sup> dealing with the distribution of D-Ser in human CNS found that the D-Ser concentration in the cerebrum is around 10-fold higher than in the brainstem, cerebellum and spinal cord. Within the cerebrum, the highest concentration is found in neocortex, putamen and hippocampus, containing 20% higher level of D-Ser than the cerebral average. At the same time 20% lower concentrations were found in corpus callosum, pineal gland, mamillary body and olfactory bulb, suggesting that D-Ser seems to be concentrated in the areas abundant in glutamatergic synapses. By comparison, Gly was detected in concentrations 10-fold of those of D-Ser, but no region-specific differences were detected. NMDARs play significant roles in many physiological processes, such as memory formation, nociception, synaptic development and plasticity, but also neurotoxicity<sup>46</sup>.

Considering that D-Ser is mainly distributed in the prefrontal cortex and hippocampus, its misregulation has been linked with several psychiatric and neurologic

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diseases such as schizophrenia<sup>5-9</sup>, amyotrophic lateral sclerosis<sup>12-14</sup> and depression<sup>10</sup>. Lately, D-Ser was even suggested to be administered as therapy (see section 1.5).

Animal and human studies<sup>47</sup> have shown that decreased levels of D-Ser lead to NMDAR hypofunction, which may contribute to the onset of schizophrenia. Moreover, DAAO, the enzyme which degrades D-Ser, seems to be activated in schizophrenia<sup>48</sup>. A recent review and meta-analysis<sup>23</sup> on the levels of D-Ser in patients suffering from schizophrenia concluded that the levels of D-Ser in brain and blood coming from those patients were lower than in healthy controls. Therefore, the NMDAR hypofunction is a justified hypothesis.

Recently, a direct link between amyotrophic lateral sclerosis/motor neuron disease (ALS) and elevated D-Ser levels has been suggested<sup>12-14</sup>. One of the causes of high D-Ser levels was identified as a pathogenic mutation in the DAAO. The mutated enzyme has been shown to have marked toxic effects in motor neurons, promoting apoptosis. At the same time, in ALS the CSF Gly levels are also high, suggesting that the clearance of both Gly and D-Ser may be impaired. The balance between the two co-agonists is probable to be complex and regulated by multiple factors.

More and more evidence suggests that neural neurotransmission via NMDAR is a factor involved in the pathophysiology of the major depressive disorder<sup>49-51</sup>. One recent study<sup>10</sup> shows that serum levels of D-Ser and L-Ser in patients suffering from major depressive disorder are significantly higher compared to healthy controls. Moreover, the ratio between L-Ser and Gly was also significantly higher, which may suggest that in depression there is an abnormal function in the synthesis and metabolism of Ser enantiomers. However, the NMDAR dysfunction in the pathology of depression is still a matter of debate and research, considering that both antagonists (e.g. ketamine) and agonists (e.g. D-Ser) have therapeutic effects<sup>52</sup>.

### 1.3.2 D-Asp

D-Asp, an endogenous amino acid found in invertebrates and vertebrates, has a neuroendocrine role in the central nervous system<sup>26</sup>, acting as a neurotransmitter. Besides its roles in the nervous system, D-Asp is involved in the endocrine system, regulating hormone synthesis and release.

It was demonstrated that D-Asp is present in high concentrations in the nervous systems of chicken, rat and human embryos<sup>26</sup>; in the nervous systems of adults it practically disappears, while it increases in the endocrine glands. The neurotransmitter role of D-Asp was highlighted by its detection in nerve endings, being capable of increasing the intracellular levels of cAMP in cultured neurons<sup>53</sup>. Moreover, some studies<sup>54,55</sup> detected specific D-Asp transporters which transfer it from the synaptic cleft to the presynaptic neurons.

Considering that the highest levels of D-Asp are found in glands and glandular tissue, they are most likely candidates for modulation by this amino acid. On top, they possess biosynthesis and degradation systems for D-Asp. It was shown that in the rat hypothalamus it promotes the release of gonadotropin releasing hormone<sup>56</sup>. In the

anterior pituitary gland acts as an excitatory molecule stimulating the secretion of prolactin, growth hormone<sup>57</sup> and luteinizing hormone<sup>58</sup>. At the same time, it has an inhibitory effect on the release of melatonin secreting hormone and oxytocin in the intermediate and posterior pituitary. Evidence shows that the stimulatory effect of D-Asp is exerted through NMDAR activity. Additionally, it is present in testes, in Leydig cells, being involved in the release of testosterone and progesterone.

Most of the efforts regarding D-Asp research have been focused on establishing D-Asp's role in the reproductive systems. A recent systematic review<sup>59</sup> on the presumed effects of D-Asp on blood testosterone levels revealed that exogenous D-Asp enhanced testosterone levels in male animal studies, while the studies on humans generated inconsistent results (mostly due to poor quality studies).

### 1.3.3 Other D-AAs

Besides D-Ser and D-Asp, various other D-AAs were found in the nervous and endocrine systems such as D-Ala, D-Cys, D-Glu, D-Gln, D-Leu, D-Pro. Few other D-AAs, (D-Tyr, D-Trp and D-Phe) could be determined from several human biological fluids such as amniotic fluid, cerebrospinal fluid, plasma and urine<sup>60</sup>. D-Leu and D-Pro were found in the brain, pineal and pituitary glands of rodents<sup>61,62</sup>.

D-Ala has been detected in multiple organs of rodents, such as brain, pituitary and adrenal glands, testes and pancreas<sup>63</sup>. In humans, small quantities of D-Ala was detected in cerebrospinal fluid<sup>64</sup>. In addition, D-Ala binds to the NMDAR, proving useful in the treatment of schizophrenia<sup>65</sup>.

Another D-AA found in human brain is D-Cys, which seems to be one of the sources of H<sub>2</sub>S in the brain. D-Cys is metabolized by DAAO to 3-mercaptopyruvate, which is then further transformed into H<sub>2</sub>S<sup>66</sup>. H<sub>2</sub>S increases the NMDAR activity by reducing disulfide bonds in the receptors. In contrast, H<sub>2</sub>S<sub>n</sub> induces the activation of transient receptor potential A1 channels in the astrocytes<sup>67</sup>, which leads to the release of D-Ser, enhancing the NMDAR-dependent long-term potentiation<sup>68</sup>. On top, H<sub>2</sub>S promotes the activity of Parkin, leading to protective effects against Parkinson's disease<sup>15</sup>.

Regarding the sources of D-Cys in the human body, it is known that a part of D-Cys is absorbed from food, but there seems to be also a source of D-Cys in the body, information which still needs to be clarified<sup>69</sup>.

One recent study<sup>38</sup> found no measurable quantities of D-Glu in perfused mouse brain tissue, at the same time L-Glu being the most prevalent amino acid. It was hypothesized that the D-Glu metabolism may be a unidirectional process, not a cyclic one (unlike to the L-Glu – L-Gln cycle). This specific removal of D-Glu from the brain might have important physiological implications, worth to be further studied.

## 1.4 D-AAs used in therapy

The involvement of some D-AAs in different pathologies was described in section 1.3. Considering that in some of those diseases there is a decreased availability of certain D-AAs (D-Ser, D-Ala, D-cycloserine), they were proposed as new therapeutic agents.

One approach to the treatment of schizophrenia consists in the potentiation of NMDAR-mediated neurotransmission. Several studies<sup>50,70-74</sup> have been conducted where D-Ser and D-cycloserine were added to the antipsychotic therapy for reducing the positive, negative and cognitive symptoms of schizophrenia. The efficacy of using D-Ser in therapy was confirmed by a recent meta-analysis<sup>23</sup>. Better outcomes were obtained if antipsychotics were combined with D-Ser than a placebo, for both positive and negative symptoms. Having the glutamatergic transmission as a target is considered one of the most promising approaches in treating schizophrenia, particularly in the early stages<sup>16</sup>.

Like D-Ser, D-Ala proved to provide significant improvement in the positive, negative and cognitive symptoms of schizophrenia due to its property of being a full agonist on the NMDA-glycine site<sup>65</sup>. D-Ala's effects on reducing the schizophrenia symptoms have the same magnitude as D-Ser or D-cycloserine.

A study<sup>11</sup> on mice reported potent anti-seizure effects of D-Leu in the context of intractable epilepsy, being found that D-Leu protects mice when administered prior to the onset of induced seizures. A later study<sup>75</sup> by the same research group concluded that D-Leu was ineffective against chronic seizures, but some efficacy was proven during the dark cycle.

One problem in administering D-AAs is their reduced bioavailability if administered orally, being quickly metabolized by DAAO. Nevertheless, therapeutic concentrations have been achieved when D-Ser was administered with DAAO inhibitors<sup>5,76,77</sup>, which may represent a new pharmacological class on their own.

## Chapter 2:

# (+) or (-)-1-(9-fluorenyl)ethyl chloroformate as chiral derivatizing agent

### 2.1. Introduction

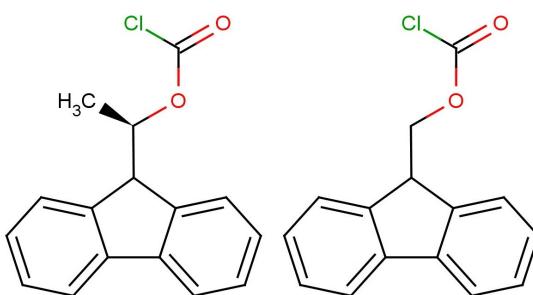
( $\pm$ )-1-(9-fluorenyl)ethyl chloroformate was first introduced as a chiral derivatizing agent (CDA) in 1987 resulting from the pioneering work done by Einarsson et al.<sup>78</sup>. Since then, it has proved to be one of the most versatile CDAs available on the market.

FLEC is structurally related to 9-fluorenylmethyl chloroformate (FMOC), the only difference being the presence of a methyl group on the acyl moiety (Fig 1). But this difference is essential, since it is responsible for the chirality of the molecule.

The (+) and (-) enantiomers of FLEC react fast and quantitatively with primary and secondary amines forming stable and non-racemizing derivatives. Therefore, the resulting diastereomers can be rather easily separated and quantified.

In most of the studies liquid chromatography was the separation technique of choice, next to a few capillary electrophoresis applications. Moreover, as a way of further improving sample throughput, it was recently proven that a full process automation (in-capillary derivatization) can also be achieved using this derivatization agent<sup>79,80</sup>.

Since its introduction FLEC was used in numerous applications for achieving or improving the chiral separation and detection of various analytes. These include the analysis of endogenous compounds in humans or other living organisms (amino acids (AAs), biogenic amines, nucleotides, peptides, hormones, toxins), pharmaceuticals ( $\beta$ -blockers, antimalarials, antidepressants, myorelaxants, drugs of abuse, antibiotics) and some environmentally relevant compounds (herbicides), summing up over 50 original research papers.



**Figure 1:** (–)-FLEC (left) and FMOC (right) structures.

Considering the long timespan since its introduction, some of the applications involving FLEC were mentioned in earlier reviews<sup>81–97</sup> or book chapters<sup>98,99</sup>, but no comprehensive review has been published until now. Few of the most notable papers, such as those from Toyo’oka<sup>93</sup>, Josefsson<sup>99</sup> and Ilisz<sup>83,89</sup> devote a distinct chapter or section to FLEC derivatization.

This chapter is aiming at illustrating the state of the art of FLEC derivatization in separation science, with a special focus on some of the practical aspects of the derivatization procedure.

## 2.2 General aspects of FLEC derivatization reaction

### 2.2.1 FLEC synthesis and purity

FLEC synthesis was first described by Einarsson et al.<sup>78</sup>. Using a chiral synthesis method, they managed to obtain a very enantiopure compound (>99%). Nevertheless, Camerino et al.<sup>100</sup> proposed an improved method for the synthesis of (+)-FLEC, since they found that the previous approach was not so efficient, with significant issues regarding the formation of multiple side-products.

The two main risks in using chiral reagents in the analysis of enantiomers are the improper enantiomeric purity of the reagent and the possibility of racemization during the derivatization. When talking about the determination of traces of one of the enantiomers in the presence of high excess of the other one, the enantiomeric purity of the derivatizing agent is a limiting factor in achieving a good sensitivity.

In order to assess the impact of these risks several methods have been developed<sup>78,101,102</sup>, all revealing that the risk of racemization is minimum and the commercially available (+)- and (–)-FLEC (>99%) are pure enough to avoid any risk for the quantification of various chiral analytes.

### 2.2.2 Derivatization principle and reaction mechanism

FLEC is mostly used in reactions with primary and secondary amines. However, it can also react with hydroxyl groups, but only under certain conditions. The derivatization reaction is fast and it takes place at room temperature, in aqueous

solutions at basic pH. Additionally, the resulting derivatization products also proved to be stable (at least one month at -20°C<sup>103</sup> or at least 5 days at room temperature<sup>104</sup>).

The reaction mechanism of FLEC with an amine is based on nucleophilic acyl substitution, where the nucleophile component is the amine. The reaction takes place under so-called Schotten-Baumann conditions (proper pH buffering and organic solvents). The reaction products are the FLEC derivatives (carbamates) and hydrogen chloride. The basic pH is needed to drive the equilibrium in the formation of amides and to neutralize the reaction by-products, while the role of the organic solvent is to insure the dissolution of the derivatizing agent. Considering that the reaction takes place in aqueous media, the excess of FLEC reacts with water (more precisely OH<sup>-</sup>) resulting in FLEC-OH, hydrogen chloride and carbon dioxide. Since FLEC is usually added in large excess, both excess FLEC and produced FLEC-OH disturb the separation and detection of the analytes. The two main ways for overcoming this issue will be discussed in more detail in section 2.2.4.5.

The structure of FLEC derivatives was confirmed by both GC-MS<sup>78</sup> and fast atom bombardment-mass spectrometry<sup>105</sup>. Prior to the GC-MS studies the methylation of the carboxyl groups was performed. The resulting derivatives showed stability at high temperatures (200°C).

For less reactive compounds (nucleophiles), such as alcohols, the FLEC derivatization can be performed in non-aqueous media (i.e. pyridine). This approach was successfully applied to the derivatization of α-hydroxy acids<sup>106</sup>.

### 2.2.3 Automatization of the derivatization process

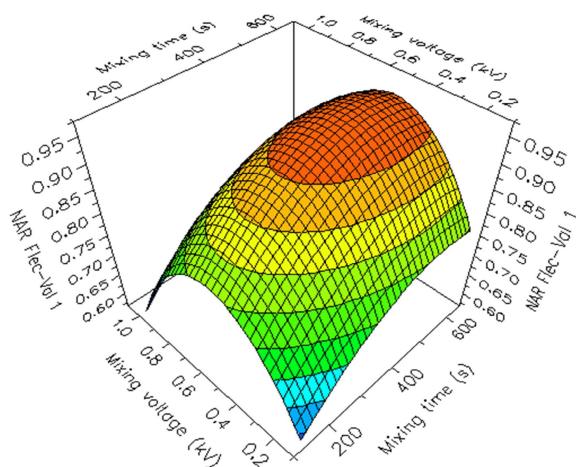
The automatization of the FLEC derivatization procedure was demonstrated in both LC and CE methods.

Lai et al.<sup>107</sup> automatized the FLEC derivatization in a LC method by programming the autosampler to mix the reagent and the analyte, wait for the reaction to take place and then inject the mixture into the chromatographic system. This methodology proved to be highly reproducible.

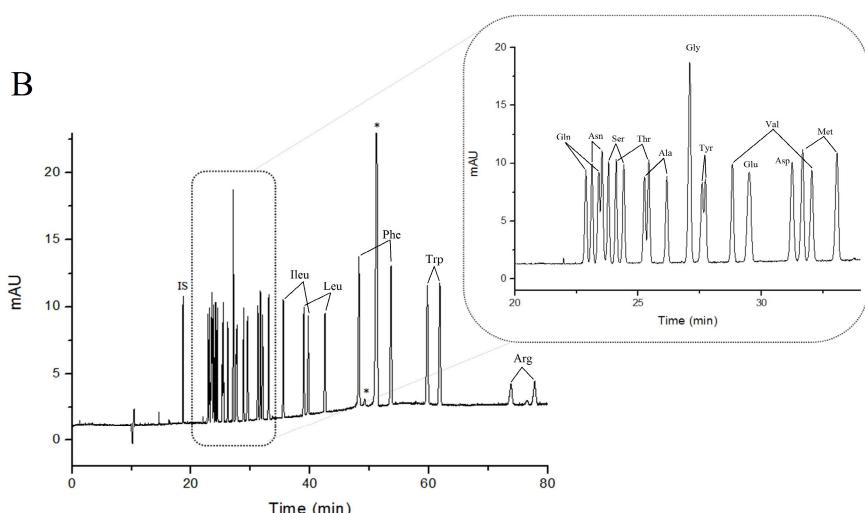
The approach using in-capillary derivatization with FLEC as labeling agent was described for the first time by Fradi et al.<sup>79</sup> for the analysis of D- and L-amino acids. The in-capillary reaction proved to be fast and quantitative, making it suitable for automatization. After some preliminary studies involving different injection and mixing techniques, the derivatization parameters were optimized by experimental design (Fig. 2). Successful labeling was achieved by the sequential injection of a sample and derivatizing agent plugs, and the application of a mixing voltage of 0.2 kV for 570 seconds. Moreover, a special washing procedure was established in-between the runs, in order to improve the run-to-run repeatability (peak area RSD values in the range of 6.6-11.6%). A similar approach was employed by Moldovan et al.<sup>80</sup> for the derivatization and separation of amino acid enantiomers by MEKC-MS. Even if the derivatization principle was identical, new challenges arose from the CE-MS coupling. The complete change of the BGE composition and the suction effect present at the capillary outlet (due

to the sheath-liquid interface) implied a full re-optimization of the derivatization parameters. In this case, the optimal derivatization conditions were found to be obtained by successive injection of the sample and the derivatizing agent followed by the application of a current of 0.1 kV for 900 s. The run-to-run reproducibility was higher when FLEC was dissolved in acetonitrile (ACN), with RSDs between 5.6 and 11.9% (except for Tyr – 30.03%). Both methods described above were proved to be applicable to samples of artificial cerebrospinal fluid.

A



B



**Figure 2. A:** Response surface plot showing the high influence of the mixing voltage and time on the derivatization response of valine enantiomers; **B:** Selectivity obtained for the separation of FLEC derivatives of amino acids by CE using in-capillary derivatization.

## 2.2.4 Practical aspects

Generally, the most important factors influencing the efficiency of the derivatization reaction are the reaction time, the quantity of FLEC and the value of pH.

### 2.2.4.1 Analyte:FLEC molar ratio

For ensuring that the derivatization reaction is quantitative, the FLEC reagent should be added in excess relative to the analyte. Among published papers, only a few<sup>104,108-111</sup> dedicate a short discussion to the optimization of the analyte:FLEC molar ratio.

We calculated the molar ratio (expressed as the ratio of the number of moles of each reactant) for the derivatization procedures, which provided the necessary data (see Tables 1 and 2). In most cases FLEC was used in excess relative to the analyte, with only two exceptions where the ratio was 1:1<sup>101,112</sup>.

Among the papers dealing with the optimization of the molar ratio, Freimüller et al.<sup>108</sup> concluded that for the derivatization of carnitine a ratio of 1:1.5 between the analyte and FLEC was sufficient to achieve a robust derivatization procedure. In contrast, for the same analyte De Witt et al.<sup>111</sup> found a ratio of 1:15 to be optimum. Large FLEC excesses of 1:20 to 1:40 were reported by Bergqvist et al.<sup>104</sup> for the derivatization of mefloquine and by Boursier-Neyret et al.<sup>109</sup> for a new quinolone derivative.

Chen et al.<sup>110</sup> reported that for the FLEC derivatives of amphetamine and amphetamine related drugs of abuse the maximum fluorescence intensity was obtained by selecting a molar ratio of 1:2; in this manner the problems related to excess of reagent were also minimized.

Having in mind the reported ratios and our own experience we recommend using FLEC in a concentration at least 10-fold higher than the expected analyte concentration.

### 2.2.4.2 Buffers and pH range

The FLEC derivatization reaction is usually carried out at a neutral or basic pH, depending on the analyte (Annexes 1 and 2). In a decreasing order of popularity, the buffers reported as being used in the derivatization procedure are: borate, carbonate, phosphate, tertbutylamine and triethylamine.

In the majority of the studies sodium tetraborate buffer was used in a broad range of concentrations, from 0.005 M<sup>80</sup> to 1 M<sup>78</sup>. The derivatization medium was usually buffered at a pH around 9 (the pK<sub>a</sub> value for boric acid is 9.2). However some publications report the use of this buffer in a rather wide pH interval, between 6.85<sup>78</sup> and 11<sup>103</sup>.

Nevertheless, pH values higher than 10 should be avoided due to the accelerated hydrolysis rate of the derivatizing reagent. Failing to do so may lead to two main drawbacks: a higher FLEC-OH peak (which may affect the selectivity) and the need of a higher FLEC concentration to guarantee an excess of reagent.

It was observed that for specific classes of analytes borate buffer was not employed. For amphetamine and amphetamine-derived drugs, the FLEC derivatization

procedures reported in literature<sup>105,110,113,114</sup> were carried out at the following pH: 7.8 (phosphate buffer), 10 (carbonate buffer) or 12 (sodium hydroxide). When using phosphate buffer the peak areas of the derivatives reached their highest values at a pH above 7.8, whereas the optimal pH using carbonate buffer was found to be between 9.5 and 10.

Carnitine enantiomers were derivatized with FLEC using tetrabutylammonium hydroxide ( $\text{TBA}^+\text{OH}^-$ )<sup>111</sup> or carbonate buffer<sup>101,108,112</sup>. When  $\text{TBA}^+\text{OH}^-$  was employed the pH was found to have a significant influence on the reaction yield, the optimal value being 7. Nevertheless, successful FLEC derivatization was also reported to take place when using water and triethylamine as reaction medium<sup>109</sup>.

Non-aqueous derivatization of reboxetine was performed by Walters et al.<sup>115</sup>. The reaction took place in acetone, for 5 minutes at 30°C. Compared to a previous reboxetine derivatization procedure performed in aqueous solution<sup>116</sup>, the non-aqueous derivatization procedure uses a nearly 30-fold lower amount of FLEC.

The impact of several buffers on the derivatization reaction of a novel adenosine antagonist was studied by Witte et al.<sup>117</sup>. The selected buffers were borate (pH 8.1), phosphate (pH 7.0), phosphate (pH 6.3) and acetate (pH 5.2). The highest yield was achieved after 2 h and it was obtained using phosphate buffers, without significant difference caused by the different pH values.

Obviously, from a practical point of view the pH value is more important for less reactive systems, involving target analytes that are weaker nucleophiles, and the use of a base can activate them by keeping them in a non-charged form. From the proton interaction perspective, the use of non-aqueous protophilic solvents may further improve the expected yield of the derivatization reaction.

#### **2.2.4.3 FLEC dissolution medium**

(+) and (-)-FLEC are commercialized as 18 mM acetone solutions, therefore acetone was the solvent of choice in the majority of the cited research papers. Acetonitrile was preferred by six research groups, mixtures of acetone with ACN or methanol (MeOH) were used in eight studies, while pyridine or dichloromethane were each used in one work (Annex 1). The advantage of acetone over ACN, as presented by Molnár-Perl et al.<sup>118</sup>, may originate from its higher polarity, increasing the labeling reaction rate of the analyte amino group. Nevertheless, similar reaction rates are expected when using either acetone or ACN.

Bergqvist et al.<sup>104</sup> recommend the use of a 55-75% proportion of organic component in the reaction mixture, as a higher percentage may lead to the precipitation of the buffer whereas lower values would lead to the precipitation of FLEC, both phenomena being to the detriment of the derivatization yield.

A special case is represented by the in-capillary derivatization<sup>80</sup>, where an improvement in reproducibility can be observed by using ACN instead of acetone as a solvent for FLEC. This may be explained by the higher vapor pressure of acetone at room

temperature, which leads to an uncontrollable solvent evaporation and variation in FLEC concentration over time.

#### **2.2.4.4 Reaction kinetics**

The rate of amino acid derivatization with FLEC was measured by the individual labeling of 10 AAs at pH 8<sup>78</sup>. Results revealed that most AAs were derivatized in 60 s, the fastest to react being the aromatic ones, while acidic amino acids (glutamic and aspartic acids) reacted more slowly. In comparison with FMOC, FLEC was found to react almost 3 times slower, probably due to a steric hindrance resulting from the additional methyl group. However, the methyl group may also exert an inductive effect over the electron density, therefore modifying the electrophilicity of the FLEC acyl moiety.

The optimization of the reaction time and temperature for the derivatization of D- and L-carnitine enantiomers has been performed in two studies<sup>108,111</sup>. However, the overall experimental conditions in the two studies were different. In the first study<sup>111</sup> when adding TBA<sup>+</sup>OH<sup>-</sup> to the derivatization medium, the reaction was found to be optimal if it took place at 80°C for 25 min. However, it is not advisable to use elevated reaction temperature because it may favor racemization.

In the second study [31], employing a carbonate buffer, the temperature effects were found to be less pronounced, while the reaction yield was higher if the reaction time was set to its maximum value (i.e. 60 min). No difference in the reaction rates for the two carnitine enantiomers was observed. This result was confirmed by another study<sup>101</sup> which indicated that carnitine enantiomers have similar reaction velocities and the fluorescence properties of the formed derivatives are identical.

The influence of the reaction time on the derivatization yield of N-6-(endo-2-norbornyl)-9-methyladenine enantiomers was studied by Witte et al.<sup>117</sup>. After 60 minutes the reaction was more than 90% complete. However, it was also noticed that the quantity of FLEC-OH increased over time, so that it would be advisable to shorten the derivatization time as much as possible, while keeping a sufficient derivatization yield. In contrast, methamphetamine and its metabolites required much longer reaction time (24 h) for achieving a high yield<sup>113</sup>.

Glufosinate was found to react faster and in a more reproducible way with FLEC if the temperature was kept at 40°C during the reaction, rather than at 25°C<sup>119</sup>.

#### **2.2.4.5 Removal of excess FLEC and produced FLEC-OH**

As discussed earlier in section 2.2.4.1, FLEC must be used in excess relative to the analyte for achieving robust results in the shortest possible time. Nevertheless, a large excess FLEC and its hydrolyzed form (FLEC-OH) give huge signals that can disturb the separation and/or the detection of the analytes, mostly when using optical detection methods (UV and fluorescence detections (FL)). Consequently, some methodologies have been developed to deal with these issues.

Two approaches were generally implemented for the removal of the excess of FLEC and its hydrolysis product: (1) The two interferents can be extracted with non-polar solvents (e.g. pentane, hexane) due to the significant hydrophobicity induced by

the fluorenyl moiety; (2) Excess FLEC can be reacted with different molecules (e.g. amantadine, 1-aminoadamantane (ADAM), hydroxyproline, glycine), resulting in derivatives with significantly different physicochemical properties that enable their fairly simple separation from the target analytes. As a note of caution, Okuma et al.<sup>103</sup> stated that in the case of amino acid derivatization the extraction should be performed within 15 minutes after the reaction took place, otherwise a significant loss of lysine would occur.

In some instances, the presence of excess FLEC and FLEC-OH was not detrimental, due to the inherent selectivity of the separation technique or detection mode. In the case of SFC separations<sup>120</sup> the excess of FLEC is eluted early, so that it does not affect the detection of the derivatives, while in CE-MS approaches<sup>80,121</sup> the detector provides the needed selectivity.

Another manner of dealing with the presence of excess FLEC was proposed by Witte et al.<sup>117</sup>. They suggested analyzing the sample one day after the derivatization reaction has taken place, because FLEC derivatives are stable, while all excess FLEC would be converted to FLEC-OH, reducing the number of peaks that may cause problems.

#### **2.2.4.6 Solid-phase derivatization**

The FLEC derivatization using solid-phase extraction (SPE) cartridges was introduced by Verdú-Andrés et al.<sup>122</sup> for the labeling of amphetamine-derived drugs and has the advantage of preconcentrating the analytes before being derivatized. Compared to the derivatization in solution the analyte response was increased by a factor of 28-58. From a practical point of view, the procedure uses the typical SPE steps followed by the derivatization reaction and desorption of the analytes. The SPE cartridge, containing a C18 stationary phase, was conditioned in two steps using MeOH/ACN and carbonate buffer (0.5%, pH 9.5). After the sample loading, a carbonate buffer wash and air drying were performed. Then, a FLEC solution (4.5 mM) was loaded, and after a certain reaction time (1-15 min) the derivatives were desorbed using acetic acid (1 M) in MeOH/ACN.

#### **2.2.4.7 Column care and practical advice**

For reversed phase liquid chromatography (RP-LC) separations it was observed that column cleaning was essential to improve the robustness of the method and ensure a maximum column lifetime<sup>108,111</sup>. The complete removal of the excess of FLEC and its hydrolysis product can be achieved by a system flushing with a relatively non-polar gradient (up to 98% ACN). It was also reported that column regeneration was required after each 100 injections in order to improve the method robustness.

Hutchaleelaha et al.<sup>105</sup> reported that the non-reacted FLEC significantly reduces the column longevity (C18). For increased column lifetime, Zoutendam et al.<sup>123</sup> advised to add a supplementary step after the completion of the derivatization reaction, in order to neutralize excess FLEC with another amine (see section 2.2.4.5).

## 2.2.5 Selectivity and sensitivity compared to other derivatization agents

The most important advantage of FLEC over other CDAs is its fast and quantitative reaction at room temperature (Table 1).

Regarding the comparison of different chiral reagents for the analysis of D- and L-amino acids, Brückner et al.<sup>124</sup> stated that in terms of selectivity (+) and (-)-FLEC are the only alternatives to OPA for resolving complex mixtures of amino acid enantiomers.

Houben et al.<sup>125</sup> compared the absorptivity of primary amines derivatized with five different CDAs, OPA-NAC, OPA-NAP, GITC, Marfey's reagent and FLEC, at five different wavelengths. FLEC showed the highest absorption in the lower-UV region (i.e. 200, 214, 254 and 280 nm) where unfortunately, various potential interferents are likely to absorb (mostly at 200 and 214 nm). In contrast, derivatization with Marfey's reagent resulted in high absorptivity at 340 nm, but some features make it less desirable, such as the elevated temperature required for derivatization and long reaction times.

