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