A comparison between the selectivities obtained with FLEC, NEC, DNPA and GITC was made by Shimada et al.<sup>126</sup> when performing derivatization and RP-LC chiral separation of baclofen derivatives. The resolutions values were satisfactory for all CDAs. However, upon addition of  $\gamma$ -cyclodextrin to the mobile phase, the resolution of FLEC- and GITC-derivatives increased significantly (more than 2 fold for FLEC).

Wan et al.<sup>127</sup> and Chan et al.<sup>128</sup> compared the separation of diastereomers (formed by derivatization with FLEC) with the direct separation ( $\beta$ -CD, HP- $\beta$ -CD and  $\gamma$ -CD) of enantiomers (formed by derivatization with FMOC) by CE. Either approach was able to resolve around 80% of the analytes, but the indirect approach offered higher separation efficiency. Moreover, compared to other CDAs (Marfey's reagent and GITC), the concentration of sodium dodecylsulphate (SDS) required for the resolution of FLEC-AA derivatives was much lower.

**Table 1. Comparative reaction kinetics of different CDAs for the derivatization of AAs**

CDA	pH	Reaction time (min)	Temperature (°C)	Ref.
Reagents based on activated carboxylic acids	(S)-NIFE	11	20	r.t. 129
	(S)-TBMB-COOH (S)-MNB-COOH	9	30	r.t. 130-132
	1,3-benzodioxole-4 or 5-carboxylic acid	n.-aq.	30	r.t. 133
Reagents based on chloroformates	FLEC	7-11	<1-15	78-80,103,134 -136
	APOC	8.5	10	r.t. 137
Reagents based on isothiocyanates	GITC	n.r.	15-40	r.t. 138,139
	FDAA	n.r.	60-90	35-40 139,140
	CDITC	n.r.	120	60 141
	(±)-DBD-PynCS and (±)-NBD-PynCS	n.r.	20	55 142
	DANI	n.r.	120	60 143
Reagents based on N-haloarylamino acid derivatives	FDVA, FDPA, FDIA, FDLA	n.r.	60	37 144
	s-triazine analogs	n.r.	60	80 145
Reagents based on o-phthalaldehyde + chiral thiol	OPA-NAC	9.4	15	r.t. 146
	OPA-IBLC OPA-IBDC	10.4	2	n.r. 147,148

Abbreviations: r.t.: room temperature; n.r.: not reported; n.aq.: non-aqueous; APOC: (±)-1-(9-anthryl)-2-propyl chloroformate; CDITC: N-[2-isothiocyanato)cyclohexyl]-6-methoxy-4-quinolinylamide; DANI: 1,3-diacetoxy-1-(4-nitrophenyl)-2-propyl isothiocyanate; (±)-DBD-PynCS: (±)-4-(3-isothiocyanatopyrrolidin-1-yl)-7-(N,N-dimethyl-aminosulfonyl)-2,1,3-benzoxadiazole; FDAA: (1-fluoro-2,4-dinitrophenyl)-5-L-alaninamide; FDIA: 1-fluoro-2,4-dinitrophenyl-5-L-isoleucine amide; FDLA: 1-fluoro-2,4-dinitrophenyl-5-L-leucine amide; FDPA: 1-fluoro-2,4-dinitrophenyl-5-L-phenylalanine amide; FDVA: 1-fluoro-2,4-dinitrophenyl-5-L-valine amide; FLEC: (±)-1-(9-fluorenyl)ethyl chloroformate; GITC: 2,3,4,6-tetra-O-acetyl-β-D-glucopyranosyl isothiocyanate; (S)-MNB-COOH: 2-β-naphthyl-2-methyl-1,3-benzoxadiazole-4-carboxylic acid; (±)-NBD-PynCS: (±)-4-(3-isothiocyanatopyrrolidin-1-yl)-7-nitro-2,1,3-benzoxadiazole; (S)-NIFE: (S)-N-(4-nitrophenoxy carbonyl)phenylalanine methoxyethyl ester; OPA-IBDC: o-phthalaldehyde/N-isobutyryl-D-cysteine; OPA-IBLC: o-phthalaldehyde/N-isobutyryl-L-cysteine; OPA-NAC: o-phthalaldehyde/N-acetyl-L-cysteine; (S)-TBMB-COOH: (S)-2-t-butyl-2-methyl-1,3-benzodioxole-4-carboxylic acid.

## 2.2.6 Sample preparation

Some reported methods have been applied to biological samples, which may require specific clean-up before derivatization.

The analysis of D-amino acids from different tissues of crustaceans and mollusks was of interest for several authors<sup>103,134,149–151</sup>. The general approach before the derivatization step was to collect the samples, homogenize them and precipitate the proteins. After centrifugation and neutralization, the supernatant was ready for derivatization.

The sample preparation for the analysis of N-methyl-D-aspartate (NMDA) and N-methyl-L-aspartate (NMLA) in tissues of bivalves was described by Todoroki et al.<sup>152</sup>. Although similar to the procedure described above, it has an additional extraction step using an ion-exchange resin. Therefore, after clean-up the sample contained only N-methyl-aspartate and acidic amino acids.

For the chiral analysis of amino acids in mouse macrophages, Kato et al.<sup>136</sup> used a simple approach for sample preparation. The cells were ultrasonicated and then the proteins were precipitated. After centrifugation the supernatant was used for derivatization.

The CE analysis of L- and D-amino acids in CSF has been performed by Prior et al.<sup>121</sup> without any specific sample preparation other than pH adjustment prior to the derivatization procedure. Some in-capillary derivatization procedures for analyzing L- and D-amino acids were also tested on artificial CSF<sup>79,80</sup> after a simple dilution.

In general, for the analysis of chiral pharmaceuticals in biological media, specific extraction procedures were used, depending on their structure and the sample matrix. Liquid-liquid extraction was found to be the method of choice for the isolation of propranolol from whole blood<sup>153</sup>, mefloquine and atenolol from plasma<sup>104,154</sup> and for the separation of methamphetamine and its metabolites in urine<sup>113</sup>. SPE was preferred to isolate two novel compounds (S 12024<sup>109</sup> and PGE-9509924<sup>123</sup>) from plasma.

## 2.3. State of the art in the separation of FLEC derivatives

### 2.3.1 Chromatographic and electrophoretic chiral separations

FLEC was designed as a chiral analog of FMOC, a well-known and widely used derivatizing agent. FLEC derivatives are stable, highly fluorescent and can be easily separated using chromatographic and electrophoretic techniques.

#### 2.3.1.1 Chromatographic separations

Chromatographic separations (LC and SFC) of FLEC derivatives were used for the chiral analysis of some endogenous molecules, pharmaceuticals and herbicides.

All but one of the reported LC chiral separations were achieved using the reversed phase mode. The stationary phases employed were C18 (75% of the reported methods) and C8 (25%).

The mobile phases used for the analysis of amino acid derivatives usually comprised two common components: a tetrahydrofuran (THF) based organic part (sometimes mixed with ACN) and an aqueous buffer at an acidic pH (3.8-6). Some authors stated that THF was needed to desorb some of the analytes, while the mobile phase pH seemed to greatly influence the selectivity of the method. In contrast with the methods developed for amino acids, almost all LC methods for the chiral analysis of other analytes make use of ACN as the organic component of the mobile phase, while keeping an acidic pH for the aqueous phase component.

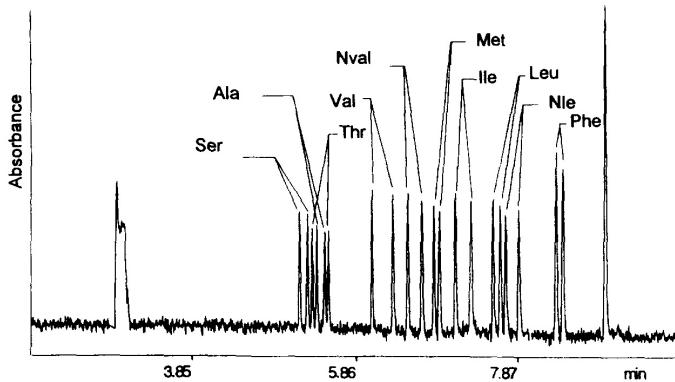
The only reported NP-LC method was applied to the separation of (+)-FLEC-reboxetine diastereomers using three stationary phases (silica, cyano and a chiral CSP: cellulose tris(3,5-dimethylphenylcarbamate)) with an ethanol/heptane based mobile phase<sup>115</sup>.

### **2.3.1.2 Electrophoretic separations**

The separation of FLEC derivatives by capillary electrophoresis was chosen by around 20% of the authors cited in this chapter. The preferred CE mode was MEKC (6 papers), followed by CZE (4 papers) and CEC (1 paper). The studied classes of analytes were amino acids, beta-blockers and peptides.

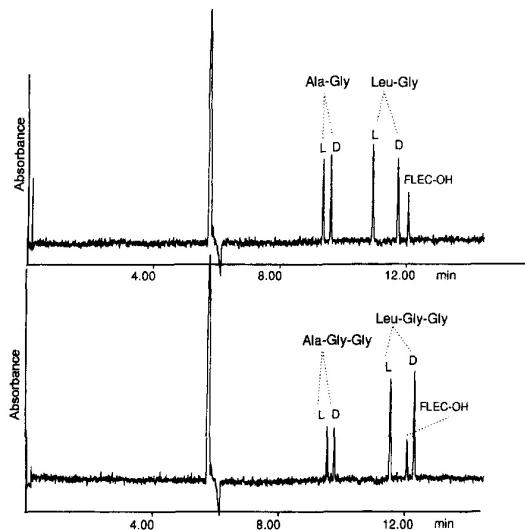
In spite of its lower popularity, the main advantage of CE compared to LC for chiral separations is the high efficiency of the former, which makes baseline separation possible even at low  $\alpha$  values.

In order to improve resolution, a common practice is to add an organic modifier to the BGE. It usually extends the migration window by inducing a decrease in electroosmotic flow. The first reports on the use of such a BGE for the MEKC separation of FLEC derivatives were presented by Chan et al.<sup>128</sup> and Wan et al.<sup>127</sup> (Fig. 3). For the majority of the analyzed amino acids an important positive impact on resolution was observed by adding 10 - 15% organic modifier to the BGE. Higher concentrations (20%) led to lower resolution, probably due to reduced partitioning of the FLEC derivatives into the micelles. A particular case is represented by Tyr and Lys derivatives whose chiral separation requires higher concentrations of organic modifiers (30% and 50%, respectively). This separation is assumed to be due to the use of a BGE with an SDS concentration close to its critical micellar concentration. The same positive effect on chiral resolution was observed by Fradi et al.<sup>79</sup>, using a BGE made up of tetraborate buffer and SDS. On the contrary, Moldovan et al.<sup>80</sup> and Prior et al.<sup>121</sup> reported a negative influence of the organic modifier on chiral resolution using a MEKC buffer containing ammonium perfluorooctanoate.



**Figure 3. Separation of 10 FLEC-AA derivative pairs by MEKC.**  
(Reproduced from Ref. <sup>127</sup>)

It was suggested that for the separation of di- and tripeptides<sup>155</sup> (Fig. 4) the presence of 15% isopropyl alcohol (IPA) may speed up the mass transfer of hydrophobic solutes to the micelles, demonstrating further the benefits of adding an organic modifier to the BGE.



**Figure 4. Separation of di- and tri-peptides by CE after FLEC derivatization.** (Reproduced from Ref. <sup>155</sup>)

### 2.3.1.3 Elution/migration order

The order of elution or migration is mainly relevant when one of the enantiomers is found in large excess relative to the other one (e.g. the analysis of D-amino acids in biological samples).

Einarsson et al.<sup>78</sup> reported that in their RP-LC method the (+)-FLEC-D-amino acids eluted first. Later, Wan et al.<sup>127</sup> and Chan et al.<sup>128</sup> observed in their electrophoretic separations that the (-)-FLEC-D-amino acid derivatives migrated first, while a reversal

of the migration order was easily achieved by replacing (-)-FLEC with its enantiomer. The same phenomenon has been reported for LC separations of carnitine<sup>101,112</sup> and secondary amino acids<sup>152</sup>.

#### 2.3.1.4 Detection modes

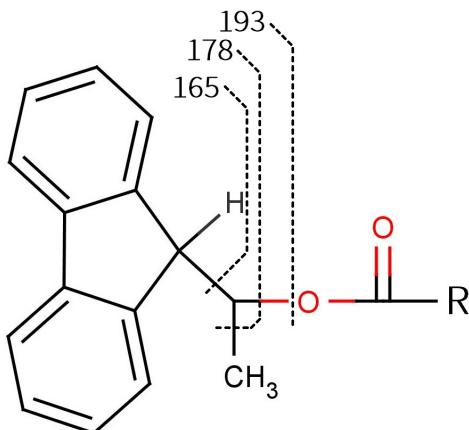
FLEC derivatives are highly fluorescent thanks to the fluorenyl moiety. Therefore FL detection was selected in many studies. The excitation and emission spectra of (+)-FLEC-Ala were studied by Chan et al.<sup>128</sup>, revealing that this derivative had an excitation maximum around 265 nm, whereas the emission maximum was found at 310 nm. The same authors made a comparison between UV and laser induced fluorescence (LIF) detection, concluding that by using LIF the sensitivity could be increased up to 100 times. A similar increase in sensitivity was observed by Lai et al.<sup>107</sup> for the analysis of propranolol derivatives (UV - 254 nm; FL - exc. 265 nm, em. 345 nm).

No significant differences were found between the fluorescence quantum yield of FLEC and FMOC<sup>78</sup>. On the contrary, a difference in fluorescence response was reported<sup>104</sup> for the (-)-FLEC-(SR)- and (-)-FLEC-(RS)-mefloquine diastereomers<sup>104</sup>, the (RS)-mefloquine derivative response being found to be four times higher. The opposite could be observed by using (+)-FLEC as derivatization reagent.

Mass spectrometry was employed in several studies<sup>80,105,113,119,121,123</sup>, either for elucidating the structural features of FLEC derivatives, or as detection method for these derivatives by hyphenation with LC or CE.

The confirmation of the structure of FLEC derivatives was one area in which mass spectrometry proved to be crucial. Mass spectrometric analyses were performed by using fast-atom bombardment ionization<sup>105,113</sup> and ESI-MS<sup>117</sup>. Besides the molecular ions, FLEC related fragments were detected in most cases, the most abundant being m/z=193, resulting from the cleavage of the alkyl-oxygen bond (Fig. 5). The LC-MS analysis of FLEC-DL-glufosinate was performed by Hori et al.<sup>119</sup>, confirming that the derivatives were formed through a single-molecule amide binding.

The use of MS as detection method was employed both in LC and CE. LC-MS was used for the detection of some novel compounds<sup>117,123</sup>. In one of the studies<sup>123</sup>, both MS and FL were used, giving comparable results with similar linear ranges, both exhibiting good precision and accuracy. The advantages of MS detector over FL detection would be a shorter analysis time, higher selectivity and a slightly better LOD (i.e. 5 ng/mL vs. 10 ng/mL). Of course, an increase in sensitivity of at least 100 fold should be expected when using MS compared to UV.



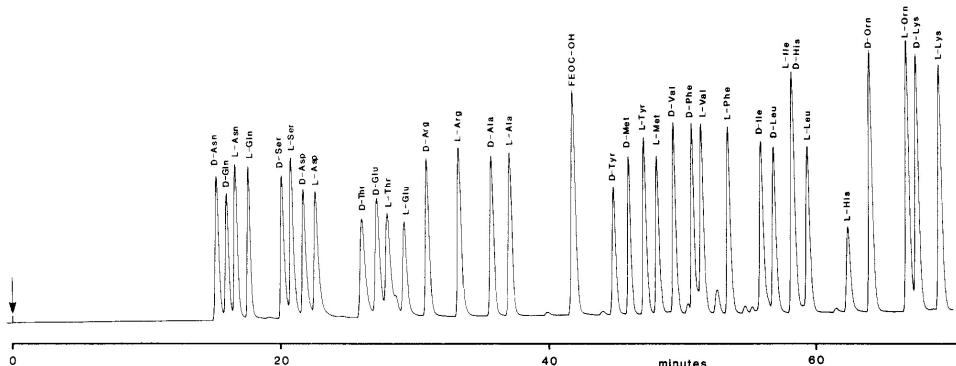
**Figure 5.** MS fragmentation pattern of FLEC derivatives.

Two MEKC-MS methods<sup>80,121</sup> were developed for analyzing D- and L-amino acids. The detection mode enabled a significant reduction of the analysis time because the chemoselectivity provided by the MEKC system was no longer a requirement for all analytes, once the desired chiral selectivity was reached. In terms of sensitivity, one MEKC-MS approach<sup>121</sup> reported LODs around 30 times lower than a similar UV approach<sup>79</sup> when separating the same analytes.

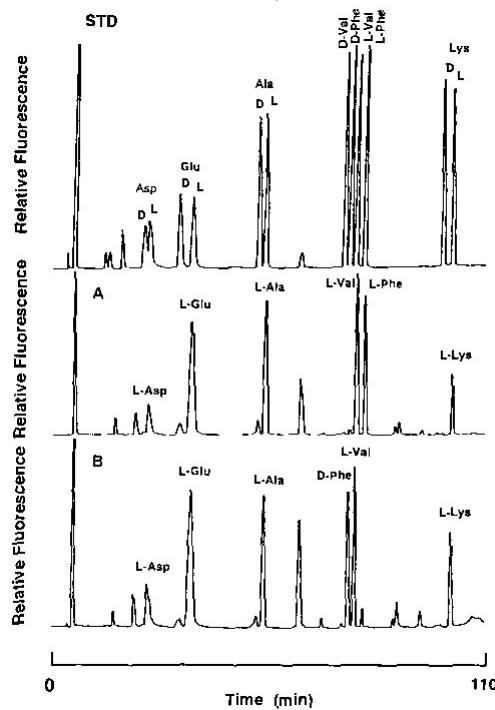
### 2.3.2 FLEC derivatization for the chiral analysis of amino acids and peptides

Amino acids have been and still remain one of the most studied classes of molecules. They represent an interest from a biological point of view for their involvement in most of the physiological processes. From an analytical point of view, even the non-chiral separation of these molecules poses a challenge since they are difficult to detect using accessible detection methods, such as UV and FL, most of them lacking chromophore or fluorophore moieties in their structure. Therefore, FLEC derivatization offers a new way to tune their properties in order to achieve better selectivity and detectability.

The first paper<sup>78</sup> that reported the use of FLEC as derivatizing agent describes the successful chiral separation of primary and secondary amino acids, imino acids and  $\beta$ -blockers. Seventeen pairs of primary amino acid derivatives were separated in a single run using RP-LC (Fig. 6). The analysis of the secondary amino acids was performed only after the primary ones were derivatized (with OPA/mercaptoethanol) and removed from the sample. Therefore, the separation and detection of the secondary amino acids was achieved without any interference from the primary ones. This method was later used<sup>156,157</sup> for the analysis of amino acids in different matrices (Fig. 7).



**Figure 6.** RP-LC separation of 17 pairs of FLEC-D- and FLEC-L-AAs derivatives. (Reproduced from Ref. 78)



**Figure 7.** Hydrolysis of crustacean hyperglycemic hormone (CHH-I – A; CHH-II – B) of *Procambarus clarki*, analyzed by LC after derivatization with FLEC. (Reproduced from Ref. 157)

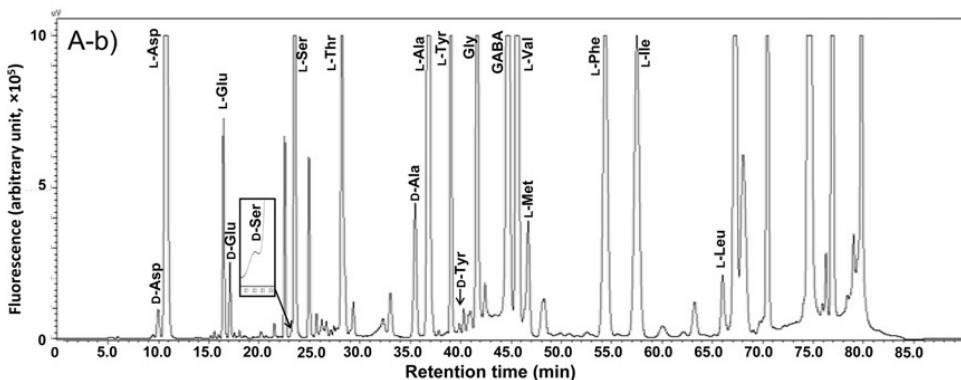
In a later work, Todoroki et al.<sup>152</sup> studied the separation of NMDA- and NMLA-FLEC derivatives, observing that the addition of thiols (during OPA/thiol procedure) severely affected the formation of FLEC derivatives, which was probably caused by the reaction of FLEC with the more reactive thiol groups.

In the RP-LC method used for the analysis of the FLEC derivatives of NMDA and NMLA<sup>152</sup> isocratic elution was performed with a mobile phase containing a high percentage of aqueous buffer (sodium acetate, pH 5.6), achieving chiral resolution of the

two diastereomers. In spite of the very long analysis time (more than 100 min), sensitivity in the range of nmol/g of wet tissue was obtained. A similar approach was later used by Tarui et al.<sup>158</sup> for the determination of N-methyl-D-glutamate and N-methyl-L-glutamate in various mollusks and other invertebrates.

A landmark paper on the separation of FLEC-AA derivatives was published in 1994 by Okuma et al.<sup>103</sup>. The separation was carried out by RP-LC using three different gradient programs (3 different runs) in order to resolve most of the analytes. The outcome was the chiral separation of 19 amino acids out of a mixture of 25 with LODs in the nM range. The development of this method was followed by several other applications by the same research group<sup>134,135,149–151</sup> concerning the analysis of D-amino acids from different species of mollusks and crustaceans.

The LC analysis of D- and L-amino acids in sake was performed by Gogami et al.<sup>159</sup> (Fig. 8). After the derivatization reaction took place, the excess FLEC was removed using ADAM. The sample was then chromatographed on an ion exchange stationary phase, achieving chiral resolution for 6 pairs of amino acids, with LODs ranging from 0.01 µM to 20 µM. Since the excitation and emission wavelengths that were used in this study (exc. 340 nm, em. 450 nm) were not ideal for FLEC derivatives, it is expected that even lower LODs could be obtained by use of a FL detector with the appropriate settings (see section 3.1.4).



**Figure 8. Analysis of naturally occurring D-AAs in sake beverages (Partially Reproduced from Ref. <sup>159</sup>)**

A few years later, the same group reported the analysis of D- and L-amino acids as FLEC derivatives in mouse macrophages<sup>136</sup>, but this time the separation was performed using a commercially available kit (no information regarding the stationary phase).

The diastereomers of several FLEC derivatized  $\alpha$ -hydroxy acids have been separated by RP-LC, in a work reported by Fransson et al.<sup>106</sup>. They concluded that the separation of these rather hydrophobic derivatives can be controlled by adjusting the buffer pH, but besides a powerful organic modifier (THF) is needed to desorb the analytes and the excess reagent.

FLEC derivatization was also employed for the analysis of cardioactive peptides isolated from tissues of cephalopods<sup>160</sup>. The method was based on the determination of the chirality of the amino acids after hydrolysis of the peptides. For this purpose, FLEC-AA derivatives were monitored with an amino acid analyzer, confirming the presence of D-Ser and D-Phe in the structure of the peptides.

A method for the identification of the carboxy terminal amino acid of a protein has been developed based on a principle of racemization and hydrolysis of this C-terminal amino acid<sup>161</sup>. The resulting hydrolysate was subjected to FLEC derivatization and then the analytes were separated by RP-LC using a stepwise elution with three mobile phases containing different proportions of sodium acetate, ACN and THF. Using this method, 39 derivatives were separated within a very long timeframe of 170 min. The approach presents several limitations: C-terminal Gly or Pro cannot be determined, there is no discrimination between glutamic acid and glutamine and between aspartic acid and asparagine, and it is impossible to racemize C-terminal Ser or Thr.

The first CE separation of FLEC derivatives was reported by Wan et al.<sup>127</sup> and was developed for the chiral separation of amino acids. The MEKC system consisted of a borate-phosphate buffer, SDS and acetonitrile. The separation parameters were optimized using an experimental design. Although there was a high variability in the optimal separation conditions for each analyte, it was concluded that a BGE with a low content of SDS (20 mM) and high pH (9.2) would be necessary. Under these conditions the chiral separation of 15 out of 16 amino acids was achieved within 15 minutes. Using a similar indirect approach<sup>155</sup>, the same team reported the diastereomeric separation of 9 di- and tripeptides (Fig. 4). It was found that the selectivity is mainly dependent on the hydrophobicity of the diastereomers; therefore, the more hydrophobic the diastereomer, the lower the needed SDS concentration. Moreover, the selectivity is dominated by the FLEC moiety and the amino acid directly attached to FLEC. Another necessary condition for applying successfully the indirect method for the analysis of peptides is that the distance between the chiral centers of FLEC and the analyte should not be too long. This method failed to provide the separation of peptides having Gly as the first amino acid.

A similar MEKC approach was employed by Chan et al.<sup>128</sup> for the chiral separation of 7 amino acids. The BGE had a similar composition, being formed of phosphate or borate buffer, SDS and acetonitrile. As the detection of FLEC derivatives was made by LIF, a higher sensitivity was achieved. The pH was found to play an important role in achieving chiral resolution, regardless of the buffer used. Therefore, buffers containing HEPES, phosphate or borate adjusted to the same pH values in the 6.8-8.5 range gave similar results.

Fradi et al.<sup>79</sup> described the automatization of the FLEC derivatization procedure and the separation of a mixture of 19 amino acids. The separation was achieved by use of a BGE made up of tetraborate buffer (pH 9.2), SDS and IPA. The optimum BGE composition was determined by using an experimental design. It was observed that the SDS concentration was positively correlated with the chiral resolution (except for Phe

and Arg), while the impact of IPA on resolution was specific to each amino acid. Ultimately the optimum BGE composition was found to be 40 mM tetraborate buffer, 21 mM SDS and 8.5% IPA.

Recently, the separation of FLEC-AA derivatives by MEKC-MS was described by Moldovan et al.<sup>80</sup> and Prior et al.<sup>121</sup>. Considering the special requirements of mass spectrometry, MEKC-MS approaches have always been difficult to implement. In these works, a semi-volatile pseudostationary phase was used, containing perfluorooctanoic acid (PFOA) as surfactant. An important constraint was the maximum current (50 µA) allowed by the manufacturer for the CE-MS interface, making the achievement of the desired selectivity a more challenging task. Nevertheless, by employing a BGE made of PFOA at basic pH (9.5), chiral selectivity was achieved for 14 pairs of FLEC-AA derivatives in a reasonable timeframe (37 minutes).

In none of the conditions described above, the chiral separation of acidic amino acids (i.e. aspartic acid and glutamic acid) could be achieved. At a basic pH, they have a net charge of (-2), leading to an electrostatic repulsion from SDS (or PFOA) micelles. Chan et al.<sup>128</sup> consider that an increase in the SDS concentration could lead to their chiral separation, achieving partial separation of FLEC-glutamic acid diastereomers using a BGE formed of 150 mM SDS and 5 mM borate buffer. However, they could achieve the chiral separation of the two amino acids by using a buffer with an acidic pH (citrate buffer, pH 4.4). Their separation is especially relevant from a metabolomic perspective, since the D form of aspartic acid is recognized as a neurotransmitter in the central nervous system and an increase in its concentration seems to play a role in some neurological diseases (e.g. schizophrenia, Alzheimer's disease<sup>162,163</sup>).

One particular study<sup>120</sup> shows the applicability of SFC for the separation of FLEC derivatized amino acids. Considering the presence of a residual carboxylic function in the structure of the derivatives it was necessary to add a counter ion (an amine) in the organic modifier. FLEC derivatives of Val, Ala and Met could be separated on a C18 stationary phase using a mixture of methanol, water and methylamine as modifier in the mobile phase. In contrast to RP-LC, the less polar compounds were found to elute first, followed by those having an additional ionizable group.

### 2.3.3 FLEC derivatization for the chiral analysis of pharmaceuticals

Another domain of interest in which FLEC was found to be applicable is the analysis of pharmaceuticals, since many of them are primary or secondary amines. Among these compounds, chiral analysis of cardiovascular drugs ( $\beta$ -blockers, antiarrhythmic agents), muscle relaxants, antidepressants and antimalarials was investigated. The separation of drug enantiomers can often be desirable, since it is well known that their pharmacological activity may be totally different or one of them can be toxic or produce side effects.

The first application of FLEC derivatization in pharmaceutical analysis was performed by Einarsson et al.<sup>78</sup>. They achieved the chiral separation of several cardiovascular drugs by using LC methods. In the following years, several

studies<sup>107,153,154</sup> have been aimed at the analysis of β-blocker enantiomers in different matrices.

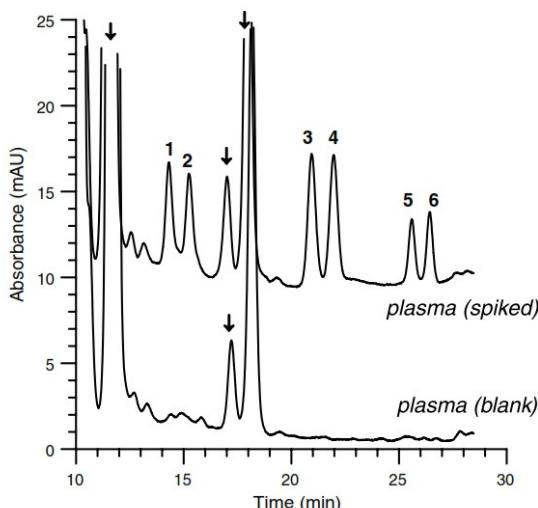
For pharmacokinetic studies, Roux et al.<sup>153</sup> developed a LC method for propranolol enantiomers in human blood. The separation took place on an achiral stationary phase, using the reversed-phase mode. A very low LOQ of 0.5 ng/mL was reached using a FL detector. Later, a similar method was described by Lai et al.<sup>107</sup>; the novelty came from the implementation of a new automatized system (see section 2.2.3) in order to increase the efficiency and reproducibility of the analytical procedure. Atenolol derivatives have also been separated after isolation from rat plasma by a RP-LC method using a C18 stationary phase and ACN/sodium acetate buffer as mobile phase<sup>154</sup>.

For the separation of FLEC derivatives of baclofen, a skeletal muscle relaxant<sup>126</sup>, a C8 stationary phase was used together with a γ-cyclodextrin (γ-CD) based mobile phase. The addition of the CD led to a 2-fold increase in resolution (from 1.75 to 3.45).

Three studies<sup>115,116,164</sup> were published regarding the LC separation of FLEC-reboxetine derivatives, this drug being used as an antidepressant. Although the molecule possesses two chiral centers, due to chiral synthesis only two enantiomers are present and need to be separated. The first study<sup>116</sup>, focused on the development of a RP-LC method capable of separating the FLEC derivatives of the two enantiomers, was based on isocratic elution with almost equal proportions of aqueous and organic mobile phase components, delivered at a rather low flow-rate (0.5 mL/min). This resulted in a quite long analysis time (60 min), achieving a chiral resolution of 1.1 and a LOQ of 1 ng/mL in plasma. The same method was later used<sup>164</sup> for the assessment of reboxetine pharmacokinetics in mouse and rat plasma and brain. Later, Walter et al.<sup>115</sup> developed a faster NP-LC method for the analysis of the same drug, able to resolve the FLEC derivatives of the two enantiomers ( $Rs=1.25$ ) in less than 16 minutes, while keeping sensitivity at a level similar to that of the previously reported method. For achieving this result, they used three stationary phases connected in series; the first one was silica for separating FLEC-reboxetine derivatives from the internal standard, then the second and third stationary phases were cyano and cellulose tris(3,5-dimethylphenylcarbamate) for the chiral resolution of FLEC-reboxetine diastereomers and elimination of derivatization byproducts.

The chiral separation of amphetamine and amphetamine related drugs as FLEC derivatives was reported by several authors<sup>105,110,113,114,122,165</sup>. These studies were conducted to meet the increasing demand of toxicological and forensic analyses. It is well known that the enantiomers of these drugs of abuse have different actions; for example, D- or (+)-methamphetamine has a greater activity on the central nervous system. The first attempt to separate the FLEC-derivatized enantiomers of methamphetamine by RP-LC was made by Chen et al.<sup>110</sup>. A C18 column was used together with a mobile phase consisting of ACN/phosphate buffer (0.05 M), enabling good chiral separation ( $\alpha=1.04$ ). Moreover, ephedrine and pseudoephedrine, which are methamphetamine precursors, can be analyzed using the same method. Another LC

method for the analysis of amphetamine and methamphetamine in rat serum was developed by Hutchaleelaha et al.<sup>105</sup> using again a C18 stationary phase and a mobile phase made of acetate buffer/ACN/THF. The same method was later used by Sukbuntherng<sup>113</sup> for the quantitation of methamphetamine enantiomers and their metabolites in urine. The same kind of RP-LC system was developed by Campíns-Falcó et al.<sup>114</sup> and Verdú-Andrés et al.<sup>122</sup>, using a similar stationary phase (C18) and a mobile phase consisting of MeOH/acetate buffer 0.05M. Chiral resolution was achieved for FLEC derivatives of amphetamine, methamphetamine, ephedrine, pseudoephedrine, 3,4-methylenedioxymamphetamine (MDA), 3,4- methylenedioxymethamphetamine (MDMA) and 3,4-methylenedioxymethylamphetamine (MDE) within 30 minutes (Fig. 9).



**Figure 9. Analysis of amphetamine-related designer drugs by RP-LC after derivatization with FLEC. Analytes: 1 R-MDA; 2 S-MDA; 3 R-MDMA; 4 S-MDMA; 5 R-MDE; 6 S-MDE. (Reproduced from Ref. 122)**

A method designed for the determination of (SR)- and (RS)-enantiomers of mefloquine, an antimalarial agent, was found to be a highly suitable investigational tool for pharmacokinetic studies<sup>104</sup>. Baseline resolution of FLEC derivatives was achieved by RP-LC, as well as a very low LOD of 50 nM by using small amounts of plasma (100 µL).

The enantiomers of a new and original compound (S 12024) with cognitive enhancing properties have been separated (Rs=0.7) by RP-LC after FLEC derivatization<sup>109</sup>. The method proved to be robust and sensitive for the analysis of biological samples in a pharmacokinetic study.

A stereoselective analytical procedure for a novel non-fluorinated quinolone (PGE-9509924) was developed by Zoutendam et al.<sup>123</sup>. This new compound was found to be active against multiple drug resistant bacteria. Both LC-MS-MS and LC-FL methods were developed, both offering the same sensitivity. Nevertheless, the LC-MS-MS method was chosen for further studies due to its higher selectivity. A mixture of methanol and acetonitrile was selected as the organic part of the mobile phase, since the two solvents

used separately did not offer a suitable peak shape, nor chiral selectivity in a reasonable amount of time.

The separation of FLEC-carnitine derivatives by RP-LC was studied by De Witt et al.<sup>111</sup>. One of the main experimental factors influencing the retention and resolution was the pH of the mobile phase, the maximum resolution being reached at pH 7. The LC separation of FLEC derivatized carnitine enantiomers has also been reported by Vogt et al.<sup>101</sup>. The strong influence of the pH on resolution was again highlighted, but in this case the best results were obtained at a lower pH (2.6). Later, Freimüller et al.<sup>108</sup> developed and validated a similar RP-LC method for the quantification of the same analytes.

The CE separation of FLEC-carnitine derivatives was also performed by De Witt et al.<sup>111</sup>. Baseline separation was achieved using a phosphate buffer (pH 3.4). Vogt et al.<sup>101</sup> also separated FLEC-carnitine derivatives by CE using a BGE made up of 50 mM phosphate buffer and 20 mM TBABr (pH 2.6). The addition of TBABr seems to play an essential role in achieving chiral separation, the resolution being positively correlated with the amount of TBABr. The analysis time and resolution obtained by CZE are similar to those obtained by LC, but unfortunately the limits of detection are two times higher.

A CEC method was successfully developed by Aturki et al.<sup>166</sup> for the indirect chiral resolution of several β-blocker agents. Three C18 stationary phases were evaluated, while the mobile phase composition was optimized. The best chiral separation was obtained when the mobile phase consisted of borate buffer (pH 8)/ACN – 20/80 (v/v). This methodology proved to offer good selectivity for the FLEC derivatized enantiomers in a fairly short amount of time (18 min).

### 2.3.4 Other applications

FLEC derivatization has been applied to the chiral analysis of glufosinate<sup>119</sup>, a nonspecific phosphorous-containing amino acid-type herbicide. Chiral resolution was achieved by RP-LC, with a very good sensitivity (LOQ=5ng/mL).

For the study of a novel adenosine antagonist, Witte et al.<sup>117</sup> developed RP-LC methods using both UV and MS detection. Considering that resolution of the analyte could not be achieved by direct separation using a chiral selector, FLEC derivatization provided a good alternative. Baseline separation of the FLEC derivatives was achieved using a mobile phase consisting of ACN and ammonium acetate (2 mM).

Tóth et al.<sup>167</sup> developed a RP-LC method for the determination of the absolute configuration of some oxytocin analogues. The synthesized molecules contained a D-Trp in position 2, and the derivatization with FLEC could be implemented in order to achieve chiral separation.

## 2.4. Conclusion

Over the last 30 years FLEC proved to be a very versatile chiral derivatization agent. Due to the formation of hydrophobic diastereomers, excellent enantioselectivity can be achieved using conventional instrumentation, without the need of expensive chiral selectors. Moreover, it is highly advisable to use it for increasing the UV or FL detection sensitivity for certain classes of molecules, which do not possess chromophores or fluorophores in their structure (e.g. most amino acids).

Its major advantages over other derivatization agents are fast reaction kinetics and the simple derivatization procedure. Quantitative derivatization reactions can be expected if the chiral reagent is used in at least 1:10 molar excess relative to the analyte.

The applicability of FLEC derivatization was attested for a wide range of molecules present in different matrices. No need for special sample pretreatment was reported in the cited studies, other than those usually employed for analytical methods.

Even if FLEC's relatively high price makes it less popular among CDAs, the cost issue may be tackled by decreasing the FLEC consumption by implementing automated techniques for labeling the analytes, such as in-capillary derivatization when using CE.



## **PERSONAL CONTRIBUTION**



## HYPOTHESES and OBJECTIVES

The chiral analysis of amino acids is becoming more and more necessary. Therefore, new sensitive and efficient tools need to be developed in order to get the maximum of information out of a sample in the shortest time possible.

The main objective of this research was to design new and efficient methods for the analysis of D- and L-amino acids in biological fluids. This objective was reached by implementing several strategies using different separative techniques. A common feature of all the developed methods was the derivatization of amino acids with (+) or (-)-FLEC. The reaction products are pairs of diastereomers, which can be separated using achiral separation media.

The first approach<sup>80</sup> used for achieving the objective was to develop a completely automatized analysis setup using a micellar electrokinetic chromatography system coupled with mass spectrometry for analyzing the D- and L- forms of all proteinogenic amino acids. This way, the method would benefit not only from the high peak efficiency of the capillary electrophoresis, but also from the sensitivity and selectivity of mass spectrometry. In order to automatize the process, the capillary was intended to be used as a reaction chamber for the derivatization of amino acids. The sample and derivatizing reagent would be automatically injected, the reaction would take place in the capillary and then the separation would occur.

The goal for the second method<sup>168</sup> was to develop a targeted analysis method for five biologically relevant amino acids. Among them were two acidic amino acids (DL-Asp and DL-Glu), for whose separation of the FLEC diastereomers by capillary electrophoresis was reported only once<sup>128</sup>. In the review paper which comprises Chapter 2 of this work, we noticed that generally the resolution of FLEC derivatives of the amino acids was dependent on pH (both using CE and LC techniques). Considering this, a side objective for this study was to clearly determine the influence of pH on the migration of the diastereomers.

Third study's objective was to progress the knowledge in the separation of FLEC amino acid derivatives by liquid chromatography. As presented in Chapter 2, all separation methods for FLEC-AAs involved similar conditions: a RP C4, C8 or C18 stationary phase and a THF based mobile phase. The use of THF, which is a nonpolar protic solvent, as mobile phase component proved to be critical in obtaining resolution on the mentioned stationary phases. Nevertheless, THF is usually regarded as detrimental to the LC instruments, due to its ability to dissolve and degrade the different plastic (PEEK tubing) and rubber parts of the LC instruments. Moreover, even if good separations were obtained for all D- and L-FLEC-AAs, usually the analysis time took very long (around 100 min.). Considering all this, our objective was to evaluate the selectivity

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of other types of RP stationary phases for FLEC-DL-AAs, while using only common organic solvents and buffers as mobile phases. In order to shorten the analysis time and gain in selectivity, a mass spectrometry detector was employed.

Considering that all the developed methods are meant to be applicable to biological samples, another goal of the research was to develop, if needed, specific sample preparation methodologies.

## Chapter 3:

# A micellar electrokinetic chromatography - mass spectrometry approach using in-capillary diastereomeric derivatization for fully automatized chiral analysis of amino acids

### 3.1 Introduction

Derivatization procedures are often implemented for better sensitivity and selectivity when analyzing certain categories of compounds. The most common derivatization procedure in CE is the off-line procedure, also called pre-capillary derivatization, whereby the chemical reaction is performed manually outside the capillary. Having in mind that the volume needed for the actual CE analysis is a few nanoliters, the pre-capillary approach generates an important waste of sample and reagent. Thus, the automation of the whole derivatization process is an important step to be developed and standardized in order to save time, improve method reliability and reduce the consumption of reagents and samples. Indeed, the use of in-capillary derivatization brings a significant gain in time by reducing the duration of sample preparation and also by offering the possibility of implementing a continuous workflow. Nevertheless, some criteria need to be fulfilled: the reaction has to be fast and quantitative, the reaction by-products should not interfere with the separation and all reaction products should be compatible with the detection method (e.g. MS detection).

Among the available chiral derivatization reagents, FLEC was considered. It was first reported by Einarsson et al.<sup>78</sup> for the chromatographic analysis of amino acids. Since then, (+) and (-) FLEC have been used for the LC chiral separation of different molecules, such as amphetamines<sup>114</sup>, herbicides<sup>119</sup> and amino acids<sup>103,159,169</sup>. Its application in CE is still rather limited, with only few studies involving the chiral separation of beta-blockers<sup>166</sup> and amino acids<sup>79,127</sup>. As the derivatization reaction takes place in a few minutes at room temperature, we considered this chiral reagent suitable for automatization of the derivatization step and we previously developed an MEKC-UV method using this approach<sup>79</sup>.

A recent review<sup>170</sup> on amino acid analysis highlighted some important CE-MS studies published over the last three years. When using MS, it is well known that there

are important reagent compatibility constraints so that it can be challenging to achieve the desired selectivity. Usually, the separation methods for chiral analysis (used in food analysis, pharmaceutical industry or bioanalysis) make use of a chiral selector<sup>171,172</sup>. When using a chiral derivatizing agent, the addition of chiral selectors to the BGE is not only superfluous, but the selectivity can also be tuned more easily. One way of improving the separation of amino acids by CE-MS is the use of a semi-volatile surfactant such as ammonium perfluorooctanoate. Indeed, it was reported that this might offer a significant improvement in terms of selectivity compared to ammonium acetate alone<sup>173</sup>.

Some metabolomic studies on neurodegenerative diseases showed that D-amino acids are of particular interest<sup>174,175</sup>. Some of them (D-Ala, D-Gln, D-Ser) were found to play a role in bacteria<sup>176-179</sup> and their presence in mammals was supposed to be simply due to ingestion or the metabolism of commensal bacteria. Nevertheless, the presence of endogenous free D-amino acids was also demonstrated in the brain of humans and other mammals<sup>174,175</sup>. Among them, D-Asp was found in large quantities during the embryonic phase of brain development<sup>180</sup> and for normal subjects it was established to have a neuroendocrine role<sup>26</sup>; also, D-Ala, D-Pro and D-Glu were identified as being endogenous in the mammal brain<sup>181,182</sup>. In a recent paper<sup>183</sup> it was stated that D-Ser seems to be synthesized and released by the neurons. Also, the distribution of D-Ser is similar to that of the N-methyl-D-aspartate receptors, which suggests its role as neurotransmitter<sup>184</sup>. All these studies highlight the importance of the separation of the amino acids enantiomers for further quantification in biological samples. In the only study performed over the last 3 years on the chiral analysis of amino acids by CE-MS, Sánchez-Hernández et al.<sup>185</sup> described the use of pre-capillary derivatization with FMOC and vancomycin as chiral additive in a partially filled coated capillary.

In the present study, we aimed at developing a fully automated method for separating and quantifying D- and L-amino acids by CE-MS. We used as starting point the very promising results previously obtained in our laboratory using the in-capillary labeling technique for the chiral separation of amino acids by CE-UV<sup>79</sup>. The adjustment of the method to make the BGE compatible with MS detection changed completely the selectivity and brought new challenges for the in-line derivatization procedure. In this paper, the optimization of the MS-related parameters and the automatized derivatization procedure are described.

## 3.2 Material and methods

### 3.2.1 Chemicals and reagents

All the chemicals used in this research were of analytical grade purity. Racemic amino acid standards of Ala, Arg, Asn, Asp, Glu, Leu, Ile, Phe, Pro, Trp, Val and D- and L-enantiomers of Thr were purchased from Sigma-Aldrich (Steinheim, Germany). Gln was provided by Aldrich (Milwaukee, WI, USA), Ser was made by Merck (Darmstadt, Germany), His and Tyr by Fluka (Steinheim, Germany). Gly was purchased from Fisher Scientific (Loughborough, UK) and Met from Certa (Braine-l'Alleud, Belgium). N-methylmorpholine and APFO were provided by Sigma-Aldrich, 2-butanol and 25% ammonia solution by Merck (Hohenbrunn, Germany), isopropanol (IPA) by VWR Chemicals (Fontenay-sous-Bois, France), sodium tetraborate and formic acid were from Acros Organics (Geel, Belgium). Sodium dodecylsulfate (SDS) was obtained from Fisher Scientific.

The derivatization agent, (-)-1-(9-Fluorenyl)ethyl chloroformate 18 mM solution in acetone, was purchased from Sigma-Aldrich (Steinheim, Germany).

The water used in this work was either of Milli-Q purity, obtained with a Milli-Q water purification system (Millipore, Switzerland) or water for LC-MS provided by Biosolve (Valkenswaard, the Netherlands).

All solutions were prepared in class A glassware. The solutions were filtered using 0.2 µm cellulose filters obtained from Macherey-Nagel (Düren, Germany).

### 3.2.2 Instrumentation

All experiments were carried out on Agilent 3D-CE systems (G1600AX) (Agilent Technologies, Waldbronn, Germany). MS detection was achieved using an Agilent XCT Ion-Trap instrument controlled by means of MSD Trap Control 5.3 Software and the MS data was analyzed using DataAnalysis (both provided by the same manufacturer). The CE acquisition and primary data analysis were performed using Agilent Chemstation (Rev. B.03.01 and B.04.01). The hyphenation between the CE and MS instruments was achieved using an ESI sheath-flow interface. The sheath liquid (SL) was delivered using a syringe pump manufactured by KD Scientific (Holliston, MA, USA).

### 3.2.3 Solution preparation

Amino acid stock solutions (1 mM) were prepared by dissolving the appropriate quantity of each amino acid in 5 mM NaTB. Further dilutions were made in order to achieve the desired concentrations. MS compatible BGE solutions containing APFO were prepared by dissolving the required amount of APFO in water and adjusting the pH to 9.5 with ammonia solution (25%).

For the part of the study performed with UV detection the BGE solutions consisting of NaTB, sodium dodecyl sulfate and IPA were prepared by dissolving the required amount of SDS in 40 mM NaTB and then adding the appropriate percentage of organic modifier.

The aCSF was prepared by a protocol developed by Durect Corporation (Cupertino, USA)<sup>186</sup>. Two aqueous solutions (500 ml each) were prepared. The first one was made by dissolving in water NaCl (8.66 g), KCl (0.224 g), CaCl<sub>2</sub>·2H<sub>2</sub>O (0.206 g) and MgCl<sub>2</sub>·6H<sub>2</sub>O (0.163 g), while the second one contained Na<sub>2</sub>HPO<sub>4</sub>·7H<sub>2</sub>O (0.214 g) and NaH<sub>2</sub>PO<sub>4</sub>·H<sub>2</sub>O (0.027 g). The final aCSF was prepared by mixing the two solutions. The samples were prepared by spiking Ala, Ser, Val, Glu, Met and Phe in aCSF, followed by a 1:1 dilution with 5mM NaTB and addition of 10% (v/v) acetonitrile.

The FLEC solution for the in-capillary derivatization was prepared by 2:1 dilution of the 18 mM FLEC solution with 5 mM NaTB.

### 3.2.4 Electrophoretic method

All experiments were carried out using bare fused-silica capillaries (50 µm ID; total length of 80.5 cm long), provided by Polymicro Technologies (Phoenix, AZ, USA).

Before use, each new capillary was conditioned by flushing 15 min with 1 M NH<sub>4</sub>OH, 15 min with water and 15 min with the running buffer. At the beginning of each day the capillary was conditioned by flushing 15 min with 1 M NH<sub>4</sub>OH and then 15 min with water. Before each run the capillary was preconditioned by flushing 3 min with 1 M NH<sub>4</sub>OH, 2 min with water and 5 min with BGE.

The BGE was composed of 150 mM APFO adjusted with NH<sub>4</sub>OH to pH 9.5. During the analysis a voltage of 25 kV was applied (ramped in one minute), with a maximum system-limited current of 50 µA. The capillary was thermostated at 15°C.

### 3.2.5 Derivatization procedure

The in-capillary derivatization for the CE-UV part of the study was performed according to the protocol described by Fradi et al.<sup>79</sup>. For the CE-MS coupling, the in-capillary derivatization was carried out by injecting a sample plug for 15 s followed by another plug of the derivatization agent (FLEC - 12 mM) for 7.5 s. The mixing was achieved by applying a voltage of 0.1 kV for 900 s. All plugs were injected hydrodynamically using a pressure of 30 mbar.

If not otherwise stated, the pre-capillary derivatization was performed by mixing the sample and derivatization agent in equal amounts. The reaction time was 40 min; the resulting solution was diluted twice with water.

### 3.2.6 MS parameters

The sheath liquid was composed of IPA:H<sub>2</sub>O:FA (90:10:0.1) (v/v/v) and was delivered at a flow rate of 2.5 µL/min. The capillary tip was positioned one unit inside the sprayer needle.

The MS detection was performed in positive mode using the following parameters: capillary voltage: -5000 V, dry temperature: 300°C, nebulizer: 138 mbar, dry gas: 4 L/min, octopole RF amplitude: 168.9 Vpp, capillary exit: 48 V, skimmer: 29.7 V, Oct 1 DC: 11.7 V, Oct 2 DC: 1.8 V, max accumulation time: 300 ms, ICC target: 50000, scan interval: 200-650 m/z.

The FLEC derivatives were detected using the following m/z values: Gly – 312.0; Ala – 326.0; Ser – 342.1; Pro – 352.0; Val – 354.0; Thr – 356.0; Leu & Ile – 368.0; Asn – 369.0; Asp – 370.0; Gln – 383.1; Glu – 384.0; Met – 386.0; Phe – 402.0; Arg – 411.0; Tyr – 418.0; Trp – 441.0; His – 628.0 (His – doubly derivatized).

### 3.2.7 DoE and data analysis

The MS detection signal optimization was performed by response surface modeling (quadratic model) using a face centered central composite design with four quantitative factors (mixing time – Mt; mixing voltage – MV; sample plug – Sp; derivatization agent plug – Dp), three center points and thirteen responses (peak areas of the extracted ion chromatogram expressed as relative signal intensities of the 13 pairs FLEC derivatives of the studied amino acids), consisting of a total of 27 experiments. The design of experiments and data interpretation were carried out with the aid of Modde PRO 11 (MKS Data Analytics Solutions, Umea, Sweden).

The data analysis, which involved area measurements, comprised total peak areas, meaning that the cumulated areas of the enantiomers were evaluated.

The limits of detection (LODs) were determined based on the standard deviations of the responses and its slopes. According to the ICH guidelines<sup>187</sup>, the LODs (and LOQs) can be calculated using equations (1) and (2).

$$\text{LOD} = 3.3\sigma/S \quad (1)$$

$$\text{LOQ} = 10\sigma/S \quad (2)$$

Where  $\sigma$  = the standard deviation of the response; S = the slope of the calibration curve.

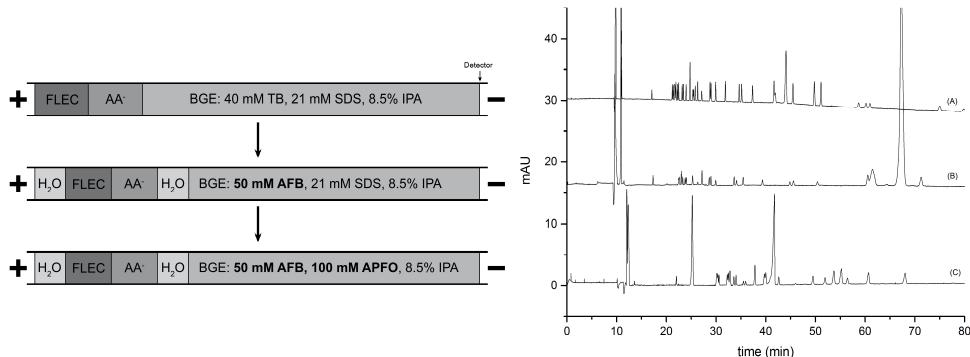
## 3.3 Results and discussion

The main goal of this research was to develop for the first time a fully automated MEKC-MS method for the chiral analysis of amino acids using in-capillary derivatization. The starting point was the previous work done by Fradi et al.<sup>79</sup>, who developed a MEKC-UV method with in-capillary derivatization using (-)-FLEC and a BGE consisting of NaTB, SDS and IPA.

### 3.3.1 MEKC-UV method adjustment for MS detection

Since NaTB and SDS are not suitable for MS detection, they were replaced by MS compatible components (Fig. 10). Considering that the derivatization agent reacts with primary amines, a suitable buffering system around pH 9 was necessary. Therefore, N-methylmorpholine<sup>188</sup> was investigated as a MS compatible buffer component. It offers the advantages of being volatile and has buffering capacity around the desired pH range ( $pK_a=7.6$ ). The buffer was tested at 10 mM concentration in combination with 21 mM SDS and 8.5% IPA. This BGE offered interesting separation capabilities but it did not provide reproducible results probably due to its low buffering capacity (data not shown).

Consequently, ammonium formate (AFB) was tested as electrolyte even if ammonium salts are generally not recommended because they are prone to react with FLEC. In order to minimize the contact between the derivatization agent and the ammonium ions during the reaction time, water plugs (3s, 30 mbar) were introduced at the edges of the derivatization zone. This approach proved to be effective as shown in Fig. 10A and B.



**Figure 10. Electropherograms of the in-capillary derivatized amino acids obtained by MEKC-UV. Selectivities obtained by replacing the BGE components with MS compatible ones. (A) BGE: 40 mM tetraborate buffer with 21 mM SDS and 8.5% IPA; (B) BGE: 50 mM ammonium formate buffer with 21 mM SDS and 8.5% IPA; (C) BGE: 50 mM ammonium formate buffer with 100 mM APFO and 8.5% IPA.**

The next step was to replace SDS. Indeed, it has already been proven that SDS induces important ionization suppression<sup>189</sup>. Even though partial filling techniques<sup>190</sup> allow the use of SDS with MS detection, they proved to be difficult to be implemented. Consequently, APFO was selected to replace SDS, as it is a semi-volatile surfactant which does not induce important ionization suppression in positive ion mode<sup>173</sup>. However, the selectivity offered by APFO appeared to be quite different from that obtained with SDS (Fig. 1B and C). Considering that all components of the BGE were MS compatible (50 mM AFB with 100 mM APFO and 8.5% IPA), the subsequent analyses were made using CE-MS coupling. Even if the method transfer from UV to MS detection was in principle straightforward, some important parameters were still to be optimized.

### 3.3.2 CE-MS studies

#### 3.3.2.1 ESI related technical observations

The CE instrument was coupled to the mass spectrometer via a sheath liquid interface. The main parameters to be optimized were the sheath liquid composition, SL flow, nebulizing gas pressure and dry gas flow. The SL composition was selected from a previous work in which APFO was also used in the BGE<sup>173</sup>. Since the nebulizing gas is known to produce a significant suction effect at the capillary tip<sup>191-193</sup>, it was set at the minimum value, 138 mbar.

Another important parameter related to the spray quality is the positioning of the capillary tip. Even if the manufacturer advises to position the capillary tip outside the

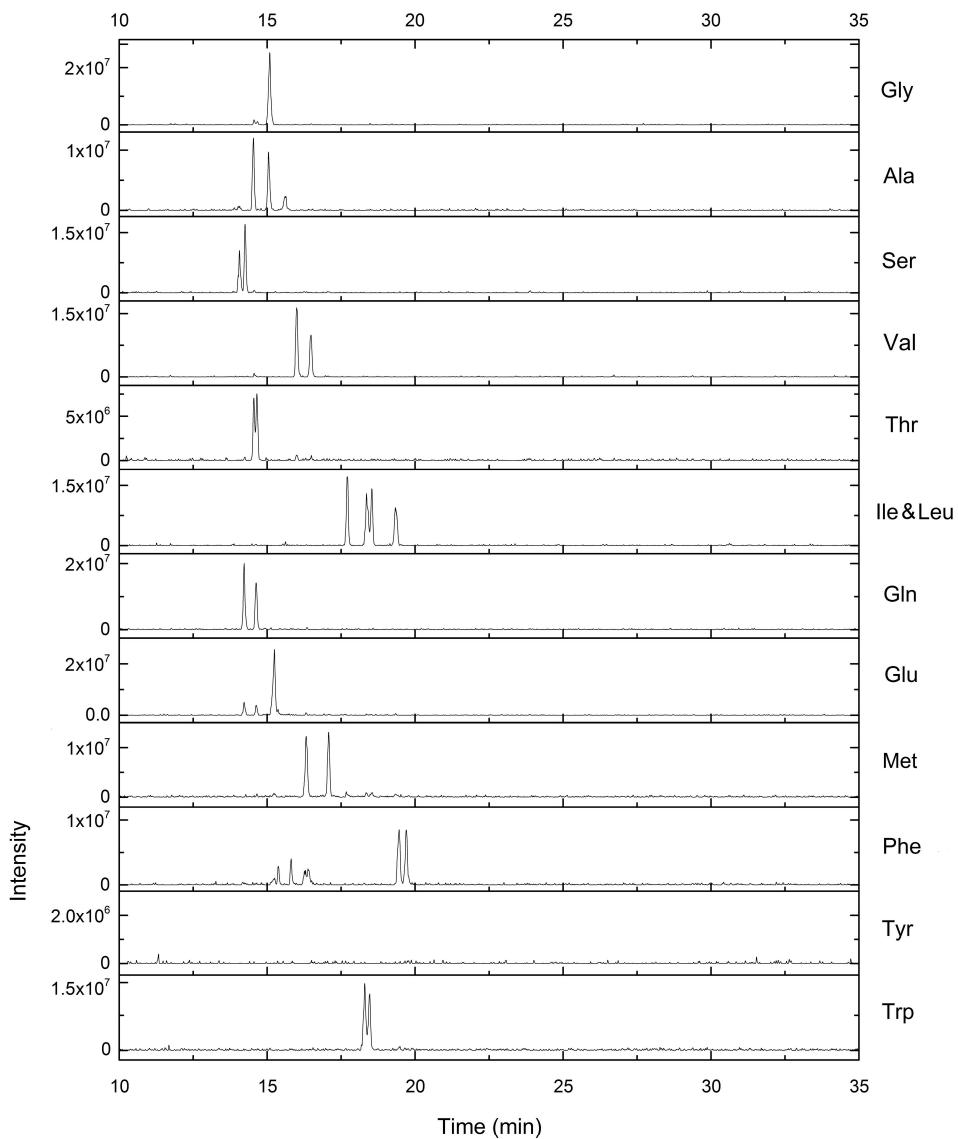
sprayer, we obtained a more stable spray with the capillary tip inside the probe, as previously observed<sup>194</sup>.

### **3.3.2.2 BGE optimization for chiral separation**

It should be noted that pre-capillary derivatized samples were used during the optimization of the BGE composition.

The BGE that provided the best results in the CE-UV mode, consisting of 50 mM AFB, 100 mM APFO, 8.5% IPA, was tested using the CE-MS coupling. Low resolution and long migration times were obtained due to the system limitation of the current to 50 µA. In order to decrease the conductivity of the BGE, the percentage of the organic modifier was increased to 12% but this led to very long migration times (>100 min), which is not appropriate for routine analysis. Consequently, in order to decrease the current and to save the analysis time, AFB was removed from the BGE as well as the organic modifier. Indeed, the latter was found only useful for improving chemoselectivity but not chiral selectivity. As MS detection was used, the efforts were concentrated on chiral selectivity.

Baseline resolution was achieved for the diastereomers of Ala, Val, Leu, Ile, Gln, Met and Phe using 100 mM APFO. By increasing APFO concentration to 150 mM, Ser diastereomers were also completely resolved (Fig. 11). Thr, Asn and Trp diastereomers were partially separated in a reasonable timeframe (35 min). An increase of APFO concentration over 150 mM led to lower resolution (due to high conductivity and current limitation).

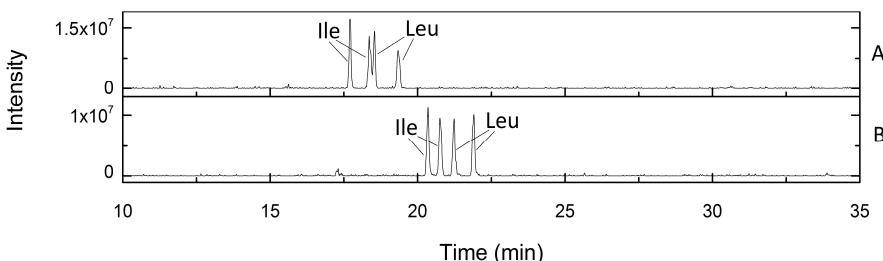


**Figure 11. Electropherograms of the pre-capillary derivatized amino acids obtained by MEKC-MS. Selectivity shown by a BGE composed of 150 mM APFO ( $\text{pH} = 9.5$ ). Derivatization reaction time: 40 min.**

As shown in Figure 11, the diastereomers of leucine and isoleucine were completely separated but the chemoselectivity between these two isobaric amino acids was not satisfactory. To solve this particular problem, a BGE consisting of 120 mM APFO and 1% 2-BuOH, was found to be appropriate to separate the four isomers (Fig. 12).

### 3.3.2.3 Optimization of the in-capillary derivatization procedure

The sensitivity of the method is directly influenced by the derivatization efficiency. It is well known<sup>79</sup> that this procedure is influenced by multiple factors, mainly related to the injected quantities of sample and reagent and the mixing of the two plugs. In order to evaluate the impact of these factors, an experimental design was implemented. The factors selected for this study were the mixing time (Mt), mixing voltage (MV) and the size of the two plugs, i.e. the FLEC plug size (Dp) and the sample plug size (Sp).

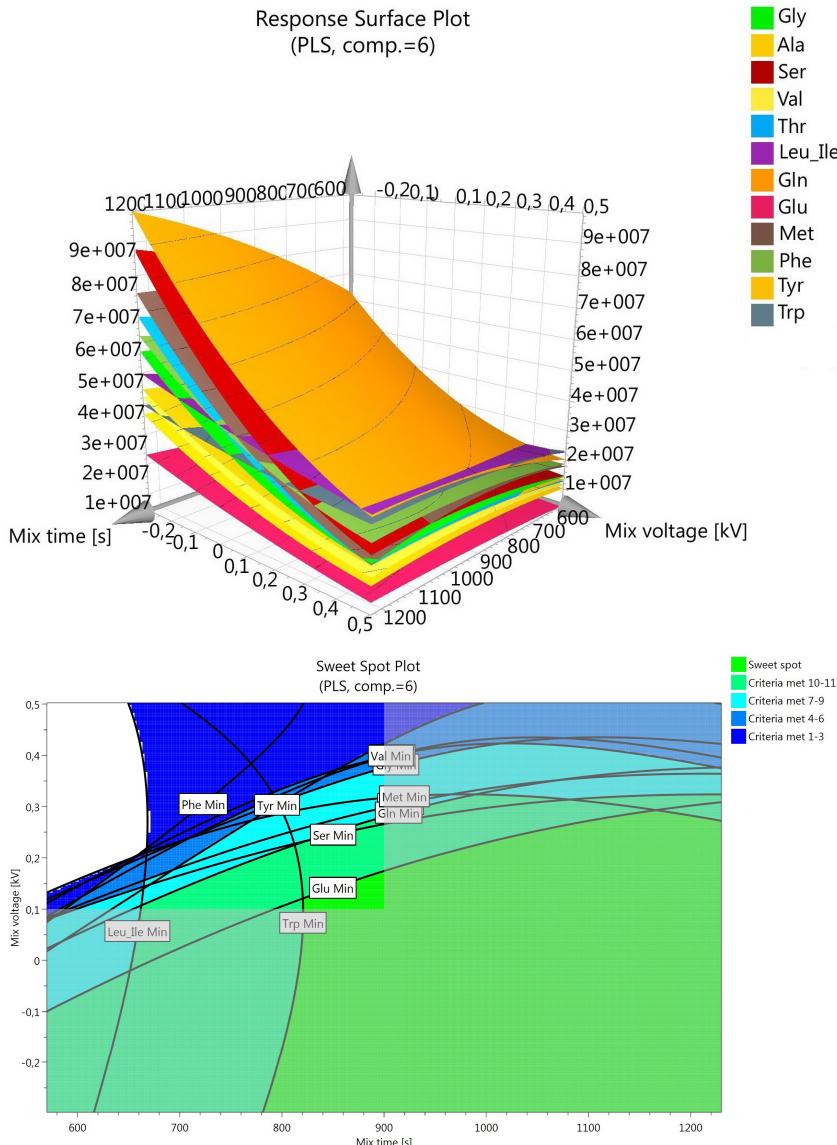


**Fig. 12. Separation tuning of Leu and Ile derivatives. BGEs: (top) 150 mM APFO (pH = 9.5); (bottom) 120 mM APFO (pH = 9.5) with 1% of 2-BuOH.**

The design of experiments was carried out on pre-screened ranges for the four quantitative variables ( $Mt = 240 - 900$  s;  $MV = 0.1 - 0.9$  kV;  $Sp = 5 - 15$  s (30mbar);  $Dp = 5 - 10$  s (30 mbar)) in which relevant data was expected to be obtained, allowing to avoid constraints from the experimental space and being a good compromise between time effectiveness, system stability and detection sensitivity. The MV interval was also chosen because previous studies<sup>79</sup> had suggested that the derivatization efficiency was higher at lower voltages. The FLEC plug size (Dp) interval was selected since the experiments performed with a longer plug (above 300 mbar·s) led to current disruption. The matrix contained 15 model terms (the four variables, their quadratic and interaction terms and a free term). The normal probability plots (residuals of a response vs. the normal probability distribution) indicated that there were no outliers in the experimental matrix (data not shown). The model was fitted by Partial Least Squares, followed by model pruning taking into account the goodness of fit ( $R^2$ ) and the fraction of the variation of the response predicted by the model according to cross validation ( $Q^2$ ) simultaneously for all the investigated responses.

The summary of statistics for the obtained model indicates a good fit for all responses ( $R^2$  between 0.71 – 0.85), and a useful model with a fair prediction power ( $Q^2$  between 0.34-0.53). The values obtained for model validity ( $> 0.25$ ) in all cases ensure a correct model (no lack of fit and a model error in the same range as the pure error) with no outliers or other transformation problems. For Trp (model validity 0.12) the lack of fit was artificial, due to a reproducibility parameter very close to unit, which is

not representative of the true experimental error. The obtained reproducibility values (between 0.65-0.99) demonstrate a very low variation of the replicates compared to the overall variability and represent the variation of the response under the same conditions (pure error) at the center points compared to the total variation of the response.



**Figure 13. Response surface (A) and sweet spot plot (B) around the optimal values.**

The fitting of the model by PLS also indicated a strong correlation between the responses (data not shown) and of course the effects of all the variables on the

responses. Thus, an overview of the normalized regression coefficients (divided by the standard deviation of their respective response) reveals the influence (sign and intensity) of the variables on each individual response. The strongest influence on the relative ionic abundance of the amino acid derivatives is exerted by two linear terms ( $Sp$ ,  $Mt$ ), two interaction terms ( $Mt^*MV$ ,  $Mt^*Sp$ ) and two quadratic terms ( $Dp^*Dp$ ,  $MV^*MV$ ).  $Sp$ ,  $Mt$  and  $Mt^*Sp$  show a positive relationship, whereas  $Dp^*Dp$  and  $Mt^*MV$  show a negative correlation with the investigated responses. As expected, longer sample plugs and mixing times led to an increase of the MS signal, but the model also reveals the influence of some interaction terms (i.e.  $Mt^*MV$ ,  $Mt^*Sp$ , etc.). It is interesting to note the quadratic effect of  $Dp^*Dp$ : a shorter derivatization plug leads to a lower yield of the FLEC derivative, whereas a too long plug may lead to a lower mixing efficiency, resulting in both cases in lower MS signals (responses). It is also worth mentioning that higher MS signals were observed at lower mixing voltages and higher mixing times, an effect described by the  $Mt^*MV$  interaction term.

The optimal in-line derivatization conditions yielding the highest MS signals for all investigated amino acids was predicted by the fitted response surfaces and corresponding sweet spot plot (Fig. 13A and B) and led to the following set points: 0.1 kV for the mixing voltage, 900 s for the mixing time, 15 s for the sample plug and 7.5 s for the FLEC plug with a factor contribution of 27%, 62%, 7.4% and 3.6%, respectively. The obtained model was externally validated by running experiments at the predicted levels of the variables. The response signals for the majority of the amino acids were found to be inside the prediction intervals (Annex 3) and reproducible (RSDs for peak areas below 12%), except for Tyr (Table 2).

Using the optimal derivatization and separation conditions, an extra set of 5 amino acids (Pro, Asn, Asp, Arg and His) was analyzed (Figure 5). The peak efficiency for Pro, Arg and His was observed to be lower than for the other amino acids. This occurs for different reasons: Arg contains a guanidine functional group that probably interacts strongly with the negatively charged surfactant, His is doubly derivatized (more lipophilic) leading to long migration times while Pro peak shape may be influenced by its nonpolar cyclic side-chain. Asp's interaction with the micelles is probably diminished because of its two negative charges, leading to no enantioresolution (similarly to Glu).

Therefore, using the described technique we could separate ( $Rs \geq 1$ ) the (-)-FLEC derivatives of 13 amino acids within a timeframe of 37 minutes.

### ***3.3.2.4 Pre-capillary versus in-capillary derivatization***

In order to compare pre- and in-capillary derivatization efficiency, the pre-capillary approach was designed to simulate that employed in the optimized in-capillary derivatization. The reactants were added in the same proportion (sample:FLEC – 2:1). During the reaction time (15 min), the sample was mixed using a vortex mixer. For these particular derivatization conditions, it was observed that the in-capillary approach generally offers a higher rate of derivatization (with some exceptions – see Table 2) and similar resolutions (except Phe - not derivatized within 15 min). The differences in derivatization rate may be explained by two different mixing mechanisms (turbulent flow mixing vs. counter-current electromigration). The mixing taking place in the in-capillary approach is clearly susceptible to increase the fraction of efficient intermolecular collisions.

Using the in-capillary derivatization technique the chiral selectivity was similar to that obtained with the pre-capillary approach (Figs. 11 and 14). Though, a small increase in the migration times and slightly lower enantioresolution was observed for some amino acids. These phenomena are believed to be due to the differences in current intensity during the first minutes of the analysis, caused by the different conductivities of the injected plugs and also by the diffusion processes that probably take place during the derivatization procedure. Moreover, Tyr enantiomers were successfully derivatized and baseline separated, which was not the case using the pre-capillary approach. These differences may indicate different reaction kinetics for Tyr or an enhancement of the reaction rate by the electromixing, which was performed inside the capillary.

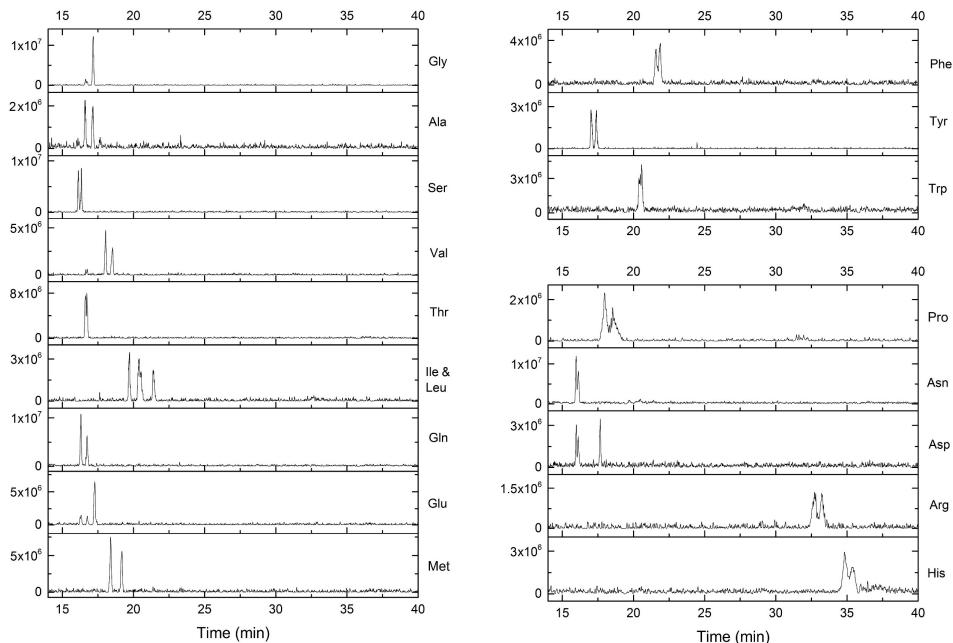
In terms of linearity, the method proved to be linear in the range of low micromolar concentrations (LOQ-60 µM). The estimation of LODs and LOQs for the studied amino acids can be found in Table 2. These limits were found to be similar to those obtained using other CE-MS approaches<sup>195,196</sup>.

**Table 2: Performance parameters for each studied amino acid**

AA	Peak area RSD <sup>a</sup> (%)	IC vs PC <sup>b</sup> (%)	Resolution IC/PC <sup>c</sup>	Linearity <sup>d</sup> (R <sup>2</sup> )	LOD <sup>e</sup> (μM)	LOQ <sup>f</sup> (μM)
Gly	10.75	5.46	-/-	0.9983	4.85	14.71
Ala	9.74	-2.36	3.1/2.6	0.9998	9.46	28.66
Ser	9.11	3.04	1.4/1.4	0.9973	4.03	12.21
Val	4.49	48.98	1.8/1.6	0.9972	2.99	9.05
Thr	7.10	26.91	0.5/0.7	0.9997	4.19	12.69
Leu & Ile	11.09	65.98	2.0 & 4.0/ 2.8 & 3.7	0.9868	7.05	21.37
Gln	8.07	-20.62	2.2/2	0.9962	9.02	27.34
Glu	5.63	-84.84	-/-	0.9963	6.16	18.67
Met	9.82	70.10	2.9/4	0.9937	5.83	17.66
Phe	11.93	100.00	0.5/-	0.9922	8.44	25.57
Tyr	30.03	100.00	1.7/-	0.9489	6.89	20.89
Trp	7.63	77.33	-/0.8	0.9916	11.92	36.11

<sup>a</sup>The peak area RSD in the optimized conditions (n=5);<sup>b</sup>In-capillary vs pre-capillary derivatization rates (peak area IC-peak area PC/peak area IC);<sup>c</sup>Resolution differences using in-capillary vs. pre-capillary approaches (pre-capillary reaction time: 15 min);<sup>d</sup>Linearity coefficients obtained for the concentration range of LOD to 60μM;<sup>e,f</sup>Estimated LODs and LOQs.

\*IC – in-capillary; PC – pre-capillary



**Figure 14. Electropherograms of the in-capillary derivatized amino acids obtained by MEKC-MS in the optimized conditions; BGE: 150 mM APFO (pH = 9.5).**

Compared to the pre-capillary approach, the in-capillary derivatization has some clear advantages, such as the small quantities of sample and reagent that are used. Moreover, the remaining part of the sample can be used for further analyses. The in-capillary approach is suitable for a continuous workflow, with minimal intervention from the analyst, thus reducing the possible sources of errors.

### 3.3.2.5 aCSF samples

The optimized derivatization procedure was tested on aCSF samples. A set of 6 amino acids was chosen, being composed of Ala, Ser, Val, Glu, Met and Phe. The aCSF was spiked with these amino acids and then diluted two times with 5mM NaTB, in order to reach a pH of 9, mandatory for the derivatization step. For these samples, the recoveries (compared to the standard samples) were found to be between 13% and 19%, which was considered to be unsatisfactory. The low yield was assumed to be caused by the high ionic strength of the sample plug (due to the high concentration of salts). This issue was solved by adding 10% acetonitrile (v/v) to the samples. In this way, the recoveries were found to be between 73.7% and 94.5%. These results are given in Table 3. With respect to migration times and resolution values, no changes were observed. Having in mind these results the method can be considered to be suitable for CSF sample analysis.

**Table 3.** RSDs obtained analyzing samples of aCSF spiked with amino acids ( $n=4$ ). Recoveries are calculated by comparing peak areas obtained on aCSF samples and standard samples

AA	$t_M$ RSD (%)	Peak area RSD (%)	Recovery (%)
Ala	0.28	14.52	93.71
Ser	0.23	13.80	76.66
Val	0.26	14.43	94.07
Glu	0.24	23.18	73.74
Met	0.33	6.59	74.61
Phe	0.74	22.03	94.47

### 3.4. Conclusions and perspectives

The use of a fully automated in-capillary derivatization method has proven to be suitable for the chiral analysis of amino acids by CE-MS. An experimental design approach was applied for the optimization of the in-capillary derivatization procedure. It also revealed the correlations between the variables and the analytical responses, which might help to provide a better understanding of this particular derivatization process.

The separation of diastereomeric FLEC derivatives ( $Rs \geq 1$ ) was achieved for 14 amino acids. APFO showed interesting selectivity but could be used only in a limited concentration range due to maximum current allowed with this interface. It was also observed that organic modifiers have a negative impact on chiral selectivity, but they can be useful for tuning the chemoselectivity after optimization of their type and concentration. Moreover, the method was found to be suitable for biological sample (CSF) analysis.

Additional efforts have to be made for the chiral separation of some amino acids for which a baseline resolution was not yet achieved. Higher sensitivity could be expected using a more sensitive mass spectrometer, such as a triple quadrupole.



## Chapter 4:

# Capillary electrophoresis-mass spectrometry of derivatized amino acids for targeted neurometabolomics - pH mediated reversal of diastereomer migration order

### 4.1 Introduction

Not so long ago it was a widely accepted hypothesis that the occurrence of D-amino acids in mammals was related either to their assimilation from the ingested food or to the metabolism of commensal bacteria. Along with the development of sensitive analytical techniques, a revolutionary discovery identified the presence of small quantities of D-Ser in the mammalian brain<sup>39,179,197</sup>. Since then, extensive research has been carried out in order to elucidate the role played by D-amino acids (D-AAs) in mammalian organisms.

Over the last years, several reviews<sup>17-21,198</sup> have been published describing the role, synthesis and metabolism of D-amino acids in the central nervous system, endocrine system and peripheral tissues. Among the D-amino acids found in brain, the endogenous levels of D-Ser and D-Asp have been reported to be the most significant<sup>181</sup>. The role of D-Ser as a co-agonist of *N*-Methyl D-Aspartate (NMDA) receptors has been well established, but much less is known about the role of D-Asp. A recent study<sup>162</sup> suggests that D-Asp is also an NMDA receptor agonist, found in high quantities during the brain development stages. The relevance of these D-AAs stems from their involvement in the pathogenesis of neurological and neurodegenerative diseases such as epilepsy (D-Ser)<sup>199,200</sup>, schizophrenia (D-Ser, D-Asp)<sup>1,23,201</sup>, depression (D-Ser)<sup>10</sup>, Alzheimer's disease (D-Ser)<sup>25,202</sup>, amyotrophic lateral sclerosis (D-Ser)<sup>12,13</sup>. Even though D-Ser and D-Asp have been extensively studied, additional work is required in elucidating their participation in the numerous signaling pathways.

A few other D-AAs, such as D-Glu, D-Gln, D-Asn, D-Ala, D-Pro and D-Leu have also been detected in mammalian tissues or biological fluids<sup>61,203,204</sup>, but their role still remains to be determined. In a recent study, Weatherly et al.<sup>38</sup> reported no detectable amount of D-Glu in brain or blood. At the same time the predominant D-amino acid was D-Gln, suggesting that D-Gln is a product of D-Glu metabolism. Unlike the L-glutamate – L-glutamine cycle, the D-Glu removal pathway seems to be unidirectional.

The analysis of D-amino acids is usually carried out by liquid chromatography (LC), either using chiral stationary phases or employing chiral derivatization of the analytes<sup>64</sup>. Even if the robustness and applicability of LC methods are widely accepted, capillary electrophoresis proved to be another valuable technique due to its cost effectiveness and its high separation efficiency, showing high application potential for chiral analysis and determination of D-amino acids<sup>182,205-209</sup>.

Most of the amino acids are difficult to be detected due to their low molecular mass and the lack of chromophore or fluorophore moieties. To enhance their detectability, derivatization is customary, which usually also helps improving their separation selectivity. Among the chiral derivatization reagents, (+) or (-)-FLEC turns out to be one of the most versatile<sup>210</sup>. It reacts in a fast and quantitative manner with primary and secondary amines, making it an excellent choice for amino acid derivatization. The micellar electrokinetic chromatography (MEKC) separation of FLEC-DL-AAs has been previously described in several studies, using UV, fluorescence or mass spectrometric (MS) detection<sup>78,80,127</sup>. However, the resolution of FLEC derivatives of DL-Asp and DL-Glu was not achievable using MEKC. Their separation was described only once, using CZE, by Chan et al.<sup>128</sup>.

The objective of the present research was to develop a CE-MS method for the targeted quantitation of biologically relevant D- and L-amino acids. Also, this study comes as a complement to the in-capillary labeling method previously developed in our laboratory<sup>80</sup>, which lacked the ability of separating the enantiomers of the two acidic amino acids, Asp and Glu. Therefore, in this paper we investigated the CE enantioseparation of five FLEC-DL-AAs (Ser, Asn, Asp, Gln and Glu) and developed a sample preparation technique suitable for their analysis in cerebrospinal fluid. Moreover, insights of the separation mechanism are also presented.

## 4.2 Materials and method

### 4.2.1 Chemicals and reagents

All the chemicals used in this research were of analytical grade purity. Amino acid standards of DL- and L-Asp, DL- and L-Glu, DL- and L-asparagine, fluorenylmethyloxycarbonyl-D-asparagine (FMOC-D-Asn), BSA and D-glucose were purchased from Sigma-Aldrich (Steinheim, Germany). DL- and L-Gln was provided by Aldrich (Milwaukee, WI, USA), DL-Ser was from Merck (Darmstadt, Germany), while L-Ser was from Alfa-Aesar (Karlsruhe, Germany). Ammonia solution 25% (m/m) was purchased from Merck (Hohenbrunn, Germany), acetic acid, formic acid and ethyl acetate from VWR Chemicals (Fontenay-sous-Bois, France), sodium tetraborate from Acros Organics (Geel, Belgium). Water, MeOH and ACN used in this work were of LC-MS grade, provided by Biosolve (Valkenswaard, the Netherlands).

The derivatization agent, (+)-FLEC 18 mM solution in acetone, was purchased from Sigma-Aldrich (Steinheim, Germany).

The sorbents used for the solid phase extraction sample pretreatment were: Oasis HLB and MCX (both 1 cm<sup>3</sup>, 30 mg) (Waters Corporation, Milford, MA, USA), Bond Elut-C18 (1 cc, 100 mg) and SampliQ SCX (1 cm<sup>3</sup>, 30 mg) (Agilent Technologies, Waldbronn, Germany).

#### 4.2.2 Instrumentation

The CE instrument used for this study was an Agilent 3D-CE system (G1600AX) (Agilent Technologies, Waldbronn, Germany). The hyphenation with the MS detector (Agilent XCT Ion-Trap) was achieved using the ESI sheath-flow interface provided by the same manufacturer. Data acquisition was handled by means of MSD Trap Control 5.3 Software, while the MS data was analyzed using DataAnalysis (both software packages provided by Agilent Technologies). The CE-UV signal acquisition and data analysis were performed using Agilent Chemstation (Rev. B.03.01 and B.04.01). The sheath liquid (SL) was delivered using a syringe pump manufactured by KD Scientific (Holliston, MA, USA).

#### 4.2.3 Electrophoretic separation

All CE-MS experiments and the CE-UV preliminary studies were carried out using bare fused-silica capillaries (50 µm I.D., 375 µm O.D.; total length of 80 cm), provided by Polymicro Technologies (Phoenix, AZ, USA).

New capillaries were conditioned by flushing 15 min with 1 M NH<sub>4</sub>OH, 15 min with water and 15 min with the running buffer. At the beginning of each day the capillary was conditioned by flushing 10 min with BGE. A 5 min flushing with BGE was performed between runs.

For the analysis in optimized conditions, the BGE consisted of 150 mM acetic acid, adjusted to pH 3.7 with NH<sub>4</sub>OH. A separation voltage of 30 kV was applied (ramped in one minute) with the capillary thermostated at 45°C. Also, a 17 mbar negative pressure was applied on the inlet vial to overcome the suction effect of the nebulizing gas present at the capillary outlet. The samples were injected hydrodynamically for 20 s using 50 mbar.

For the determination of the effective electrophoretic mobility ( $\mu_{\text{EFF}}$ ) of Asp and Glu derivatives, the experiments were performed on a polyvinyl alcohol (PVA) coated capillary (50 µm I.D., 375 µm O.D., 56 cm effective length, 64.5 cm total length; Agilent Technologies, Waldbronn, Germany) using UV detection at 230 nm. The BGE was formed of 50 mM acetic acid adjusted to the desired pH with NH<sub>4</sub>OH and the separation temperature was settled at 20 °C. On the pH interval between 3 and 3.6, the separation was performed at the short end of the capillary, therefore 30 kV (ramped in 0.5 min) were applied. For the rest of the pH interval (i.e. 3.8-7.5), the separation was carried out using the long end of the capillary by applying -30 kV (ramped in 0.5 min). Between runs, the capillary was flushed 5 min with BGE.

The limits of detection were estimated based on the standard deviations of the responses and the linear regression slopes. According to the ICH guidelines <sup>187</sup>, the limit

of detection (LOD) and the limit of quantification (LOQ) can be calculated using equations (1) and (2), respectively.

$$\text{LOD} = 3.3\sigma/S \quad (1)$$

$$\text{LOQ} = 10\sigma/S \quad (2)$$

Where  $\sigma$  = the standard deviation of the response; S = the slope of the calibration curve.

#### 4.2.4 MS parameters

The SL was composed of MeOH:H<sub>2</sub>O:NH<sub>4</sub>OH (50:50:0.1) (v/v/v) and was delivered at a flow rate of 3 μL/min. The capillary tip was positioned 0.1 mm outside the probe.

The MS detection was performed in negative mode using the following parameters: capillary voltage: 4000 V, dry temperature: 300 °C, nebulizer: 41.36 kPa (6 psi), dry gas: 4 L/min, octopole radio frequency amplitude: 115.6 Vpp, capillary exit: -91.0 V, skimmer: -28.4 V, Oct 1 DC: -6.8 V, Oct 2 DC: -1.45 V, max accumulation time: 50 ms, ion charge control (ICC) target: 200 ms, scan interval: 300-400 m/z.

The FLEC derivatives were detected as singly charged anions, using the following m/z values: Ser – 340.0; Asn – 367.0; Asp – 368.0; Gln – 381.0; Glu – 382.0. No smoothing of the electropherograms was performed. Each data point was obtained as an average of three measurements, unless otherwise stated.

#### 4.2.5 Derivatization and SPE

Amino acid stock solutions were prepared by dissolving the appropriate quantity of each amino acid in water. The usual derivatization procedure that was used during the first stages of method optimization consisted of diluting the amino acid stock solutions with 5 mM sodium tetraborate buffer pH 9.2 (NaTB) to the desired concentration, then 60 μL were mixed with 40 μL of ACN and 20 μL of FLEC solution (18 mM in acetone). The sample was homogenized on a vortex mixer for one hour prior to analysis.

The artificial cerebrospinal fluid's ionic composition was achieved by implementing a protocol developed by Durect Corporation (Cupertino, USA)<sup>186</sup>. Additionally, 0.4 mg/mL of BSA and 0.65 mg/mL of glucose were added in order to closely mimic the CSF composition. Spiked aCSF samples were prepared by adding the desired concentration of the five amino acids in aCSF.

The derivatization procedure used for the analysis of spiked aCSF samples consisted in mixing 100 μL of aCSF sample with 10 μL of 30 mM NaTB pH 9.2 (for pH adjustment), followed by the addition of 90 μL FLEC solution (6 mM in acetone:ACN - 1:2, v/v). The samples were homogenized on a vortex mixer for one hour. After derivatization, the samples were diluted to 0.5 mL with water and SPE was performed.

The FLEC derivatives were extracted from the sample using a hydrophilic-lipophilic balance (HLB) SPE sorbent. Before sample loading, the cartridge was conditioned with 1 mL of MeOH and 1 mL of water. After the loading of the sample, the

cartridge was sequentially washed using 1 mL of aqueous MeOH (15%, v/v) and 1 mL of water. The FLEC derivatives were eluted by 2x0.25 mL of MeOH (containing 0.1% NH<sub>4</sub>OH), the eluent was evaporated and the sample was reconstituted in 40 µL of ACN:water (1:1, v/v).

The evaluation of the matrix effect and the extraction efficiency was done according to a method described by Matuszewski et al.<sup>211</sup> and later improved by Marchi et al.<sup>212</sup>, involving the comparison of peak areas of 4 samples (named A, B, C, D) (see Supplementary Information S4). For the standards A to D, a stock solution of 400 µM FLEC-DL-amino acids was prepared. The neat standards (A) were prepared by spiking the analytes in ACN:water (1:1, v/v). The B samples represent the post-extraction matrix standards and they were prepared by reconstituting the extracted matrix with the neat standard A. The pre-spiked matrix standards (C) were prepared by extracting aCSF samples spiked with the appropriate concentration of analytes, while the neat extraction standards (D) were prepared similarly to the C standards, with the aCSF being replaced by water. Both C and D standards were reconstituted in ACN:water (1:1, v/v).

In this particular case, considering that the derivatization process taking place in the matrix may affect the final yield, a fifth standard was analyzed. It involved the spiking of the matrix (aCSF) with underderivatized AAs followed by their derivatization. The sample was then extracted and reconstituted in ACN:water (1:1, v/v).

All 5 standards were prepared in triplicate at a single concentration level (40 µM for each DL-amino acid). The internal standard (I.S.) (FMOC-D-Asn) was added at the reconstitution step.

Upon the normalization of the DL-peak area with the I.S. peak area, several parameters were calculated: matrix effect (ME), process efficiency (PE), extraction recovery (ER), extraction yield (EY) and derivatization efficiency (DE) as follows:

$$\text{ME: } \frac{\text{B}}{\text{A}} \times 100 \quad (3)$$

$$\text{PE: } \frac{\text{C}}{\text{A}} \times 100 \quad (4)$$

$$\text{ER: } \frac{\text{C}}{\text{B}} \times 100 \quad (5)$$

$$\text{EY: } \frac{\text{D}}{\text{A}} \times 100 \quad (6)$$

$$\text{DE: } \frac{\text{E}}{\text{C}} \times 100 \quad (7)$$

## 4.3 Results and discussion

### 4.3.1 Preliminary investigation by CE-UV

In a previous work<sup>80</sup> we demonstrated the effectiveness of FLEC derivatization for the chiral separation of AAs forming diastereomeric pairs that could be resolved in non-chiral BGE. Using the optimized experimental conditions (use of ammonium perfluorooctanoate as surfactant in the BGE), all AA isomers were separated except Asp, Glu and Trp. Considering the important role of D-Asp in the brain, further investigations were undertaken using an acidic BGE by CE-UV. In contrast with Chan et al.<sup>128</sup> we were not able to separate the FLEC derivatives of DL-Asp and DL-Glu using a citrate buffer (pH 4.4). However, promising results were obtained using 60 mM acetic acid adjusted to pH 4.0 with NH<sub>4</sub>OH (data not shown).

### 4.3.2 Method transfer to MS and BGE optimization

The MS parameters were first optimized by infusing derivatized samples (10 µg/mL). Considering that the analytes were already negatively charged in the BGE, the negative electrospray ionization mode was chosen. Two organic solvents (MeOH and ACN) and two basic modifiers (NH<sub>4</sub>OH and NH<sub>4</sub>F) were tested for the optimization of the SL composition. The best results in terms of sensitivity were obtained using a SL made up of MeOH:H<sub>2</sub>O:NH<sub>4</sub>OH at 50:50:0.1 (v:v:v). The method transfer from CE-UV to CE-MS was straight forward, but shorter migration times and lower resolution values were observed compared to CE-UV. This phenomenon is usually caused by the suction effect created by the nebulizing gas at the capillary outlet. This issue was addressed successfully by applying a negative pressure (-17 mbar) at the inlet vial during the run (Annex 4).

In order to achieve the separation of the diastereomers of the 5 AAs in a single run, an investigation of the influence of the BGE pH on resolution was carried out using a BGE made of 60 mM acetic acid adjusted to pH with NH<sub>4</sub>OH. The chiral resolution was measured for the 5 pairs of FLEC-derivatives within the 3.0-7.0 pH interval (Fig. 15).

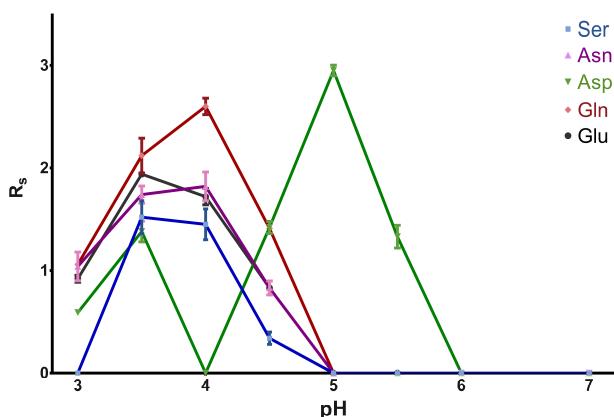


Fig. 15: Influence of BGE pH on resolution of FLEC-DL-AAs, BGE: 60 mM acetic acid (pH adjusted with NH<sub>4</sub>OH), 20°C, 30 kV.

It could be observed that all analytes, except Asp, behave similarly showing a maximal resolution between pH 3.5 and 4.0 and a loss of resolution from pH 5.0. In contrast, a complete loss of resolution was obtained for Asp diastereomers at pH 4.0, followed by an improvement of resolution up to pH 5.0. This particular behavior is expected when a reversal of the migration order (RMO) of the two diastereomers occurs. This aspect was further explored by analyzing a mixture of FLEC-D-AAs and FLEC-L-AAs in 1:3 ratio measuring their effective electrophoretic mobilities ( $\mu_{\text{EFF}}$ ) in the 3.0–7.0 pH range (Annex 5). For the three monocarboxylic amino acids (Ser, Asn, Gln) the  $\mu_{\text{EFF}}$  reached a plateau around pH 5, indicating that the carboxyl moiety was fully ionized. Compared to the non-derivatized amino acids that have pK<sub>a</sub> values around 2 for their carboxylic group<sup>213</sup>, the acidity of this group on the FLEC diastereomers seems to be reduced, its ionization taking place at higher pH values. Such changes may be explained by rearrangements of the local electron density induced by the presence of an electrophilic moiety (carbamate) in  $\alpha$ -position. On the other hand, the  $\mu_{\text{EFF}}$  of the dicarboxylic amino acids (Asp, Glu) further increased with pH as the second carboxyl moiety became ionized, reaching a plateau at pH > 6.

In order to better understand the mechanism of RMO for the Asp diastereomers (Figs. 16 and 17), the  $\mu_{\text{EFF}}$  of these derivatives was measured on a PVA-coated capillary (Fig. 16). The two inflection points corresponding to the two pK<sub>a</sub> values could be clearly observed around 3.6 and 5.1, which is in good agreement with the pK<sub>a</sub> values of FMOC-Glu determined earlier<sup>214</sup>.

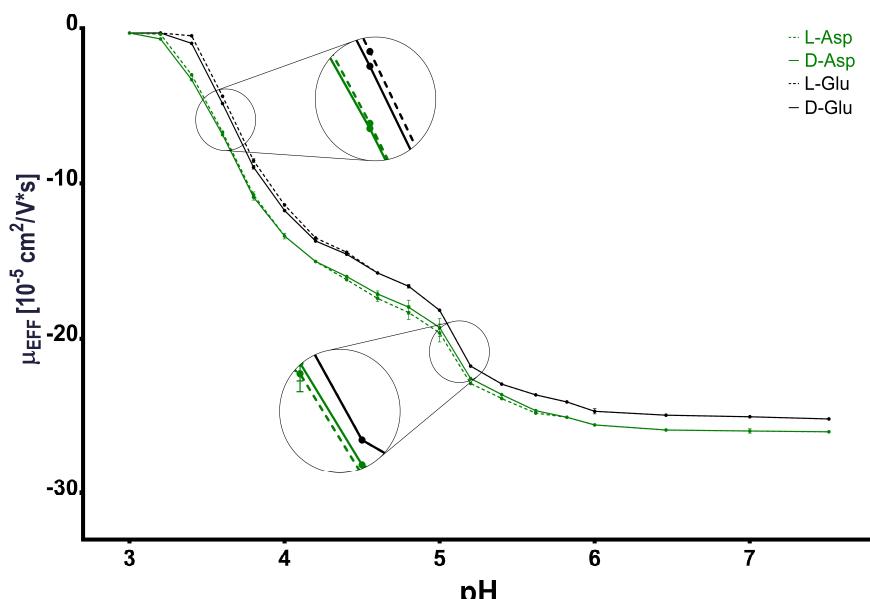
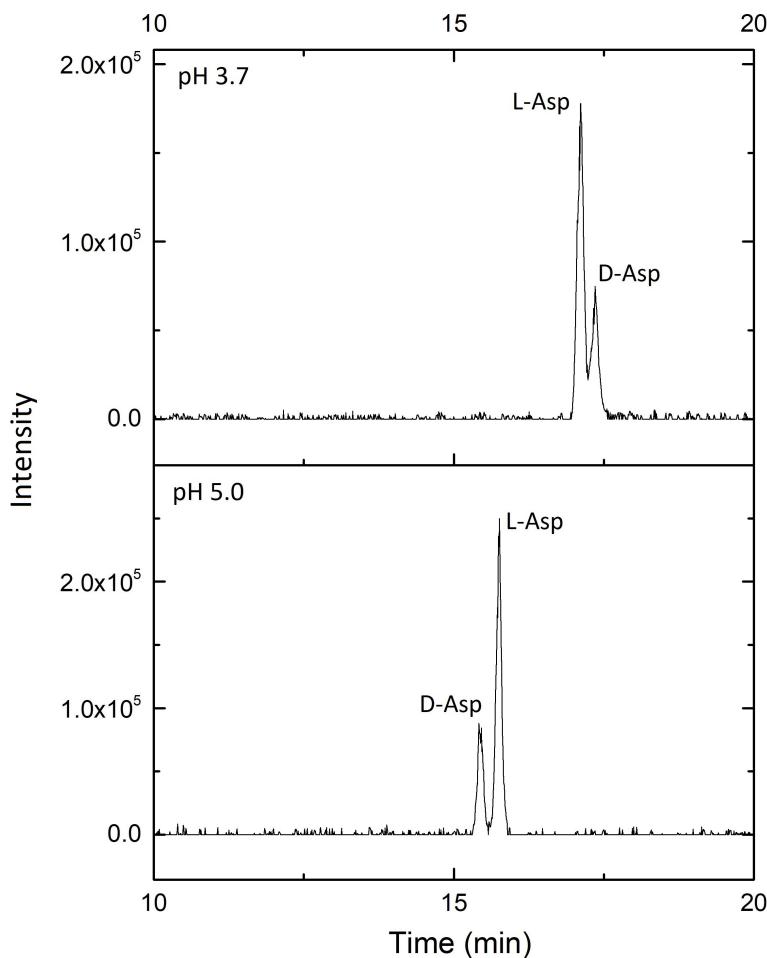


Fig. 16: Influence of BGE pH on the  $\mu_{\text{EFF}}$  of the FLEC derivatives of the two acidic amino acids (Asp and Glu) using a PVA coated capillary; BGE 50 mM acetic acid (pH adjusted with NH<sub>4</sub>OH), 20°C, -30 kV. For the first two pH values (i.e. 3 and 3.2), the migration time was above 90 minutes, therefore the corresponding  $\mu_{\text{EFF}}$  data points plotted above were calculated using the 90 minutes threshold.

The RMO can be explained by a slight difference in the pK<sub>a</sub> of the second ionizable moiety (the distal carboxyl) for the two Asp diastereomers. This pK<sub>a</sub> difference may result from the proximity of the second carboxyl group to the carbamate moiety and/or from other intra-molecular interactions. Even though a RMO was also expected for Glu diastereomers, it was not observed under those experimental conditions (Fig. 16). This is probably due to the longer side chain (additional methylene group) of Glu compared to Asp, which may decrease the impact of the derivatization with FLEC on the pK<sub>a</sub> value of the distal carboxylic group.



**Fig. 17: Reversal of the migration order of FLEC-Asp derivatives.** Sample: 10  $\mu\text{M}$  (+)-FLEC-D-Asp and 30  $\mu\text{M}$  (+)-FLEC-L-Asp; BGE: 60 mM acetic acid (adjusted to pH 3.7 or 5.0 with NH<sub>4</sub>OH), 45°C, 30 kV.

As can be observed from Fig. 15, the only pH range in which chiral resolution is achievable for all amino acids is around pH 3.5. Further BGE optimization led to a pH value of 3.7, which offers a satisfactory chiral separation of all analytes in a single run. Higher temperatures led to shorter analysis time while maintaining the resolution, 45°C being selected as suitable value. Further increase in resolution was observed when the BGE concentration was increased from 60 to 150 mM. Even though higher BGE concentrations may lead to ionization suppression, such an effect was not observed. Under these conditions, the maximal resolution for Asp derivatives was 1.4. For all the other AA diastereomers, baseline chiral resolution values were obtained (Table 4).

Nevertheless, if baseline resolution is needed for the Asp diastereomers, a second analysis can be performed using a BGE at pH 5.0.

Using (+)-FLEC derivatization and a pH 3.7 BGE, the L-amino acid derivatives of all 5 analytes migrated first. The migration order reversal is readily achievable by derivatizing the analytes with (-)-FLEC<sup>210</sup>. As described above, the RMO for D- and L-Asp derivatives can be also achieved by increasing the BGE pH. The need for a RMO can be relevant in certain situations (i.e. quantification of small amounts of analyte in the presence of a high amount of another), including targeted metabolomics.

**Table 4: Performance parameters of the analytical method and the extraction procedure**

AA	R <sub>s</sub>	Linearity (R <sup>2</sup> ) <sup>a</sup>	LOD (μM)	LOQ (μM)	ME (%) <sup>b</sup>	PE (%) <sup>c</sup>	ER (%) <sup>d</sup>	EY (%) <sup>e</sup>	DE (%) <sup>f</sup>	Peak area RSD <sup>g</sup>
<b>Ser</b>	2.0	0.9993	0.19	0.60	97.5	86.1	88.3	91.6	91.4	7.8
<b>Asn</b>	2.2	0.9990	0.18	0.56	96.2	86.2	89.6	89.2	88.9	5.2
<b>Asp</b>	1.4	0.9957	0.23	0.72	96.5	45.0	46.6	3.0	81.5	11.6
<b>Gln</b>	2.8	0.9976	0.21	0.62	89.5	81.9	91.5	86.9	94.0	9.1
<b>Glu</b>	2.1	0.9954	0.36	1.11	91.6	56.6	61.8	7.9	93.7	9.6

<sup>a</sup> Concentration range 1 to 100 μM per enantiomer; <sup>b</sup> Matrix effect; <sup>c</sup> Process efficiency; <sup>d</sup> Extraction recovery; <sup>e</sup> Extraction yield; <sup>f</sup> Derivatization efficiency; <sup>g</sup> Peak area RSD (n=6).

### 4.3.3 Method suitability for biological samples

As the method was intended to be used for the analysis of CSF samples, its suitability was tested using aCSF. The electrolyte composition of aCSF was adjusted<sup>186</sup> to resemble endogenous CSF. Besides its ionic content, CSF also contains significant amounts of proteins and glucose. For obtaining an aCSF composition as close as possible to that of real CSF, BSA and glucose were added in order to reach the normal values for proteins (15-45 mg/dL) and glucose (65 mg/dL)<sup>215</sup>.

#### 4.3.3.1 Initial tests

The first tests were performed by injecting pre-capillary derivatized aCSF samples spiked with amino acids, without any other sample pretreatment, as this approach proved to be efficient in other cases<sup>80,216</sup>. The analysis of these samples revealed that the sample composition had a big impact on peak shape. Even though the derivatization seemed to take place successfully (the target masses could be detected), the peak shapes were very bad, chiral resolution being lost for all analytes (Annex 6). The same phenomenon was observed when analyzing aCSF samples free of proteins and glucose, therefore we could conclude that the probable cause was the high salt content.

#### **4.3.3.2 Extraction optimization**

For improving peak shape and, thus, achieving satisfactory chiral separation, a procedure for the extraction of amino acids from the matrix had to be implemented.

Two approaches were considered: (1) a desalting procedure applied directly on the aCSF sample and (2) an extraction procedure for the aCSF sample after derivatization.

The desalting procedure, in spite of the challenging nature of selectively extracting small polar molecules (i.e. amino acids) from a salt rich matrix, was investigated using strong cation exchange and mixed mode sorbents. Even though the analytes were retained on both stationary phases and the excess of inorganic cations could be washed out, this procedure did not lead to peak shape improvement probably because of the remaining anions (data not shown).

In the second approach, two SPE sorbents were considered: reversed phase (C18) and hydrophilic-lipophilic balance (HLB). A generic protocol was used for both stationary phases, which consisted of conditioning with pure MeOH and 5% (v/v) MeOH in water, sample loading, wash with 5% (v/v) MeOH in water and elution with 95% (v/v) MeOH in water. The eluent was evaporated and then the sample reconstituted in 40 µL of ACN:water (1:1, v/v). It is worth mentioning that the derivatized sample was diluted to 500 µL with water in order to decrease its organic solvent content (initially 45%, v/v). Under these conditions, the analytes were successfully retained only on the HLB sorbent. On the C18 sorbent, the derivatives of the acidic amino acids were not retained at all.

For the optimized elution of the analytes from the HLB stationary phase several solvents were evaluated (Fig. 18a). At first MeOH, ACN and ethyl acetate were compared, with MeOH offering higher analyte recovery. Considering that the ionization state of the analytes usually plays a role in the retention mechanism on the stationary phases, ammonia was tested as a pH modifier in combination with MeOH and ACN. Once again, the MeOH based solvent gave the best results.

In the optimized extraction procedure, the SPE cartridges were conditioned with 1 mL of MeOH and 1 mL of water, followed by sample loading. The washing step was carried out with 1 mL of 15% (v/v) MeOH in water, followed by 1 mL of water. The analytes were eluted using two times 250 µL of MeOH with 0.1% (v/v) ammonia. The eluent was evaporated using a concentrator at 60 °C. The sample was reconstituted in 40 µL of ACN:water (1:1, v/v) and then analyzed.

#### **4.3.3.3 Extraction efficiency and matrix effect evaluation**

For a complete characterization of the extraction process, an evaluation method<sup>211,212</sup> was applied. With this strategy it is possible to determine whether the matrix constituents interfere with the sample preparation or analysis by comparing five different samples (see Section 4.2.5 and Annex 7). From this data several parameters can be calculated (cf. equations 3-7), such as the matrix effect (ME), process efficiency

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(PE), extraction recovery (ER), extraction yield (EY) and derivatization efficiency (DE). The results are summarized in Table 4 and Fig. 18b.

It is well known that the remaining matrix components after SPE may have an effect on analyte ionization, either enhancing it or suppressing it. According to this evaluation method, a ME value of 100 % represents the absence of the matrix effect<sup>211</sup>. In this case the obtained ME values (89.51-97.51 %) reflect marginal ionization suppression.

The process efficiency (PE) is a way to characterize the whole extraction process, while the extraction recovery (ER) represents evaluating the “true” recovery, free from any matrix effect. The values that were obtained for both PE and ER represent a 2.2- to 4.4-fold increase in peak area. This was expected and it is due to the preconcentration step taking place during the SPE process (200 µL initial sample to 40 µL reconstituted sample). Therefore, compared to the expected 5-fold increase in MS response, the PE and ER are between 45 % and 91 %.

Marchi et al.<sup>212</sup> considered that the extraction recovery may be influenced either by interfering compounds or by the sample preparation itself. In order to differentiate these two factors, a fourth parameter was introduced, extraction yield (EY). In this case, the peak areas of a neat extraction standard (D) are compared with those of the neat standard (A). An unexpected result was obtained in the case of the FLEC derivatives of the two acidic amino acids (Asp and Glu), their extraction yield being very low (3 % and 7.9 %). The retention of these analytes on the HLB sorbent may be influenced by their increased hydrophobicity caused by intra-molecular interactions (H-bonds).

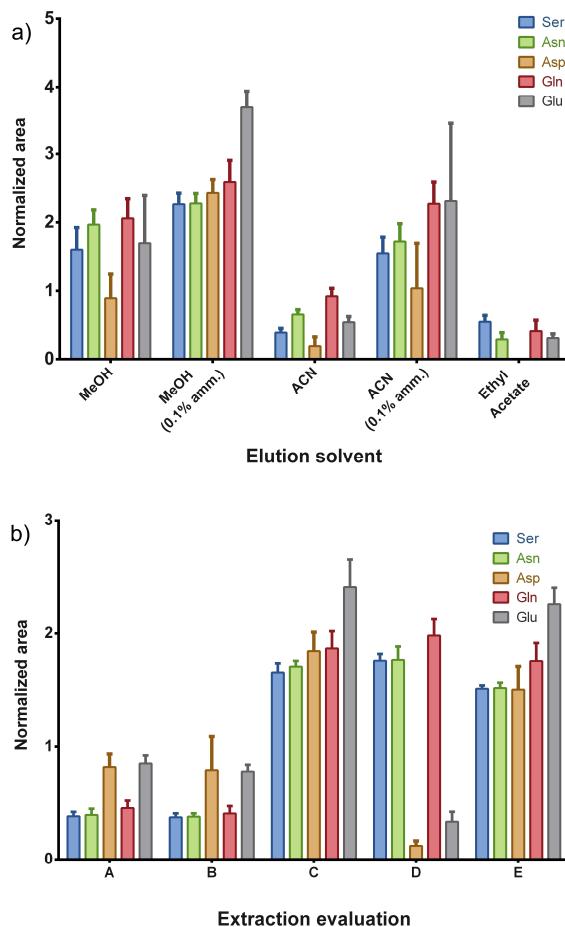
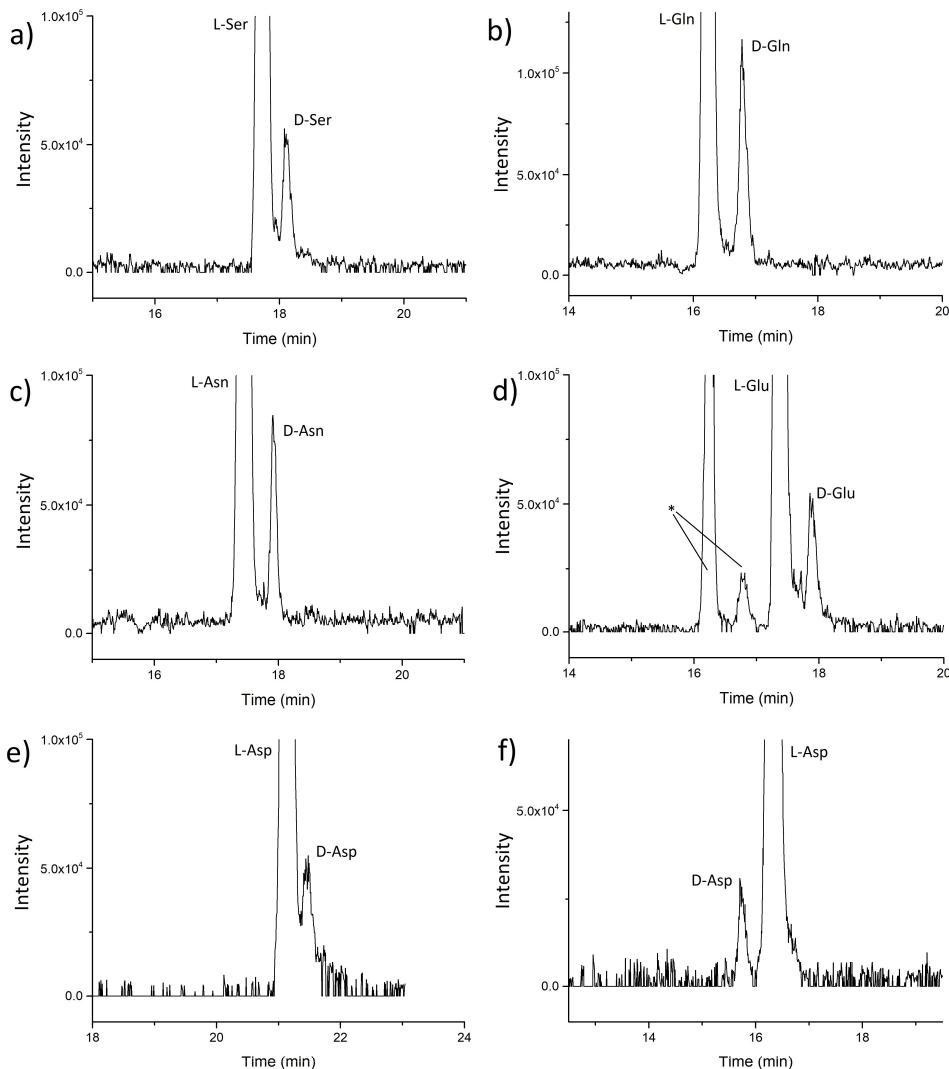


Fig.18: SPE extraction of FLEC-AAs from aCSF

a) Influence of elution solvents. (n=3)

b) Evaluation of matrix effect and extraction efficiency (A - water spiked with standards; B - matrix spiked after SPE; C - matrix spiked before SPE; D - water spiked before SPE; E - derivatization in matrix, before SPE). (n=3)

Considering that for real samples the derivatization will take place in the matrix (i.e. CSF) an extra sample (E) was analyzed for the complete characterization of the SPE procedure. When compared to sample C, the calculated derivatization efficiency (DE) was over 80 %. Analyses performed in the developed separation conditions and using the final SPE procedure shows that the desired selectivity was achieved (Fig. 19).



**Fig. 19: Determination of 1  $\mu$ M of D-AAs in the presence of an excess of 50  $\mu$ M of L-AAs in aCSF. a)-e): BGE: 150 mM acetic acid (adjusted to pH 3.7 with NH<sub>4</sub>OH), 45°C, 30 kV; f) BGE: 60 mM acetic acid (adjusted to pH 5.0 with NH<sub>4</sub>OH), 45°C, 30 kV  
\* <sup>13</sup>C isotopes of Gln (in Glu electropherogram)**

The method response function was evaluated in the range of 1 to 100  $\mu$ M (per enantiomer, after SPE of aCSF samples) and was found to be linear. The LODs and LOQs were estimated using equations (1) and (2), the results being summarized in Table 4. The developed method offers adequate sensitivity for the analysis of the targeted

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analytes in aCSF<sup>217</sup>. Compared to other CE-MS methods<sup>195,196</sup> the obtained sensitivity is similar or better for some of the analytes.

#### 4.4 Conclusion

A CE-MS method was developed for the analysis of five biologically relevant DL-amino acids after derivatization with (+)-FLEC. The separation of DL-Ser, DL-Asn, DL-Asp, DL-Gln and DL-Glu was achieved in around 22 minutes. The resolution of AA diastereomers was found to be highly dependent on the BGE pH and a reversal of the migration order was observed for Asp derivatives. The optimized pH for achieving the separation of all amino acids in one run was 3.7.

An SPE extraction procedure was successfully developed and fully characterized, demonstrating an efficiency above 80 % for the whole process (derivatization and extraction).

The analytical method shows good linearity and the limits of detection and quantification are in the sub- $\mu\text{M}$  region, suitable for metabolomics studies.



## Chapter 5:

# Selectivity evaluation of phenyl based stationary phases for the analysis of FLEC-DL-amino acids by UHPLC-MS

### 5.1 Introduction

The chiral analysis of D- and L-AAs is becoming increasingly relevant as the metabolic roles of D-AAs are clarified. For example, nowadays it is well documented that several D-AAs are involved in neurotransmission, among which D-Ser's role is well characterized as a coagonist of the NMDA receptors<sup>1</sup>. Due to their localization, these excitatory receptors play significant roles in many physiological processes such as memory formation, nociception, synaptic development and plasticity. Considering their metabolic roles, the misregulation of D-AA levels has been linked to several psychiatric and neurologic diseases, such as schizophrenia<sup>5-9</sup>, amyotrophic lateral disease<sup>12-14</sup> and depression<sup>10</sup>.

The efforts in developing new analytical tools for the analysis of D- and L-AAs in biological samples (brain tissue, plasma, CSF, urine etc.) has been focused mainly on using chromatographic and electrophoretic techniques. Considering the low concentrations of D-AAs expected in those samples (nM to  $\mu$ M in CSF<sup>64</sup>,  $\mu$ M in serum<sup>10</sup>, low nmol/g in brain tissue<sup>45</sup>), sensitive detection methods are desirable (FL, MS). Moreover, for achieving the desired selectivity and sensitivity, derivatization is often employed due to the small molecular mass of the amino acids and the lack of chromophores or fluorophores in their structures.

The chromatographic approaches developed for the chiral analysis of amino acids have been diverse, ranging from complex 3D-LC<sup>60</sup> employing both chiral and achiral stationary phases to more simple approaches using a single C18 stationary phase<sup>64</sup>. The chiral separation of amino acids by CE has been usually carried by CZE or MEKC and further addition a chiral selector in the background electrolyte<sup>218-220</sup>. Lately, advances in CE-MS analysis have been reached by automatizing the whole analytical process<sup>221</sup> by developing an in-capillary derivatization procedure with a chiral derivatizing agent.

Over the last 30 years (-) and (+)-1-(9-fluorenyl)ethyl chloroformate has proved to be one of the most versatile chiral derivatization agent for the analysis of D- and L-

AAs. Excellent chiral selectivity can be achieved using both LC and CE techniques, the derivatization reaction is fast and the resulting diastereomers are stable.

A recent review<sup>210</sup>, covering most of the publications on this topic since the introduction of FLEC in 1987, has revealed that all separations of FLEC-DL-AAs by liquid chromatography use similar conditions: a reversed phase C4, C8 or C18 stationary phase and a mobile phase containing THF in various proportions. The use of THF is claimed to be critical for achieving chiral resolution, and indeed, a good selectivity was reported for all analytes<sup>78</sup>. Nevertheless, most of the major manufacturers of chromatographic instruments recommend the use of THF only under special circumstances, considering that THF is known for degrading PEEK<sup>222</sup>. Therefore, either the proportion of THF in the mobile phase should be kept at a minimum to avoid the degradation of the PEEK tubing, either the use of stainless steel tubing is recommended. The use of THF in LC/MS should also be limited due to several other issues: it is an aprotic highly flammable solvent, so that it should be paired with an aqueous (protic) mobile phase; and last but not least, THF tends to polymerize in APCI sources, therefore the ionization needle should be cleaned more often.

The aim of this study was to find alternative strategies for the LC separation of FLEC-DL-AAs. The focus was set on the evaluation of the selectivity of two phenyl stationary phases using common solvents as mobile phase components.

## 5.2 Materials and method

### 5.2.1 Chemicals and reagents

All the chemicals used in this research were of analytical grade purity. Amino acid standards of L-Ala, L-Arg, L-Asn, DL- and L-Asp, DL-Cys, DL- and L-Glu, Gly, DL- and L-His, L-Ile, DL- and L-Leu, L-Lys, DL- and L-Met, L-Phe, L-Pro, D- and L-Thr, DL- and L-Trp DL- and L-Tyr and DL-Val were purchased from Sigma-Aldrich (Steinheim, Germany). DL-Ala, DL-Arg, DL-Asn, DL- and L-Gln, DL-Ile, DL-Phe, DL-Pro were provided by Aldrich (Milwaukee, WI, USA), DL-Ser was from Merck (Darmstadt, Germany), while DL-Lys, L-Ser and L-Val from Alfa-Aesar (Karlsruhe, Germany). L-Cys and sodium tetraborate were bought from Acros Organics (Geel, Belgium). 25% ammonia solution was purchased from Merck (Hohenbrunn, Germany) and formic acid from VWR Chemicals (Fontenay-sous-Bois, France). Water, methanol (MeOH) and acetonitrile (ACN) used in this work were of ULC-MS grade, provided by Biosolve (Valkenswaard, the Netherlands).

The derivatization agent, (+)-1-(9-fluorenyl)ethyl chloroformate, 18 mM solution in acetone, was purchased from Sigma-Aldrich (Steinheim, Germany).

### 5.2.2 Instrumentation

The LC instrument used for this study was an Agilent 1290 Infinity II UHPLC system (Agilent Technologies, Waldbronn, Germany), coupled with a 6560 Ion Mobility Q-TOF MS instrument equipped with a Dual AJS ESI source, provided by the same

manufacturer. Data acquisition and treatment were performed with the Agilent MassHunter Workstation Software (B.08.00). The design of experiments and data interpretation were carried out using Modde PRO 11 (Umetrics, Sartorius Stedim, Aubagne, France), while multivariate data analysis was performed with Simca 13.0.3 (Umetrics, Sartorius Stedim, Aubagne, France).

### 5.2.3 LC method

Two stationary phases with 1.7 µm particle size have been employed for this study. The first one was a 100x2.1mm Fortis Diphenyl fully porous column provided by Fortis Technologies (Neston, UK). The second one was a 100x2.1mm Kinetex Biphenyl Coreshell column purchased from Phenomenex (Torrance, CA, USA). The flow rates employed were the ones recommended by the column manufacturers, 0.3 mL/min for the diphenyl column and 0.4 mL/min for the biphenyl one. The elution was performed using a gradient made up of an aqueous component containing 50 mM formic acid and an organic component consisting of ACN, MeOH or IPA/ACN. The injected sample volume was 2 µL and the column compartment was thermostated at 40°C.

### 5.2.4 MS parameters

The MS detection was performed in positive ion mode. The source parameters were the following: gas temperature – 300°C, gas flow – 5 L/min, nebulizer – 35 psi, sheath gas temperature - 350°C, sheath gas flow – 11 L/min, VCap – 3500 V, nozzle voltage – 1000 V, fragmentor – 400, skimmer1 – 65, octupole RF peak - 750. The instrument was used in Q-TOF mode only and the scan was performed in the 100-1700 m/z range at a scan rate of 3 spectra/sec. Each data point was obtained as an average of two measurements, unless otherwise stated.

### 5.2.5 Derivatization and sample preparation

Amino acid stock solutions were prepared by dissolving the appropriate quantity of each amino acid in water. The stock solutions were diluted with water to the desired concentrations. For derivatization, 10 µL of 50 mM sodium tetraborate (NaTB) were added to 100 µL of AA sample for achieving a basic pH. The resulting solution was mixed with 90 µL of 6 mM FLEC solution (in ACN:acetone – 2:1), and homogenized on a vortex mixer for one hour. Before injection, the sample was diluted 1:5 (v/v) with ACN/H<sub>2</sub>O (1:1, v/v).

## 5.3 Results and discussion

### 5.3.1 Preliminary studies

In order to investigate the interest of phenyl stationary phases using solvents such as ACN, MeOH and IPA as organic modifiers for the separation of FLEC-AAs diastereomers, few preliminary assays were performed. Besides the influence of pH, the potential impact on selectivity of a few other variables was also investigated, such as the

nature of the organic mobile phase component, the slope of the gradient and the initial percentage of the organic component.

### **5.3.1.1 pH of the aqueous mobile phase component**

The first attempts to monitor the effect of FLEC-AAs ionization state on resolution were performed on the Diphenyl stationary phase, using a generic gradient (25-75% in 15 min). Three pH values of the aqueous component (2.5, 4.5, 7) were tested using ACN as organic modifier. As can be seen in Table 5, a strong influence of pH on resolution was observed for most of the FLEC-DL-AAs. An overall assessment indicates that the best results were achieved at pH 4.5, providing a baseline separation for 13 pairs of FLEC-DL-AAs and a partial resolution for four other pairs.

However, for some of the amino acid derivatives a particular chromatographic behavior was observed. For example, regardless of the mobile phase pH, no resolution was obtained for Ser diastereomers. Pro derivatives could be successfully separated at low pH (2.5), the resolution being lost at higher pH values. For other amino acid derivatives such as Leu, Ile, Arg, and the doubly derivatized Lys, His and Cys, the influence of the mobile phase pH on resolution was less important.

**Table 5. The influence of the pH of the mobile phase aqueous component on resolution (separation conditions: Diphenyl stationary phase, mobile phase: A: 50 mM ammonium acetate (adjusted to pH with NH<sub>4</sub>OH); B: ACN. Gradient: 25-75% B in 15 min)**

pH	2.5	4.5	7
Ala	0	1.4	0
Ser	0	0	0
Pro	1.7	0.85	0
Val	0	2.8	1.3
Thr	0	1.15	0
Ile	1.1	5.8	2.5
Leu	1.3	3.7	2.5
Asn	0	1.5	0
Asp	0	0	0
Arg	0.5	1.5	1.1
Gln	0	1.3	0
Glu	0	1.95	0
Met	0.9	3.3	1.6
Phe	0.8	4.2	1.8
Tyr	0.9	4.6	1.8
Trp	0	2.25	1.4
Lys	1	2.7	1.9
His	1.6	5.7	3.4
Cys	2.4	5.8	3

### 5.3.1.2 Organic phase and gradient

As mentioned above, the aim of this study was to develop an alternative LC method able to provide chiral selectivity for FLEC-DL-AAs without using THF as organic modifier. Therefore, several common organic solvents (MeOH, ACN, IPA) and some mixtures (MeOH/ACN, IPA/ACN) were evaluated in order to assess their impact on resolution. Even though the lower aliphatic alcohols, such as MeOH and IPA, are known to promote  $\pi$ - $\pi$  interactions<sup>223</sup>, they were not favorable for the separation of FLEC-DL-AAs. Comparing the different solvents, the best results were obtained using ACN. However, it would be interesting to further study the gradient time as well as its slope in order to optimize the separation.

### 5.3.1.3 Selectivity of diphenyl and biphenyl columns

Usually, the stationary phases having phenyl functionalities offer unique selectivity for aromatic compounds, through  $\pi$ - $\pi$  electronic interactions. This type of interactions is expected to take place due to the aromatic moiety (fluorenyl) present in the FLEC derivative structure. Both stationary phases used in this study feature two phenyl moieties, one important difference being that the biphenyl rings are conjugated. Therefore, shape selectivity might be altered by the different degrees of freedom in the phenyl rings rotation (Fig. 20). The angle formed by the planes of the two phenyl rings in the biphenyl moiety was reported<sup>224</sup> as being around 45°.

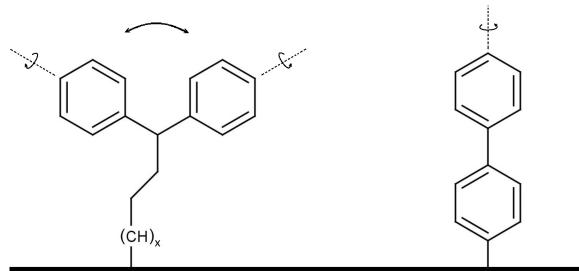
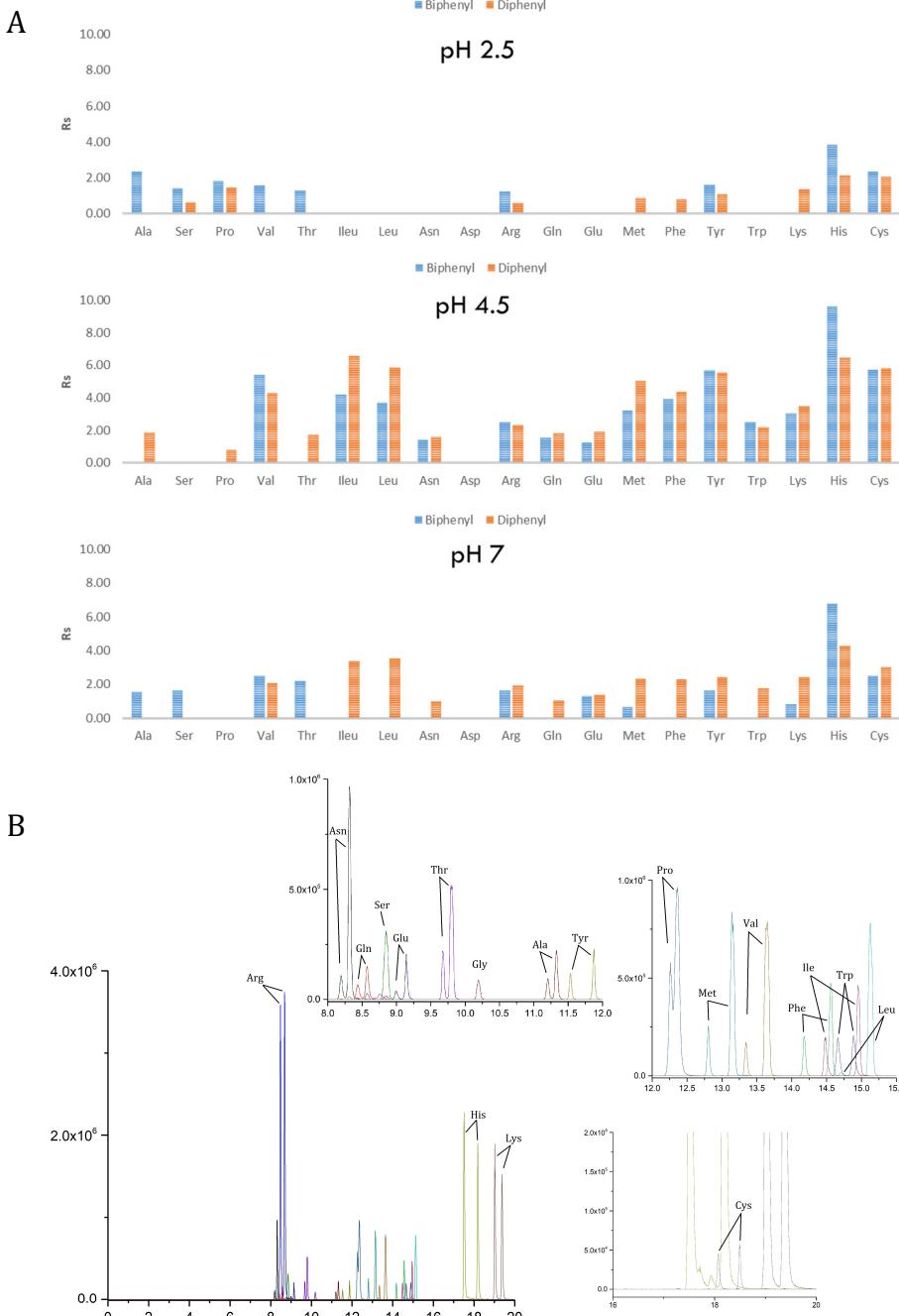


Figure 20. Diphenyl (left) vs. biphenyl (right) structures.

Considering this subtle but significant difference between the two stationary phases, several experiments were performed in order to assess whether the selectivities of the two stationary phases were different and worth further investigation. Using a gradient of 15-65% ACN in 20 min, the influence of the mobile phase pH was evaluated at 3 pH levels: 2.5, 4.5 and 7 (Fig. 21).



**Figure 21. (A) Comparison of the mobile phase pH influence on chiral resolution using Diphenyl and Biphenyl stationary phases Separation of FLEC-DL-AAs. (B) Separation conditions: Diphenyl stationary phase, mobile phase: A: 50 mM ammonium acetate (adjusted to pH 4.5 with NH<sub>4</sub>OH); B: ACN. Gradient: 15-65% B in 20 min**

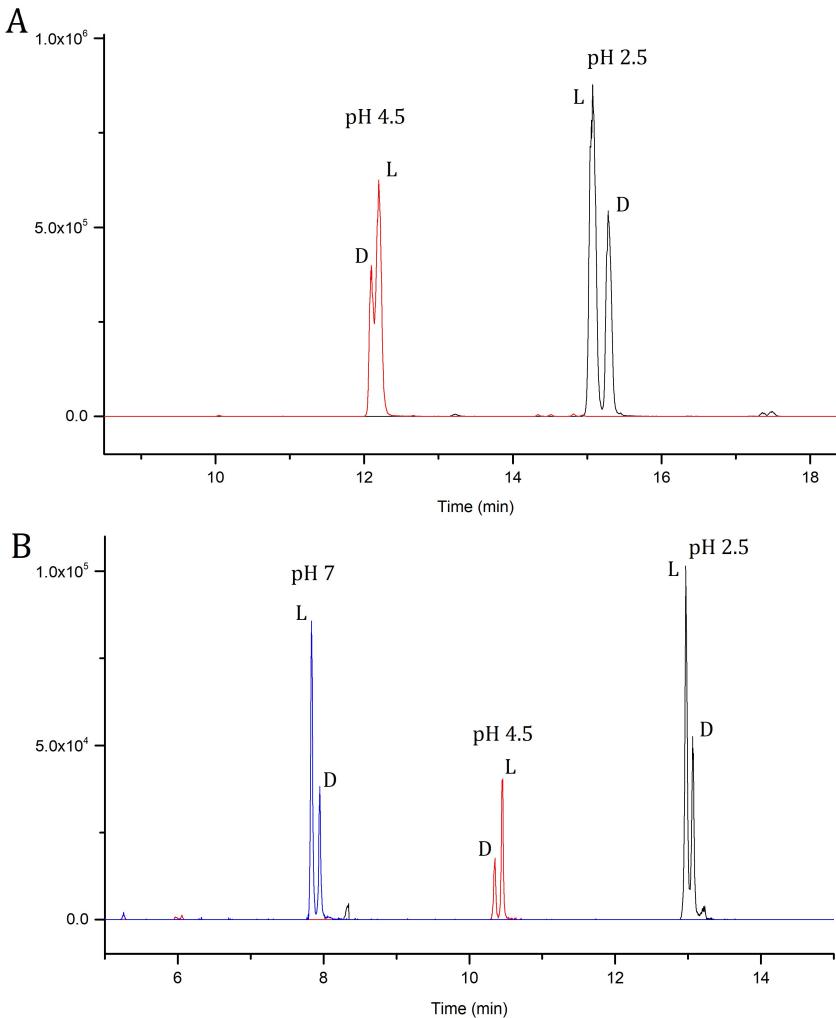
It could be easily observed that both columns provided the highest resolution values at pH 4.5, with similar selectivities. Interestingly, a certain degree of complementarity was observed for the biphenyl stationary phase, which offered significantly different diastereoselectivity at pH 2.5 compared to 4.5. Another interesting aspect is that the doubly derivatized amino acids derivatives could be separated regardless of pH or column. The elution order of the D- and L-AA derivatives was found to be the same on the two columns, with only one exception, Val at pH 4.5. In most cases the D-AA derivative eluted first (Table 6).

**Table 6. Elution order of the D- and L-AA derivatives, observed on the two stationary phases. The first eluted enantiomer is marked with "X"; red cells represent cases when there was no enantioresolution; green cells represent the two cases of reversal of elution order**

pH	Biphenyl						Diphenyl					
	2.5		4.5		7		2.5		4.5		7	
	D	L	D	L	D	L	D	L	D	L	D	L
Ala		x				x				x		
Ser		x				x				x		
Pro	x							x		x		
Val		x	x				x					x
Thr	x					x				x		
Ile			x							x		x
Leu		x						x		x		x
Asn		x						x		x		
Asp					x							
Arg	x		x		x				x		x	
Gln			x						x		x	
Glu			x		x				x		x	
Met			x					x		x		x
Phe			x					x		x		x
Tyr	x		x		x			x		x		x
Trp			x						x			x
Lys		x		x			x		x		x	
His	x		x		x			x		x		x
Cys	x		x		x			x		x		x

The first eluting enantiomer is marked with "X"; red cells represent cases when there was no chiral resolution; green cells represent the two cases of reversal of elution order

Two cases of reversal of elution order dependent on mobile phase pH were observed, one on each column. On Diphenyl column, the derivatives of D- and L-Pro eluted in reversed order at pH 5 compared to pH 2.5. (Fig 22A), whereas on Biphenyl column, a double reversal of elution order was observed for the FLEC-DL-Val derivatives. (Fig. 22B). At pH 2.5 and 7 the L- form eluted first, while at pH 4.5 the opposite elution order was obtained.



**Figure 22. Reversal of elution order under the influence of the mobile phase pH; A: FLEC-DL-Pro on Diphenyl stationary phase; B: FLEC-DL-Val on Biphenyl stationary phase.**

### 5.3.2 Optimization of FLEC-DL-AA separation

Considering the multivariate nature of chromatographic selectivity and the particularities of the two distinct stationary phases employed, it seems appropriate to make use of a statistical experimental design, or design of experiments (DoE) for screening and optimization purposes. This would provide a framework for simultaneously changing all important variables by a well-defined plan yielding as much information as possible by means of a limited number of experiments. There are many designs to choose from, the most common designs, involving qualitative and/or

quantitative variables usually include fractional factorial, Placket-Burman and D-optimal designs. All such designs incorporate the concepts of randomization and center points. The randomization of experiments is meant to eliminate any potential systematic variations, whereas the center points (at least three) are experimental runs meant to examine the center of the design region (experimental volume) defined by the range of selected variables. The center points also serve in describing any curvature in the experimental volume and to detect any variations in time during the course of experiments. The obtained results analyzed by various projection based mathematical tools could also help us draw some pertinent conclusions regarding the mechanisms underlying the chiral separation of FLEC-DL-AAs.

In order to identify an optimal experimental region providing the highest overall selectivity, potentially under one chromatographic run, as well as to pinpoint particularities in terms of FLEC-DL-AA chiral selectivity on the two stationary phases, a DoE approach was implemented, involving four significant variables: the nature of stationary phase (Col), pH, initial proportion of ACN (ACN init) and gradient time (Gt) and two responses: resolution (Rs) and retention time (Rt). The type and level/range of the studied variables are summarized in Table 7.

**Table 7. DoE variables, their characteristics and levels/ranges.**

Variable	Type	Design space				
Stationary phase	Qualitative	Diphenyl	Biphenyl			
pH	Quantitative, multilevel	2.5	3.5	4.5	5.5	6.5
Initial proportion of ACN (%)	Quantitative, multilevel	5	15	75		
Gradient time (min)	Quantitative, multilevel	15	22.5	30		

For response surface modeling (quadratic) a computer generated, D-optimal design with three center points and two replicates was employed, involving one qualitative (column type) and three multilevel quantitative variables (pH, ACN initial proportion, gradient time) with a total of 75 runs. The design region was selected based on preliminary assays performed on all amino acids in various chromatographic conditions. The generated design was balanced on the qualitative variable and it was selected from a candidate set based on the highest G-efficiency<sup>225</sup>. The data collected from the experimental design was fitted by multiple linear regression (MLR) to estimate the coefficients of the model.

### **5.3.2.1 Optimized separation conditions using $R_s$ as response**

After fitting the experimental data, a model having as response only  $R_s$  was created. The fitting of the experimental data according to the resulting model was found to be rather good for most AAs. The model significance is estimated by the  $R^2$ - and  $Q^2$ -values. An adequate measure of fit ( $R^2$ , goodness of fit), with  $R^2 > 0.7$ , has been obtained for most of the analytes, except for Thr ( $R^2=0.62$ ), Asp ( $R^2=0.23$ ) and Glu ( $R^2=0.56$ ) (Fig. 24A). The goodness of prediction ( $Q^2$ ) describes the percent variation of the response predicted by the model according to cross validation, indicating how well the model predicts new data. A useful model should have large  $Q^2$  values, where limits to adequate values is variable and may go down to  $Q^2 > 0.4$ , depending on the purpose of the model and nature of the data set<sup>225</sup>. In our case, for most AAs, goodness of prediction was between 0.55 and 0.96, the smallest values being recorded once again for Thr ( $Q^2 = 0.45$ ), Asp ( $Q^2 = -0.04$ ) and Glu ( $Q^2=0.33$ ), respectively.

Therefore, with the exception of a few AA derivatives (i.e. Asp and Glu), within the reason of a certain prediction error, such models should not only allow identifying an optimal region offering the highest overall diastereoselectivity (sweet spot) in the investigated design space for each column (Figures 24 B and C), but also predict the response values for all possible combinations of variables within the experimental region.

The predictions for best separation conditions for each FLEC-DL-AA pair are rather heterogenous (Table 8). Neither Diphenyl, nor Biphenyl columns can offer baseline separation for all analytes. Their selectivities seem complementary for some analytes (Ala, Ser, Thr, Asn, Gln and Glu), while the Asp derivatives could not be separated under these experimental conditions. On the other hand, the doubly derivatized amino acids (Lys, Cys and His), Ile, Leu, Arg, Val and Pro can be separated on both columns.

For those cases where the separation is predicted to be achievable on both stationary phases, the resolution values are generally higher on the Diphenyl column (except for Cys and His). However, the Biphenyl stationary phase is capable to offer baseline selectivity for most of the FLEC-AAs (Ala, Ser, Thr) unresolved on the Diphenyl (except Asp).

It is to be noted that baseline resolution is not achievable for the FLEC derivatives of Asp, Glu or their amidic analogues (Asn and Gln) on the Biphenyl stationary phase.

The separation pH for most analytes is predicted to be optimal between pH 4 and pH 5.5. In few cases, the optimal separation pH is 6.5 (Ala, Thr and Glu on Diphenyl; Thr on Biphenyl), while pH 2.5 is suitable to achieve separation for Ser and Pro on both stationary phases and for Ala on Biphenyl.

Chiral selectivity for Ser and Pro can be achieved on both stationary phases at low pH (2.5), with a gradient starting with the highest amount of ACN as mobile phase component (15%).

**Table 8. Predicted Rs values in optimized conditions for each FLEC-DL-AA**

AA	Diphenyl				Biphenyl			
	Rs	pH	% ACN	Time (min)	Rs	pH	% ACN	Time (min)
<b>Ala</b>	0.9	6.5	5	30	1.8	2.5	15	30
<b>Ser</b>	1.1	2.5	15	15	1.6	2.5	15	30
<b>Pro</b>	1.9	2.5	15	30	1.6	2.5	15	30
<b>Val</b>	2.9	5.5	5	30	2.2	4.5	5	30
<b>Thr</b>	0.8	6.5	15	30	2.1	6.5	11.3	30
<b>Ile</b>	6.1	4.9	15	15	3.4	4.5	5	30
<b>Leu</b>	5.7	5.1	15	15	3	4.6	8.5	30
<b>Asn</b>	1.8	5.2	12.2	25	1.2	4.1	10.6	21.2
<b>Asp</b>	0.2	4.3	15	22.5	0.2	3.7	15	22.5
<b>Arg</b>	2.8	5.1	9.3	30	2.5	4.5	10.9	30
<b>Gln</b>	1.8	5.2	11.6	25.5	1.1	4.2	11.1	21.8
<b>Glu</b>	2.1	6.5	9.6	30	1	5.9	6.2	30
<b>Met</b>	3.7	4.9	8.5	30	2.1	4.3	9	30
<b>Phe</b>	3.9	4.8	10.1	26.2	2.7	4.3	10	22.8
<b>Tyr</b>	4.3	4.8	10.4	30	4.1	4.4	10.5	30
<b>Trp</b>	2.6	5	8.6	30	1.7	4.5	9.1	30
<b>Lys</b>	3.7	5	11.8	26	2.3	4.8	10.5	25.1
<b>His</b>	7.1	4.9	11.3	30	8.2	4.5	10	22.5
<b>Cys</b>	4.5	4.9	5	27.4	4.9	4.3	15	24.8

When using the global model (on both columns, following only Rs), the optimized separation conditions for Diphenyl stationary phase, providing a predicted minimum resolution of 1.5 for AA derivatives, consisted of pH 4.9, initial percentage of ACN – 5 % and a gradient time of 30 min. In contrast, for the Biphenyl column, the chromatographic variables aiming for the targeted minimum resolution were pH 4.2, initial percentage of ACN – 6.6% and gradient time of 30 min. Using these conditions, the model predicts a maximum of 14 baseline separations of diastereomeric pairs on the Diphenyl column, while for the Biphenyl column 12 pairs are predicted to be baseline separated (Fig. 24). In comparison with the optimized separation conditions for each individual FLEC-AA pair (Table 8), on Diphenyl the baseline resolution is lost only for one diastereomer pair (Pro). At the same time, on the Biphenyl column, the baseline chiral resolution is lost for 3 FLEC-DL-AA derivatives (Ala, Ser, Pro).

AA	Diphenyl	Biphenyl
Ala	0.8	1.3
Ser	0.0	0.9
Pro	0.2	0.2
Val	2.9	2.2
Thr	0.6	1.5
Ile	5.1	3.3
Leu	3.6	2.9
Asn	1.5	0.8
Asp	0.0	0.0
Arg	2.6	2.4
Gln	1.6	0.8
Glu	1.5	0.9
Met	3.4	1.9
Phe	3.4	2.4
Tyr	3.8	3.9
Trp	2.4	1.6
Lys	3.2	1.9
His	6.3	8.4
Cys	4.4	4.1

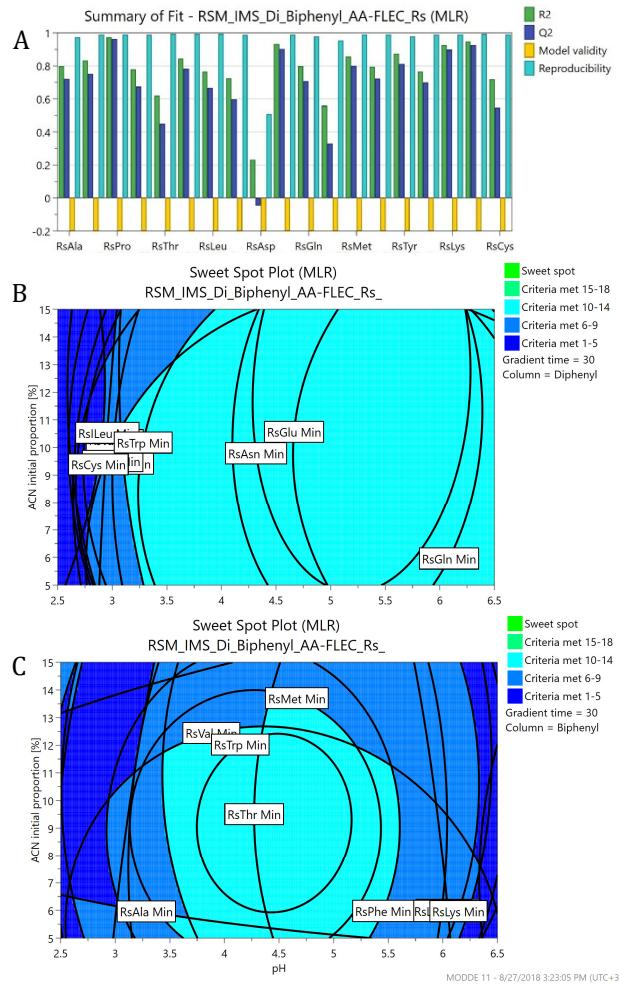


Figure 24. Left: Predicted  $R_s$  values in the optimized separation conditions; A: Summary of fit for the model resulted after the fitting of experimental data; B: Sweet spot diagram for Diphenyl column; C: Sweet spot diagram for Biphenyl column

### 5.3.2.2 Optimized separation conditions using $R_s$ and $R_t$ as response

In order to predict the best separation conditions in the shortest time possible, a new model was created using this additional constraint (both  $R_s$  and  $R_t$  used as responses). Once again, the fitting of the experimental data according to the resulting model was found to be good for all analytes (for  $R_s$ :  $R^2 = 0.54\text{--}0.96$ ; for  $R_t$ :  $R^2 = 0.96\text{--}0.99$ ), except for the resolution of Asp ( $R_s=0.18$ ).

**Table 9. Predictions of individual optimized chromatographic conditions for the chiral resolution of FLEC-AA derivatives taken separately for the two tested columns**

AA	Diphenyl					Biphenyl				
	Rs	Rt (min)	pH	% ACN	Time (min)	Rs	Rt (min)	pH	% ACN	Time (min)
<b>Ala</b>	0.5	5.9	6.5	15	15	1	4.7	5.9	15	15
<b>Ser</b>	1.1	9.5	2.5	15	15	1.1	6.6	6.5	15	30
<b>Pro</b>	1.4	11.9	2.5	15	15	1.3	9.8	2.5	15	15
<b>Val</b>	2.5	6.8	6.5	15	15	1.4	6.3	5.3	15	15
<b>Thr</b>	0.8	5.7	6.5	15	15	1.5	4.1	6.5	15	15
<b>Ile</b>	5	6.5	6.2	15	15	2.7	6.8	5	15	15
<b>Leu</b>	4.8	6.7	6.2	15	15	2.3	6.9	5	15	15
<b>Asn</b>	1.4	5.4	5.5	15	15	0.9	4.5	4.6	15	15
<b>Asp</b>	0	4.2	6.5	15	17.5	0.1	2.8	6.5	15	18.7
<b>Arg</b>	1.8	6.2	4.9	15	15	2	5.4	4.9	15	15
<b>Gln</b>	1.5	5.7	5.6	15	15	0.9	4.7	1.7	15	15
<b>Glu</b>	1.7	3.6	6.5	15	15	0.8	2.9	6.5	12.6	15
<b>Met</b>	2.8	8	5.7	13.3	15	1.8	7.2	4.9	13.3	15
<b>Phe</b>	2.2	7.8	4.9	12.6	15	2.2	7.8	4.9	12.6	15
<b>Tyr</b>	2.9	5.7	5.3	15	15	2.9	5.7	5.3	15	15
<b>Trp</b>	1.2	8	5	13.7	15	1.2	8	5	13.8	15
<b>Lys</b>	1.8	10.7	5.3	12	15	1.8	10.7	5.3	12	15
<b>His</b>	4.7	9.1	6.5	15	15	4.7	9.1	6.5	15	15
<b>Cys</b>	3.5	10.1	5.3	15	15	3.5	10.2	5.3	15	15

The predictions having a minimum Rs target of 1.5 and the simultaneous minimization of Rt are presented in Table 9. Compared to the model based only on Rs, the predicted resolution values are significantly lower for all analytes. Considering the higher pH values and the initial percentage of ACN set to maximum (in most cases), fast elution times are expected (less than 10 min). Nonetheless, baseline chiral separation is predicted for 13 pairs of FLEC-AAs on Diphenyl and 11 on Biphenyl.

**Table 10. Predictions of resolutions and retention times for the simultaneous analysis of FLEC-AA derivatives by a single run on the two studied columns**

AA	Diphenyl		Biphenyl	
	R <sub>s</sub>	R <sub>t</sub> (min)	R <sub>s</sub>	R <sub>t</sub> (min)
<b>Ala</b>	0.5	6.4	1.1	5.5
<b>Ser</b>	0	5.6	0	4.4
<b>Pro</b>	0	6.4	0	5.7
<b>Val</b>	2.7	7.5	1.5	6.6
<b>Thr</b>	0.8	6	1.1	4.9
<b>Ile</b>	5.4	6.7	2.6	6.6
<b>Leu</b>	5.2	6.9	2.2	6.7
<b>Asn</b>	1.3	5	0.7	4
<b>Asp</b>	0	4.4	0	3.9
<b>Arg</b>	1.6	6.4	2	5.3
<b>Gln</b>	1.4	5.5	0.7	4.4
<b>Glu</b>	1.6	4.5	0.7	4.1
<b>Met</b>	2.3	7.5	1.4	6.5
<b>Phe</b>	2.5	8.4	1.7	7.1
<b>Tyr</b>	2.4	6.8	3	5.8
<b>Trp</b>	1.9	8.4	1	7.5
<b>Lys</b>	2.7	11.2	1.7	10.6
<b>His</b>	4.7	10.7	6.9	10.4
<b>Cys</b>	2.4	11.5	3.7	10.4

The global optimized separation conditions offering the simultaneous resolution for most of FLEC-AA derivatives under a single chromatographic run (min R<sub>s</sub> = 1.5 at the lowest R<sub>t</sub>, Table 10) predicted by the model based on R<sub>s</sub> and R<sub>t</sub> consisted of a pH of 5.9, initial percentage of ACN of 15% and a run time of 15 min (for Diphenyl) and a pH of 5.13, initial percentage of ACN of 15% and a run time of 15 (for Biphenyl). In these conditions, the baseline chiral resolution is predicted for 12 FLEC-AA pairs on the Diphenyl column, in under 12 minutes; on Biphenyl, 10 pairs of FLEC-AA diastereomers can be separated in less than 11 minutes.

Since FLEC's first report<sup>78</sup>, chiral derivatization of amino acids using FLEC has been used in many studies. Still, only few methods<sup>78,103,161</sup> have been developed for the analysis of this class of analytes. All these analysis methods used C18 stationary phases and THF based mobile phases, obtaining chiral resolution for most of the proteinogenic amino acids in relatively long run times (between 90 and 160 min). To be noted that the chiral separation of all proteinogenic amino acids in one run was not reported in any of the mentioned studies. Compared to the previously described methods, the use of phenyl based stationary phases in combination with MS detection can offer much faster analysis times (at least 5 times faster) while keeping similar selectivity, high throughput analysis methods being desirable for metabolomics.

### 5.3.2.3 Comparison of stationary phase selectivity

As can be concluded from previous investigations, the selectivities of the two stationary phases towards FLEC-DL-AAs are significantly different. In order to further analyze the distinct separation mechanisms observed on the two columns, additional correlations between the chromatographic data set recorded for selectivity optimization and known physicochemical parameters of the amino acids (Table 7) or FLEC-AA derivatives when available (Table 8), such as the molecular weight, pKa, pI, H-index, logP and the nature of the side chain were investigated by multivariate data analysis. This was considered the most feasible alternative, considering that experimentally determined physicochemical parameters for FLEC-AA are not readily available. For better classifying similar chromatographic behavior, the amino acids were classified in several groups depending on the nature of their side chain and its charge state at pH 7, as follows: hydrophobic (Ala, Val, Ile and Leu), aromatic (Phe, Tyr and Trp), negatively charged (Asp and Glu), positively charged (Arg, Lys and His) and neutral (Ser, Thr, Asn, Gln, Met and) Cys. The remaining amino acids (Gly and Pro) could not be included in any of the above-mentioned categories, therefore were considered unique.

**Table 11. Physicochemical parameters of studied amino acids**

AA	pK <sub>a</sub> 1	pK <sub>a</sub> 2	pI	H index <sup>1</sup>	logP	Side chain <sup>2</sup>
<b>Gly</b>	2.34	9.6	5.97	-0.4	-3.21	U
<b>Ala</b>	2.34	9.69	6	1.8	-2.85	HP
<b>Ser</b>	2.21	9.15	5.68	-0.8	-3.07	N
<b>Pro</b>	1.99	10.6	6.3	-1.6	-2.54	U
<b>Val</b>	2.32	9.62	5.96	4.2	-2.26	HP
<b>Thr</b>	2.09	9.1	5.6	-0.7	-2.94	N
<b>Ile</b>	2.36	9.6	6.02	4.5	-1.7	HP
<b>Leu</b>	2.36	9.6	6.02	4.5	-1.7	HP
<b>Asn</b>	2.02	8.8	5.41	-3.5	-3.82	N
<b>Asp</b>	1.88	9.6	2.77	-3.5	-3.89	Neg
<b>Arg</b>	2.17	9.04	10.76	-4.5	-4.2	Pos
<b>Gln</b>	2.17	9.13	5.65	-3.5	-3.64	N
<b>Glu</b>	2.19	9.67	3.22	-3.5	-3.69	Neg
<b>Met</b>	2.28	9.21	5.74	1.9	-1.87	N
<b>Phe</b>	1.83	9.13	5.48	2.8	-1.38	Ar
<b>Tyr</b>	2.2	9.11	5.66	-1.3	-2.26	Ar
<b>Trp</b>	2.83	9.39	5.89	-0.9	-1.05	Ar
<b>Lys</b>	2.18	8.95	9.74	-3.9	-3.05	Pos
<b>His</b>	1.82	9.17	7.59	-3.2	-3.32	Pos
<b>Cys</b>	1.96	10.28	5.07	2.5	-2.49	N

<sup>1</sup>hydrophobicity index; <sup>2</sup>Side chain: HP – hydrophobic, N – neutral, Ar – aromatic, Neg – negatively charged, Pos – positively charged, U – unique.

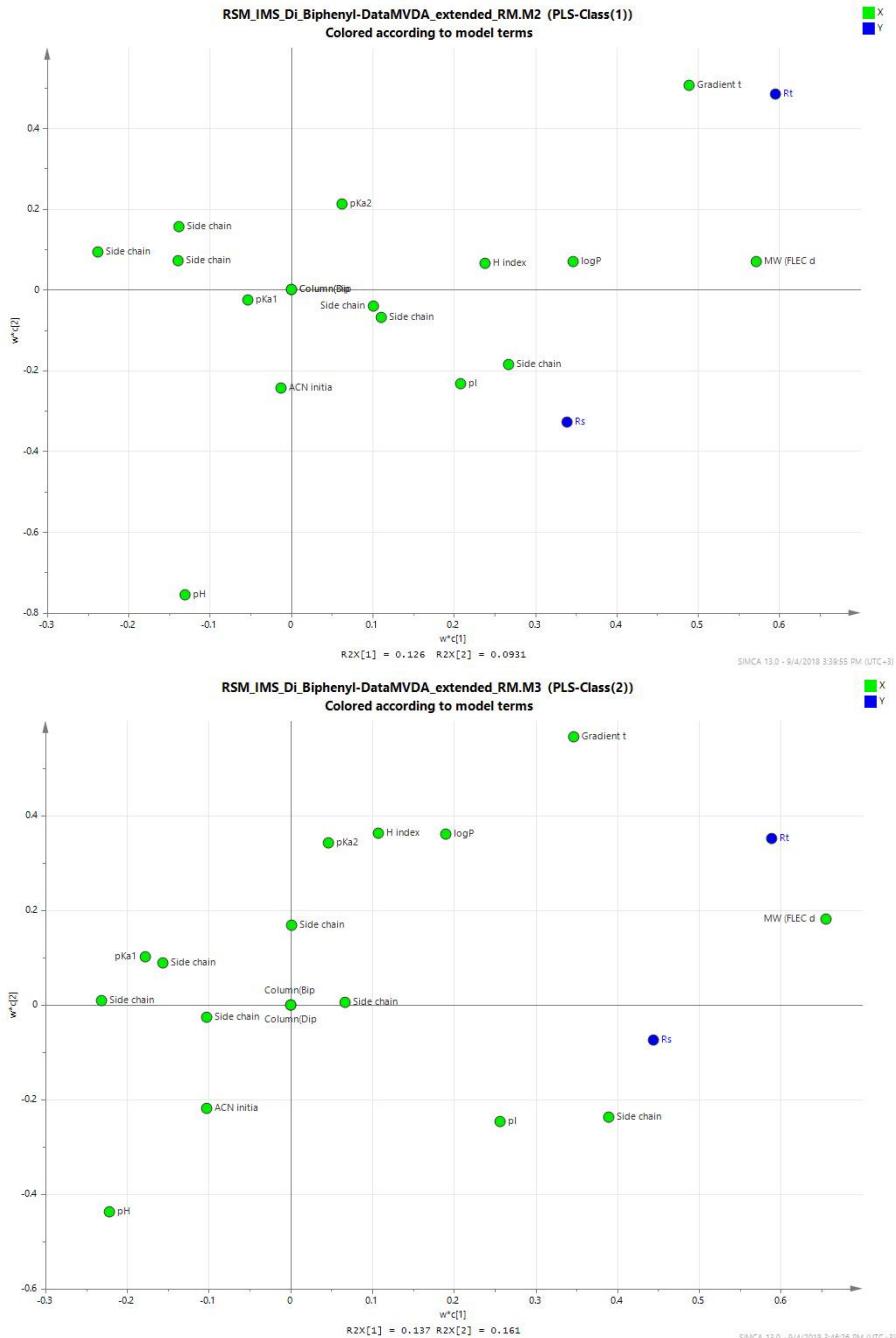
**Table 12. Parameters of the studied FLEC-AAs**

AA	MW (FLEC derivatives)	Number of derivatized moieties
<b>FLEC-Gly</b>	349.078	1
<b>FLEC-Ala</b>	363.096	1
<b>FLEC-Ser</b>	379.091	1
<b>FLEC-Pro</b>	389.117	1
<b>FLEC-Val</b>	391.123	1
<b>FLEC-Thr</b>	393.105	1
<b>FLEC-ILeu</b>	405.142	1
<b>FLEC-Leu</b>	405.142	1
<b>FLEC-Asn</b>	406.099	1
<b>FLEC-Asp</b>	407.084	1
<b>FLEC-Arg</b>	410.203	1
<b>FLEC-Gln</b>	420.115	1
<b>FLEC-Glu</b>	421.107	1
<b>FLEC-Met</b>	423.099	1
<b>FLEC-Phe</b>	439.123	1
<b>FLEC-Tyr</b>	455.128	1
<b>FLEC-Trp</b>	478.136	1
<b>FLEC-Lys</b>	618.280	2
<b>FLEC-His</b>	627.244	2
<b>FLEC-Cys</b>	631.151	2

Multivariate regression analysis by projection to latent structures using partial least square analysis (PLS) was performed on the entire recorded data set ( $N=1494$ ) involving both chromatographic columns. PLS regression provided a 4-component model ( $N=4$ ), explaining 44.5% of the variation ( $R^2X=0.445$ ) and a good predictability of the cross-validated variances ( $Q^2(\text{cum}) > 0.58$ ). Eleven X variables (column, pH, ACN initial proportion, gradient time, MW of FLEC derivative,  $pK_a1$ ,  $pK_a2$ ,  $pI$ , H index,  $\log P$ , side chain) and two Y variables as responses ( $R_s$ ,  $R_f$ ) were considered. Standard scaling and mean-centering (centered and scaled to unit variance) performed on all variables in the data pre-treatment step.

For a more detailed analysis the data set was split in two parts, based on the employed chromatographic column, creating two distinct PLS models. For the Diphenyl column the data set consisted of 714 records, the regression providing a 4-component model ( $N=4$ ), explaining 51.1% of the variation ( $R^2X=0.511$ ) and a good predictability of the cross-validated variances ( $Q^2(\text{cum}) > 0.58$ ). In the case of the Biphenyl column, 780 records were modeled, providing a 6-component model ( $N=6$ ), explaining 59.4% of

variance ( $R^2X=0.594$ ), with a slightly higher predictability of the cross-validated variances ( $Q^2(\text{cum}) > 0.67$ ).



**Figure 25.** Loading plots showing correlations between X and Y variables; top: Diphenyl column; bottom: Biphenyl column.

The loading scatter plots (Figure 25) indicate the relationship between all considered factors (X) and responses (Y), and how these variables correlate to each of the responses of interest (chromatographic selectivity and retention). X-variables with large (positive or negative) weights ( $w^*$ ) are highly correlated with the responses ( $R_s$  and  $R_t$ ). These variables with large  $w^*$  are situated far away from the origin (on the positive or negative side) on the plot. Hence, these plots indicate how the responses vary in relation to each other, which ones provide similar information and their relationship to the terms in the model.

To interpret these plots from one Y-variable, an imaginative line is drawn through the origin, while projecting other X- and Y-variables on this line. Variables opposite to each other are negatively correlated and positively correlated to variables situated near them.

Thus, considering the present loading plots for the first two PLS components ( $R^2X[1] = 0.126$ ,  $R^2X[2] = 0.0931$  with a total of 21.9% explained variation for Diphenyl column and  $R^2X[1] = 0.137$ ,  $R^2X[2] = 0.161$  with a total of 29.8% explained variation for the Biphenyl column, respectively) the X and Y variables seem to have a similar correlation structure on both stationary phases. Firstly, a positive correlation of both  $R_s$  and  $R_t$  with the MW of the FLEC-AA and its basic, hydrophobic and aromatic side chain, and a negative correlation with the acidic, neutral and unique side chain is observed. It must also be stated that AAs with a basic side chain (i.e. Lys, Hys) are also doubly derivatized with FLEC, leading to a significant increase of their MW and to a higher number of sites available for interaction with the studied stationary phases.

By a more detailed analysis of the correlation structure of the PLS loading weights additional information may be extracted identifying further similarities or potential particularities of the two studied columns.

Considering the first two PLS components, for both stationary phases, the gradient time is positively, whereas the initial proportion of ACN is negatively correlated with the FLEC-AAs retention time, being in compliance with a reverse phase elution profile. The aqueous component pH is negatively correlated with the chromatographic retention ( $R_t$ ) and recorded diastereoselectivity ( $R_s$ ). It is also worth mentioning that by the second and third PLS components ( $R^2X[2] = 0.0931$ ,  $R^2X[3] = 0.092$  with a total of 18.51% explained variation for Diphenyl column and  $R^2X[2] = 0.161$ ,  $R^2X[3] = 0.138$  with a total of 29.9% explained variation for the Biphenyl column, respectively), pH becomes positively correlated with resolution (data not shown).

This bimodal behavior was observed also during the preliminary studies (Figure 23), when the resolution of FLEC-AAs increased going from pH 2.5 to 4.5 and decreased going from pH 4.5 to pH 7.0, respectively.

All physico-chemical parameters of the unmodified AAs considered, including the hydrophobicity index (H index), pI and logP, seem to be positively correlated with the retention time and recorded resolution in case of both columns, but such correlations might have a less practical meaning, since the FLEC moiety of the derivatized AAs will significantly influence the overall chromatographic behavior or FLEC-AAs.

Thus, by the current level of multivariate data analysis more similarities than particularities in terms of the chromatographic retention and selectivity of the two stationary phases has been revealed. However, by extending the number of measured FLEC-AAs physico-chemical descriptors, and most importantly by using also certain descriptors related to the employed stationary phases, such semi-empirical mathematical models might be able to reveal certain particularities of the two adsorbents responsible for the distinctive chromatographic behavior of the derivatized AAs.

## 5.4 Conclusion

The separation of FLEC-DL-AAs was successfully achieved using phenyl based stationary phases, while employing common organic solvents as organic components of the mobile phase.

Under optimized conditions, in a single run, the Diphenyl column can provide baseline resolution for 14 (out of 19) FLEC-AA pairs, while the Biphenyl column can lead to 12 (out of 19) complete separations. Individual baseline separation conditions can be achieved for almost all FLEC-AA diastereomers pairs (except Asp) under different chromatographic conditions (different pH values of the aqueous component or different stationary phase) (Table 8).

The experimental design approach offered valuable information on the selectivity of the two stationary phases. At the same time, the recorded data was further used for understanding some correlations between the influence of different physicochemical parameters on the variance of resolution. Thus, by PLS regression of the experimental data it was possible to positively correlate the resolution and retention time with the molecular mass (most probably implying higher hydrophobicity) and, at the same time, to observe a negative correlation with negatively charged side chains. Of course, a further external validation of the models is required.

Even though conclusions and correlations could be achieved by multivariate analysis, these conditions should be treated with caution. The chromatographic behavior of the FLEC-AA derivatives is expected to be significantly influenced by the degree of derivatization (mono- or di-FLEC-AAs) and the mass ratio FLEC:AA in the AA homologous series, from the smallest (Gly) to the largest (Trp).

On both columns, pH mediated reversals of elution order were observed, as is the case for FLEC-DL-Pro on Diphenyl column and for FLEC-DL-Val on Biphenyl column. However, further experiments are needed in order to understand the mechanism underlying these phenomena.

In comparison with previously developed LC methods<sup>78,157,159</sup> for FLEC-DL-AA analysis, a much shorter analysis time (up to 5 times) is achievable, while maintaining similar selectivity.



## 6. GENERAL DISCUSSION

Capillary electrophoresis and liquid chromatography are two separation techniques that are commonly used in chiral analysis. For achieving enantioselectivity, they employ either a chiral selector, when the separation mechanism is based on a stereospecific interaction between the enantiomers and the chiral selector, or a derivatization step with a chiral derivatizing agent is performed and the resulted diastereomers are then separated based on their different physicochemical properties.

In the case of amino acids, due to their small molecular mass and most of them lack any chromophores or fluorophores, the second approach is more common. Thus, the derivatization brings two major advantages: it increases their detectability and, at the same time, offers a possibility to tune their selectivity.

One of the most versatile derivatizing agents is (+) or (-)-FLEC, which reacts quick and at room temperature with primary and secondary amines, forming hydrophobic diastereomers. Over the last 30 years, FLEC-AA diastereomers could be successfully separated both by CE and LC.

The main objective of this research was to develop new and efficient methods for the analysis of D- and L-amino acids in biological fluids, in the context of targeted metabolomics. Therefore, three studies were performed (comprised in chapters 3, 4 and 5) on both CE and LC.

The aim of the first study was to develop an automatized analysis setup for the separation and quantification of D- and L-AAs. For this, a CE-MS approach was used, and more specifically MEKC-MS.

An in-capillary derivatization procedure was developed and optimized in order to completely automatize the procedure. The process implied the subsequent injection of the sample and the derivatization agent and their mix using a low voltage. By applying a voltage during the mix, the charged molecules of the amino acids would migrate towards the derivatization agent plug, increasing the reaction yield. For a more targeted process, the optimization was performed by implementing an experimental design involving 4 variables (sample plug length, derivatization plug length, mixing time and mixing voltage).

To achieve the desired selectivity for the FLEC-DL-AAs, a semi-volatile surfactant, ammonium perfluorooctanoate (APFO), was employed as a pseudostationary phase. An increase in resolution was observed with increasing concentrations of APFO; still, the optimized BGE consisted of 100 mM APFO due to the high conductivity of the solution and the CE system current limitation to 50  $\mu$ A. In the optimized conditions, 13 pairs of FLEC-DL-AAs could be separated ( $Rs \geq 1$ ), with LODs in the low  $\mu$ M region. The developed method was tested on aCSF samples, proving its applicability.

One major drawback of this analysis method is the inability to separate the enantiomers of acidic amino acids (Asp and Glu). It is assumed that there are repulsion forces which arise at the interaction of the negatively charged acidic amino acids with the negatively charged micelles of APFO.

The second method that was developed was a targeted approach for the analysis of relevant D-AAs in the context of neurometabolomics.

The starting point were some prior observations made when analyzing FLEC-DL-AAs acidic pH and one literature study which mentioned the separation of acidic amino acids by CZE at an acidic pH. Therefore, FLEC derivatization was employed for 5 biologically relevant amino acids (D- and L- enantiomers of Ser, Asn, Asp, Gln, Glu).

A high dependency of resolution on the BGE pH could be observed. The best separation was gained by using a 50 mM acetic acid buffer, pH 3.7 (adjusted with ammonia). Using this pH, baseline separation was achieved for 4 out of 5 amino acids, the resolution for Asp being 1.4. Nevertheless, Asp diastereomers could be successfully separated using a pH 5 BGE.

One interesting phenomenon of pH dependent reversal of migration order was observed for D- and L-Asp derivatives. In order to elucidate the mechanism underlying this process, the  $\mu_{\text{EFF}}$  of both FLEC-D-Asp and FLEC-L-Asp were measured in order to calculate the pK<sub>a</sub> of those molecules. It was observed that the reversal of migration order is caused by a slight difference in the pK<sub>a</sub> of the second ionizable moiety (the distal carboxyl). At the same time, the same phenomenon could not be observed for the Glu diastereomers. Considering that the difference in the side chain of these two amino acids is just a methylene, we can conclude that probably the pK<sub>a</sub> difference between the Asp diastereomers is a result of specific intra-molecular interactions of the side chain carboxyl with the electrophile carbamate moiety.

The applicability of the method was also tested on aCSF samples. Compared to the MEKC-MS approach, the salt rich matrix of aCSF affected the enantioselectivity. Therefore, a specific SPE procedure was developed and fully characterized. The limits of detection and quantification for this method are adequate for metabolomic analyzes (below 1  $\mu\text{M}$ ).

The third study was meant to provide better LC separation tools for FLEC derivatives. Until now, all of the previously developed LC methods for the analysis of FLEC-DL-AAs used reversed phase C4, C8 or C18 stationary phases and THF based mobile phases.

Two phenyl stationary phases were evaluated for achieving the desired selectivity using mobile phases based on common organic solvents (MeOH, ACN, IPA) in combination with an aqueous phase. A close dependency of resolution on mobile phase pH was also observed. The two stationary phases showed a high level of complementarity.

For better understanding the chromatographic behavior of the analytes, an experimental design procedure was implemented. Four variables were selected with previously determined variation intervals (stationary phase, mobile phase pH, initial

proportion of ACN and gradient time) and two chromatographic responses (resolution, retention time). The resulting model predicted that the maximum 14 FLEC-DL-AAs could be baseline separation on Diphenyl in one run, while on the Biphenyl column, the maximum were 12.

The same data set was subjected to a multivariate analysis approach, in order to better understand the influence of different physicochemical parameters on the chromatographic separation. Thus, by PLS regression of the experimental data was possible to positively correlate the resolution and retention time with the molar mass and the positively charged side chains, and, at the same time to observe negative correlation with negatively charged side chains. Nevertheless, these empiric models need to be validated using an external data set.

Moreover, two cases of pH dependent reversals of elution order were documented, one on each of the stationary phases. On the Diphenyl column, the reversal of elution order of FLEC-DL-Pro derivatives was observed, whereas on Biphenyl column it was a double reversal of elution order for FLEC-DL-Val. The interactions causing these phenomena still need further investigation.



## 7. GENERAL CONCLUSIONS

Considering the implications of D-AAs in the metabolic pathways of different diseases, the separation and quantification of D- and L-amino acids is becoming more and more necessary.

In chapter 2 is presented an overview of the diastereomeric separations obtained after (+) and (-)-FLEC derivatization. This study covered most of the papers published in the last 30 years on this topic. The conclusion is that excellent diastereoselectivity can be obtained using conventional apparatus and without any chiral selectors. One of the key attributes of FLEC derivatives is their hydrophobicity, which promotes the separation using reversed phase systems. Moreover, FLEC derivatization is highly advisable for molecules which do not have chromophore or fluorophore moieties in order to increase their UV or FL detection sensitivity.

FLEC's major advantage over the other chiral derivatization agents is its fast and simple derivatization procedure. Its applicability was reported over a wide range of molecules, quantitative derivatization reactions are expected and no special sample pretreatment is needed.

The third chapter describes the development of a fully automated in-capillary derivatization method and it was proven that this approach can be suitable for the enantioseparation of amino acids by MEKC-MS. For optimizing the derivatization conditions, an experimental design methodology was implemented. This way, the different correlations between the variables and responses could be established, helping to better understand this particular derivatization process.

Using this method, enantioresolution ( $R_s \geq 1$ ) was obtained for 14 DL-AAs. These chiral separations were gained due to the interaction of FLEC-DL-AAs with the pseudostationary phase (APFO micelles), but unfortunately no resolution was obtained for the diastereomers of acidic amino acids (Asp, Glu). This is probably due to low interaction of these molecules with the micelles caused by repulsive forces between the negatively charged micelles and the analytes. It was also concluded that organic modifiers have a negative impact on the chiral resolution, but they can be used for tuning the selectivity (e.g. separate certain isobaric pairs - such as Leu and Ile). The method was tested on artificial CSF samples, proving to be suitable for analyzing this kind of biological medium.

A targeted CE-MS method was developed for the chiral analysis of five biologically relevant DL-AAs. (+)-FLEC derivatization was employed and the separation was achieved by using only ammonium acetate buffer. The chiral separation of DL-Ser, DL-Asn, DL-Asp, DL-Gln and DL-Glu was obtained in around 22 min. while the resolution was found to be highly dependent on the pH of the BGE. Moreover, a reversal of

migration dependent on the BGE pH was observed and documented for DL-Asp derivatives. After further investigations it was concluded that the reversal is caused by a discrete difference in the  $pK_a$  of the distal carboxyl of the amino acid, probably caused by intramolecular electron delocalization.

A SPE procedure was developed for achieving the separation of the targeted analytes in biological media (aCSF). The procedure was fully characterized, demonstrating the efficiency of the whole process (derivatization and extraction).

Good detection limits (below 1  $\mu\text{M}$ ) were obtained considering the analytical setup (sheath-flow CE-MS interface coupled with an old ion-trap MS instrument in negative detection mode). The performance was partly attributed to the fact that the ESI ionization was promoted by the ionization state of the analytes inside the separation capillary (negatively charged).

The last study (Chapter 5) comprised a detailed analysis of the factors that influence the diastereoseparation of FLEC-DL-AAs on two phenyl stationary phases. Several correlations have been made regarding the different variables (mobile phase pH, gradient's initial percentage of organic solvent, gradient length) and responses (resolution and retention time). The enantioselectivity was found to be closely dependent on the mobile phase pH, the two stationary phases showing significant degrees of complementarity.

No general model could be generated that predicts separation conditions suitable for baseline resolution of all analytes in one run. Still, this would be achievable by using the two stationary phases and different mobile phases.

Using PLS regression it was possible to develop some empiric models which correlate the chromatographic data with several physicochemical parameters. For example, positive correlations could be established between the resolution and retention time with the molar mass and the positively charged side chains, and, at the same time to observe negative correlation with negatively charged side chains.

One interesting aspect is the pH dependent reversal of elution order that was observed for DL-Pro and DL-Val, on diphenyl and biphenyl stationary phases, respectively. Further experiments are still needed for the understanding of the reversal of elution order mechanism and also for the validation of the PLS models by using external data sets.

## 8. Originality and innovative contributions

This PhD thesis is comprised of five chapters, two of which represent a review of the literature in two areas: the biological implications of D-AAs and the state of the art in FLEC derivatization and analysis. The other three chapters represent original research studies, focused on the analysis of D- and L-amino acids by CE-MS and LC-MS.

The second chapter consists of an exhaustive literature review of more than 70 original research papers published in the last 30 years on the topic of FLEC derivatization for chiral analysis and the subsequent analysis methods. This bibliographic study is the only work of this kind ever published on this topic.

The three research studies which were carried represent new and efficient analysis methods that have been developed for the analysis of amino acids.

The third chapter is made of the development of an innovative automated technique for the chiral analysis of amino acids by MEKC-MS. For the first time, the chiral analysis of amino acids was achieved on a MEKC-MS setup and, moreover, a new capillary labeling approach was developed and optimized. The variables affecting the derivatization were optimized using an experimental design procedure.

Chapter four represents a CE-MS method for targeted neurometabolomics. It is known that only some D-amino acids have a biological value, therefore the focus was on the chiral analysis of D- and L- enantiomers of Ser, Asp, Asn, Glu and Gln, as these amino acids play significant roles within the human body. Baseline separation was achieved for all amino acids and the sensitivity is adequate for the analysis of these D-AAs in biological fluids. An interesting discovery was made from an analytical point of view, when the reversal of migration order was observed for Asp enantiomers. For the first time, a reversal of migration order of diastereomers could be documented and the mechanism explained. By measuring the electrophoretic mobilities of the two diastereomers, it could be clearly observed that the reversal of migration order was caused by a discrete difference in the  $pK_a$  values of the second ionizable group (distal carboxyl) of the two diastereomers.

The last study, comprised in chapter five, represents a new alternative for the separation of FLEC-DL-AAs by liquid chromatography. Two phenyl stationary phases were evaluated, in combination with common mobile phases, in terms of their selectivity and performance for separating the diastereomers. Compared to the previous studies, this approach offers important advantages such as fast analysis times, excellent selectivity and use of common mobile phases (as compared to the THF based mobile phases reported in literature). Moreover, insights in the separation mechanisms were gained through correlations by multivariate analysis of the experimental data and physicochemical parameters of the amino acids.



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## **ANNEXES**



## Annex 1

Sample	Sep. method	Separation conditions	Detection	Sensitivity	(+)- or (-)- FLEC	FLEC solvent	Derivatization buffer	pH	Reaction time	t°C	Analyte: FLEC molar ratio	Matrix	Obs.	Ref.
17 primary amino acids and other amines	RP-LC	LC stationary phase: C8 Mobile phase: THF, acetate buffer pH-4.35	FL: (exc. 260nm)	n.r.	(+)	AAs: ACN/acetone Amines: acetone	AAs: borate Amines: borate	6.85 7.85	AAs: 4 min Amines: 30 min	n.r.	n.r.	Std. Sol.	Excess FLEC extracted with pentane	78
Amino acids	RP-LC	Stationary phase: C18; Mobile phase: 15 mM citric acid, 10 mM tetramethylammonium (TMA) in ACN and THF; pH-6	FL (exc. 260nm, em. 310nm)	50 nM	(+)	Acetone	Borate	11	45 s	r.t.	n.r.	Nervous tissues and eyes of crustaceans	Excess FLEC extracted with pentane (twice)	103
Ala	RP-LC	Stationary phase: C18; Mobile phase: 15 mM citric acid, 10 mM TMA in ACN and THF; pH-6	FL (exc. 260nm, em. 310nm)	n.r.	(+)	Acetone	Borate	11	45 s	r.t.	n.r.	Hard clam tissue	Analysis and derivatization done according to <sup>103</sup>	149
Amino acids	RP-LC	Stationary phase: C18; Mobile phase: 15 mM citric acid, 10 mM TMA in ACN and THF; pH-6	FL (exc. 260nm, em. 310nm)	n.r.	(+)	Acetone	Borate	11	45 s	r.t.	n.r.	Crayfish tissue	Analysis and derivatization done according to <sup>103</sup>	134
Ala	RP-LC	Stationary phase: C18; Mobile phase: 15 mM citric acid, 10 mM	FL (exc. 260nm,	n.r.	(+)	Acetone	Borate	11	45 s	r.t.	n.r.	Crab tissue	Analysis and derivatization	150

		TMA in ACN and THF; pH-6	em. 310nm)									tion done according to <sup>103</sup>		
Ala	RP-LC	Stationary phase: C18; Mobile phase: 15 mM citric acid, 10 mM TMA in ACN and THF; pH-6	FL (exc. 260nm, em. 310nm)	n.r.	(+)	Acetone	Borate	11	45 s	r.t.	n.r.	Crab tissue	Analysis and derivatization done according to <sup>103</sup>	151
NMDA	RP-LC	Stationary phase: C18; mobile phase: 0.1 M sodium acetate (pH 5.6) /ACN/THF – 86/7/7	FL (exc. 260nm, em. 315nm)	<0.1 µM	(+) and (-)	ACN/Acetone	Borate	9	15 min	50	n.r.	Bivalve tissue	Reaction terminated using cysteic acid	152
NMLA, NMDA	RP-LC	Stationary phase: C18; mobile phase: acetate buffer (pH- 5.59)/ACN/THF – 86/7/7	FL (exc. 260nm, em. 315nm)	n.r.	(+) and (-)	Acetone	Borate	9	15 min	50	n.r.	Mollusks and invertebrate tissues	Primary AAs removed by reaction with OPA. Derivatization protocol described in ref. <sup>152</sup> .	158
Amino acids	RP-LC	Stationary phase: C18; mobile phase: sodium acetate buffer (50mM)/MeOH – gradient elution	FL (exc. 263nm, em. 313nm)	n.r.	(+)	Acetone	Borate	9	30 min	40	n.r.	Sake beverages	Derivatization done according to ref. <sup>78</sup>	159
Amino acids	RP-LC	Stationary phase: unknown; mobile phase: acetate buffer (50mM, pH-4)/ACN – gradient elution	FL (exc. 263nm, em. 313nm)	n.r.	(+)	ACN/acetone	Borate	6.85	4 min	n.r.	n.r.	Mouse macrophages	Derivatization done according to <sup>78</sup>	136

<b>Amino acids</b>	RP-LC	Stationary phase: C18; Mobile phase: THF, acetate buffer (pH-5.1) – gradient elution	FL (exc. 260nm, em. 315nm)	n.r.	(+)	n.r.	Borate	6.85	4 min			Crustacean hyperglycemic hormone	Derivation done according to ref <sup>78</sup>	157
<b>Amino acids</b>	RP-LC	Stationary phase: C18; Mobile phase: 15 mM citric acid, 10 mM TMA in ACN and THF; pH-6	FL (exc. 260nm, em. 310nm)	50 nM	(+)	Acetone	Borate	11	45 s	r.t.	n.r.	Crayfish tissue	Analysis done according to ref <sup>103</sup>	135
<b>Ser</b>	RP-LC	Stationary phase: C18; Mobile phase: THF, acetate buffer pH-4.35	FL: (exc. 260nm)	n.r.	(+)	n.r.	Borate	6.85	4 min	n.r.	n.r.	Peptide hydrolysate	Derivation done according to <sup>78</sup>	156
<b>α-hydroxy acids</b>	RP-LC	LC stationary phase: C8 Mobile phase: 0.05M acetate buffer pH-3.8 – THF (60-40)	UV (265nm)	n.r.	(+)	Pyridine	Non-aqueous derivatization: Pyridine	/	5 min	n.r.	1:9	Standard solutions	-	106
<b>peptides Ocp-1, Ocp-4</b>	RP-LC	LC stationary phase: C8 Mobile phase: THF, acetate buffer (pH-4.35)	FL (exc. 260nm)	n.r.	(+)	ACN/acetone	Borate	6.85	4 min	n.r.	n.r.	Brain of Japanese octopus	Derivation done according to <sup>78</sup>	160
<b>Amino acids</b>	RP-LC	Stationary phase: C18; Mobile phase: gradient elution of sodium acetate 0.1M(pH-4)/ACN/THF –	FL (exc. 260, em. 315nm)-	n.r.	(+)	ACN/Acetone	Borate	9	15 min	50	n.r.	Peptide hydrolysate	Reaction terminated by adding cysteic acid	161
<b>Propanol</b>	RP-LC	Stationary phase: C18; Mobile phase: ACN/H <sub>2</sub> O – 75/25;	FL (exc. 260nm,	1.9 nM	(+)	Acetone	Borate	7.85	5.5 min	r.t.	1:9.76	Blood	-	153

			em. 340nm)											
<b>Atenolol</b>	RP-LC	Stationary phase: C18; Mobile phase: ACN/sodium acetate (0.01M, pH-7) – 50/50	FL (exc. 227nm, em. 310nm)	37 nM	(+)	Acetone	Borate	8.5	30 min	r.t.	1:83	Plasma	Excess FLEC removed by reaction with hydroxypr oline and extraction with pentane and dichlorome thane	154
<b>Propra- nolol</b>	RP-LC	Stationary phase: C8; Mobile phase: ACN/sodium acetate (pH-4; 0.02M) – 70/30	UV (254nm) FL (exc. 265nm, em. 345nm)	UV: 500 nM FL: 50 nM	(+)	Acetone	Borate	8.5	10 min	n.r.	1:100	Standar d solutio ns	-	107
<b>Reboxe- tine</b>	RP-LC	Stationary phase: C8; Mobile phase: 0.1M dibasic ammonium phosphate (pH-7.5) / THF – 53.5/46.5	FL (exc. 260nm, em. 315nm)	3.19 nM	(+)	ACN	Borate	8	5 min	n.r.	n.r.	Plasma	Excess FLEC removed by reaction with L-Pro and hexane extraction	116
<b>Reboxeti ne</b>	RP-LC	Stationary phase: C8; Mobile phase: 0.1M dibasic ammonium	FL (exc. 260nm, em. 315nm)	3.19 nM	(+)	ACN	Borate	8	5 min	n.r.	n.r.	Plasma	Separatio n and derivatiza tion done	164

		phosphate (pH-7.5) / THF – 53.5/46.5										according to ref. <sup>116</sup>		
<b>Reboxetine</b>	NP-LC	Stationary phase: (1) Silica, (2) cyano, (3) Chiracel OD-H; Mobile phases: (1) EtOH/heptane – 1/124, (2, 3) EtOH/heptane – 1/49	FL (exc. 260nm, em. 315nm)	6.38 nM	(+)	Acetone	-	-	5 min	30	1:4	Plasma	-	115
<b>Metamphetamine and amphetamine</b>	RP-LC	Stationary phase: C18; Mobile phase: 0.02M acetate buffer/ACN/THF – 46/39/15	FL (exc. 265nm, em. 330nm)	n.r.	(-)	Acetone	Phosphate	7.8	15-20 h, overnight	r.t.	n.r.	Serum	Excess FLEC removed by reaction with Gly and pentane extraction .	105
<b>Metamphetamine, ephedrine, pseudoephedrine</b>	RP-LC	Stationary phase: C18; Mobile phase: 0.05M phosphate buffer/ACN – 35/65	FL (exc. 295nm, em. 315nm)	77.6 nM	(+)	Dichloro methane	Sodium hydroxide	12	30 min	n.r.	n.r.	Illicit samples	-	110
<b>Metamphetamine and its metabolites</b>	RP-LC	Stationary phase: C18; Mobile phase: acetate buffer (pH-3.6)/ACN/THF – 54/25/21 or 59/26/15 (depending on analytes)	FL (exc. 265nm, em. 330nm)	33.5 nM	(-)	n.r.	Phosphate	7.8	24 h	r.t.	1:300	Urine	Excess FLEC removed by reaction with Gly and pentane	113

												or ethyl acetate extraction		
<b>Amphetamine</b>	RP-LC	Stationary phase: C18; Mobile phase: acetate buffer (pH-3.6)/ACN/THF – (60/27.5/12.5)	Electrochemical, FL (exc. 265 nm, em. 330 nm)	<i>n.r.</i>	(-)	Acetone	Phosphate	7.8	Overnight	r.t	<i>n.r.</i>	Dialysate	Derivatives extracted with pentane or ethyl acetate	
<b>Primary and secondary amphetamines</b>	RP-LC	Stationary phase: C18; Mobile phase: MeOH, acetate buffer (0.1M, pH-4.5), gradient elution	UV (265nm), FL (exc. 212nm, em. 313nm)	370 nM	(-)	Acetone /MeOH	Carbonate	10	10 min	r.t.	<i>n.r.</i>	Urine	-	114
<b>Amphetamine-derived designer drugs</b>	RP-LC	Stationary phase: C18; Mobile phase: MeOH, acetate buffer (0.1M, pH-4.5), gradient elution	UV (265nm)	37 nM	(-)	Acetone /MeOH	Carbonate	9.5	15 min	r.t.	<i>n.r.</i>	Plasma and urine	-	122
<b>Carnitine</b>	RP-LC, CE	LC: Stationary phase: C18; mobile phase: solv. A: 25%ACN, 75% 5mM TBA + 50mM KH <sub>2</sub> PO <sub>4</sub> (pH-7); solv. B: 75%ACN, 25% 5mM KH <sub>2</sub> PO <sub>4</sub>	LC: UV (260nm) FL (exc. 260nm, em. 315nm)	<i>n.r.</i>	(+)	Acetone	Tertbutylamine	7	25 min	80	1:15	Standard solutions	-	111
<b>Carnitine</b>	RP-LC	Stationary phase: C18; Mobile phase: TEA phosphate buffer/ACN – 73/27	FL (exc. 260nm, em. 310nm)	<i>n.r.</i>	(+)	<i>n.r.</i>	Carbonate	<i>n.r.</i>	1 h	45	1:1.5	Standard solutions	-	108
<b>Carnitine</b>	RP-LC, CZE	Stationary phase: C18;	LC: FL (exc. 260nm,	<i>n.r.</i>	(+) and (-)	Acetone	Carbonate	10.4	60 min	45	1:1	Microbiological	Reaction stopped with	101

		Mobile phase: TEAP (triethylamine 50mM, pH-2.6-5.2)	em. 310nm)								and phar- maceu- tical sam- ples	acetate buffer		
<b>Baclofen</b>	RP-LC	Stationary phase: C8 Mobile phase: acetate buffer/ACN – 7/12 + $\gamma$ - CD (10 mM)	UV (250nm)	n.r.	(+)	n.r.	Borate	6.85	4 min	n.r.	n.r.	Stand- ard solu- tions	Derivati- zation done according to ref. <sup>78</sup>	126
<b>Meflo- quine</b>	RP-LC	Stationary phase: C18; Mobile phase: ACN/H <sub>2</sub> O/acetic acid – 82/18/0.07	UV (263nm), FL: 10 nM em. 475nm)	UV: 250 nM FL: 10 nM	(-)	ACN	Borate	10	40 min	r.t.	n.r.	Plasma	-	104
<b>Nonflu- orinated quino- lone</b>	RP -LC	Stationary phase: C18; Mobile phases: for LC- MS: MeOH/ACN/H <sub>2</sub> O/FA – 40/40/20/0.1; for FL: MeOH/ACN/H <sub>2</sub> O/FA – 35/35/30/0.1	MS, FL (exc. 295nm, em. 530nm)	28 nM	(-)	ACN	Borate	8.2	30 min	r.t.	n.r.	Plasma	Excess FLEC was reacted with Gly	123
<b>S 12024 (quino- line deriva- tive)</b>	RP-LC	Stationary phase: RP- cyanopropyl Mobile phase: ACN/sulphuric acid (0.05%, pH-3)	FL (exc. 260nm, em. 310nm)	40 nM	(+)	ACN	Water- triethylami- ne	n.r.	1 h	r.t.	n.r.	Plasma	Excess FLEC was reacted with DL- Pro	109
<b>Glufosi- nate</b>	RP-LC	Stationary phase: C18; Mobile phase: ammonium acetate (pH-5)/ACN – 77/23	FL (exc. 260nm, em. 305nm	27.6 nM	(+)	Acetone	Borate	8.5	30 min	40	n.r.	Serum and urine	Excess FLEC extracted with ethyl acetate	119

N-6-(endo-2-norbo-myl)-9-methyladenine	RP-LC	Stationary phase: C18; Mobile phase: ACN/Acetic acid (0.1M, pH-2.9) – gradient elution	UV (270nm), MS (QQQ)	<i>n.r.</i>	(+)	Acetone	Different buffers	7	>1 h	r.t.	1:11	Standard solutions	-	117
Oxytocin analogues	RP-LC	Stationary phase: C4; Mobile phase: sodium acetate - ACN	FL (exc. 260nm, em. 315nm) MS	<i>n.r.</i>	(+)	<i>n.r.</i>	Borate	6.85	4 min	<i>n.r.</i>	<i>n.r.</i>	Standard solutions	Derivatization done according to ref <sup>78</sup>	167
Amino acids	SFC	Stationary phase: 5µm Nucleosil-100; mobile phase: CO <sub>2</sub> -H <sub>2</sub> O-MeOH-MeNH <sub>2</sub>	UV (269nm)	<i>n.r.</i>	(+)	<i>n.r.</i>	Borate	8.5	10 min	<i>n.r.</i>	<i>n.r.</i>	Standard solutions	Pentane extraction	120

Abbreviations: n.r.: not reported; r.t.: room temperature;  $\gamma$ -CD:  $\gamma$ -cyclodextrin; ACN: acetonitrile; EtOH: ethanol; FA: formic acid; MeNH<sub>2</sub>: methylamine; MeOH: methanol; TBA: tetrabutylammonium; TEAP: triethylamine phosphate; THF: tetrahydrofuran; TMA: tetramethylammonium.

## Annex 2

Sample	CE mode	Separation conditions	Detection	LOD	(+) or (-)-FLEC	FLEC solvent	Reaction buffer	pH	Reaction time	t°C	Reaction place	Analyte: FLEC ratio	Matrix	Obs.	Ref
Amino acids	MEKC	BGE: SDS, pH-9.2	UV (254nm)	n.r. <sup>a</sup>	(-)	Acetone	Borate	9	2 min	r.t. <sup>b</sup>	PC	1:3.33	Standard solutions	Excess FLEC extracted with pentane.	127
di- and tri-peptides	MEKC	BGE: SDS, pH-9.2	UV (254nm)	n.r.	(-)	Acetone	Borate	9	2 min	25	PC	1:3.33	Standard solutions	Excess FLEC extracted with pentane.	155
Ser, Ala, Val, Met, Leu, Phe, Trp	MEKC	BGE: 10mM phosphate or 5mM NaTB, 25mM SDS, 10-15%ACN	LIF (exc. 248nm, em. 310 nm)	30 nM	(+)	Acetone	Borate	8	4 min	r.t.	PC	1:11.4	Standard solutions	Excess FLEC removed by reaction hydroxyproline.	128
Amino acids	MEKC	BGE: borate buffer (40mM, pH-9.2), 21mM SDS, 8.5%IPA	UV (254nm)	5 µM	(-)	Acetone	Borate	9.2	9.5 min	r.t.	IC	1:1.6	aCSF	-	79
Amino acids	MEKC	BGE: 150 mM APFO (pH-9.5)	MS	4-11 µM	(-)	ACN	Borate	9.2	15 min	r.t.	IC	1:12	aCSF	-	80
Amino acids	MEKC	BGE: 150 mM APFO (pH-9.5)	MS	0.38 -	(+)	ACN/acetone	Borate	9.5	10 min	r.t.	PC	1:50-1:100	CSF	-	121

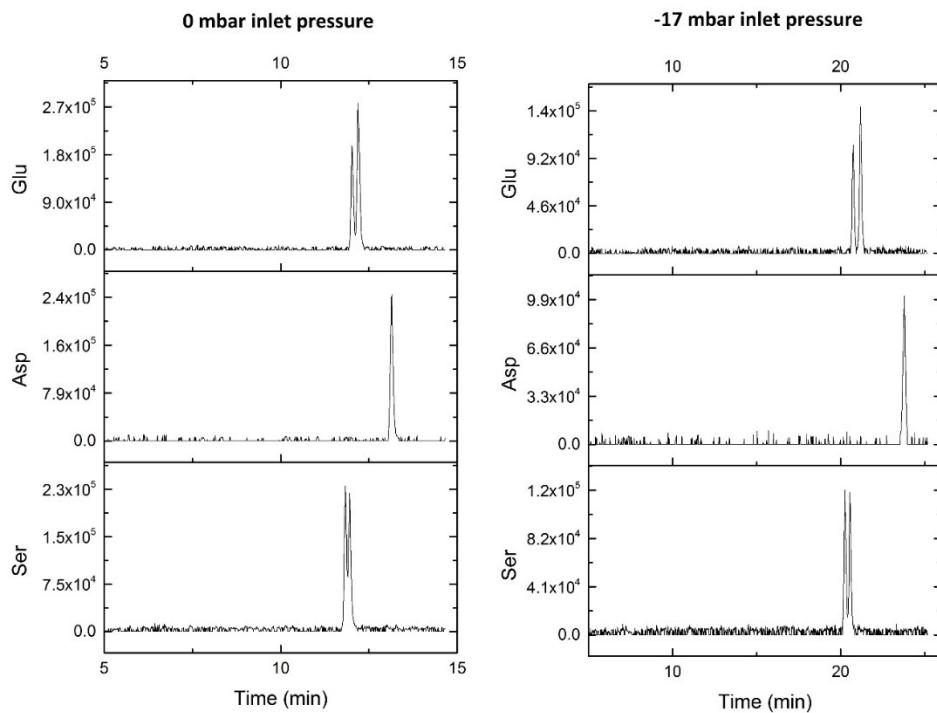
				4.62 μM											
<b>Carnitine</b>	RP-LC, CZE	BGE 50mM $\text{KH}_2\text{PO}_4$ (pH- 3.4), 14kV	CE: UV (214nm)	<i>n.r.</i>	(+)	Acetone	TBA	7	25 min	80	PC	1:15	Standard solutions	-	111
<b>Carnitine</b>	RP-LC, CZE	CE: 50 mM phosphate buffer pH-2.6, TBABr	CE: UV (200, 214, 254)	<i>n.r.</i>	(+) and (-)	Acetone	Carbonate	10.4	60 min	45	PC	1:1	Standard solutions	Reaction stopped with acetate buffer	101
<b>Carnitine</b>	CZE	CE: 50 mM phosphate buffer pH-2.6, TBABr	UV (200, 214, 254)	<i>n.r.</i>	(+) and (-)	Acetone	Carbonate	10.4	60 min	45	PC	1:1	Standard solutions	Reaction stopped with acetate buffer	112
<b>Arg-Gly</b>	CE	BGE: Phosphate buffer/SDS	UV (256nm)	<i>n.r.</i>	(+) and (-)	Acetone	Borate	9.2	10 min		PC	1:4	Standard solutions	Excess FLEC extracted with pentane.	102
<b>β-blockers</b>	CEC	St. phase: C18; 75μm ID; BGE: 5mM borate and acetate buffers, 30kV	UV (206nm)	3.83 μM	(+)	Acetone	Borate	9	10 min		PC	1:3.33	Standard solutions	-	166

Abbreviations: n.r.: not reported; r.t.: room temperature; PC: pre-capillary/pre-column; ACN: acetonitrile; APFO: ammonium perfluorooctanoate; aCSF: artificial cerebrospinal fluid; CSF: cerebrospinal fluid; NaTB: sodium tetraborate; SDS: sodium dodecylsulphate; TBA: tetrabutylamine; TBABr: tetrabutylammonium bromide;

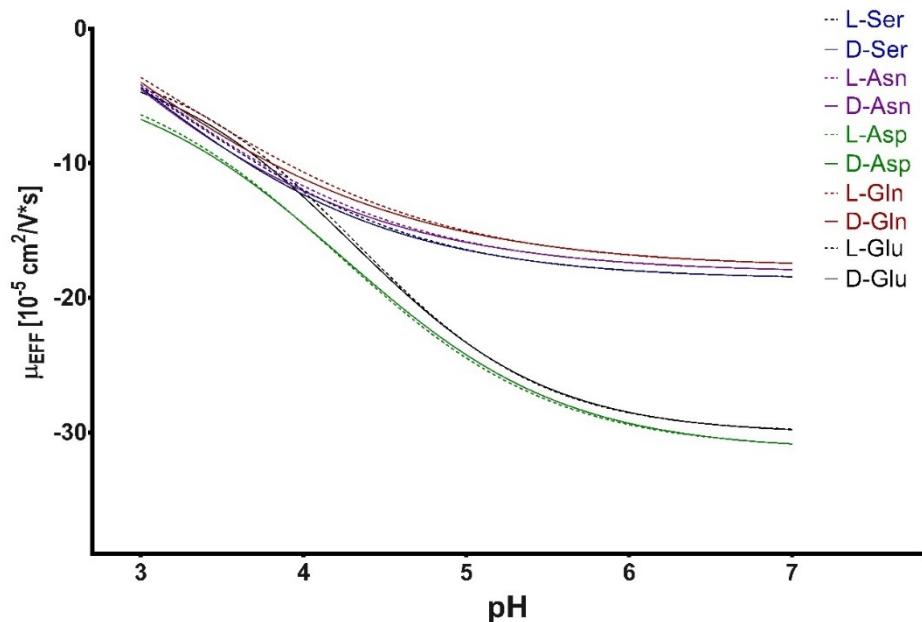
## Annex 3

*Predicted vs. experimental areas obtained in the optimized derivatization conditions for the studied amino acids*

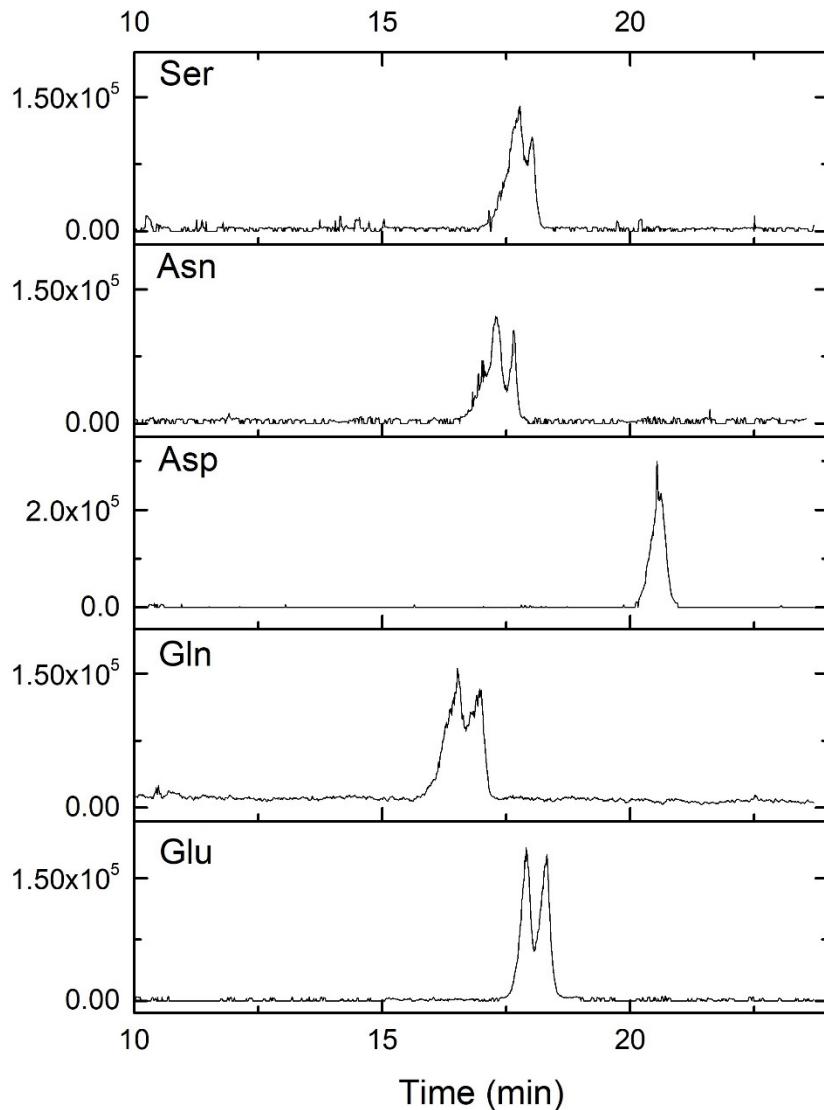
	<b>Predicted</b>	<b>Lower</b>	<b>Upper</b>	<b>Experimental</b>
<b>Gly</b>	2.78E+07	2.06E+07	3.51E+07	3.45E+07
<b>Ala</b>	1.94E+07	1.24E+07	2.65E+07	2.60E+07
<b>Ser</b>	3.86E+07	2.86E+07	4.86E+07	5.68E+07
<b>Val</b>	2.24E+07	1.52E+07	2.97E+07	2.36E+07
<b>Thr</b>	3.07E+07	2.13E+07	4.01E+07	4.59E+07
<b>Leu &amp; Ile</b>	3.28E+07	1.77E+07	4.79E+07	5.10E+07
<b>Gln</b>	4.75E+07	3.33E+07	6.18E+07	6.76E+07
<b>Glu</b>	1.15E+07	6.97E+06	1.61E+07	2.14E+07
<b>Met</b>	3.83E+07	2.48E+07	5.17E+07	4.78E+07
<b>Phe</b>	3.24E+07	2.16E+07	4.32E+07	4.11E+07
<b>Tyr</b>	2.33E+07	1.68E+07	2.98E+07	2.50E+07
<b>Trp</b>	2.75E+07	1.65E+07	3.85E+07	3.13E+07

**Annex 4**

**Impact of the application of a negative pressure at the inlet vial on migration time and resolution. BGE: 60 mM acetic acid pH 4 (adjusted with NH<sub>4</sub>OH), 20°C, 30 kV.**

**ANNEX 5**

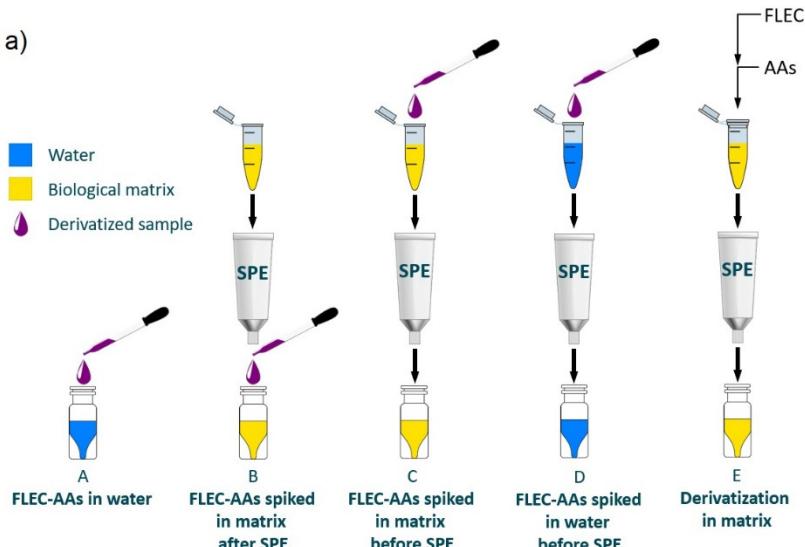
**Electrophoretic mobilities of the 5 pairs of diastereomers determined on bare fused silica capillaries.  
BGE 60 mM acetic acid (pH adjusted with NH<sub>4</sub>OH), 20°C, 30 kV.**

**ANNEX 6**

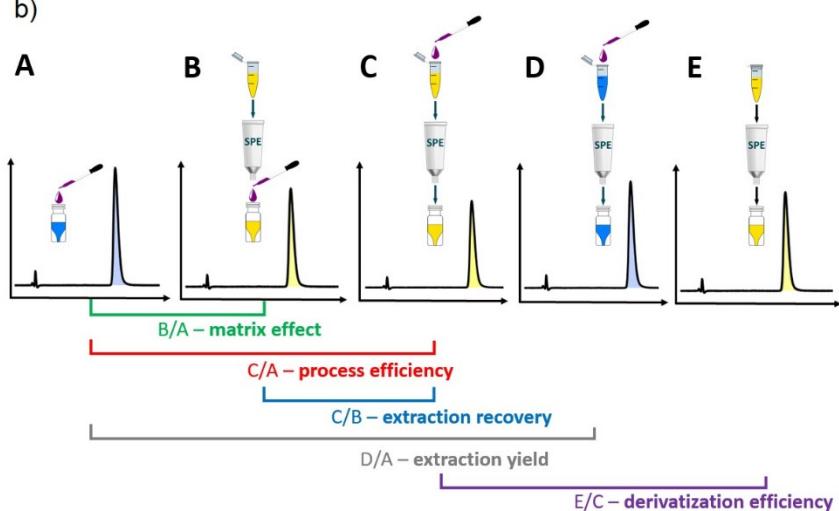
Analysis of a aCSF sample without any sample preparation. BGE: 150 mM acetic acid pH 4 (adjusted with NH<sub>4</sub>OH), 20°C, 30 kV

## ANNEX 7

a)



b)



Extraction process characterization: a) the preparation of the different samples; b) calculation of the parameters relevant for extraction characterization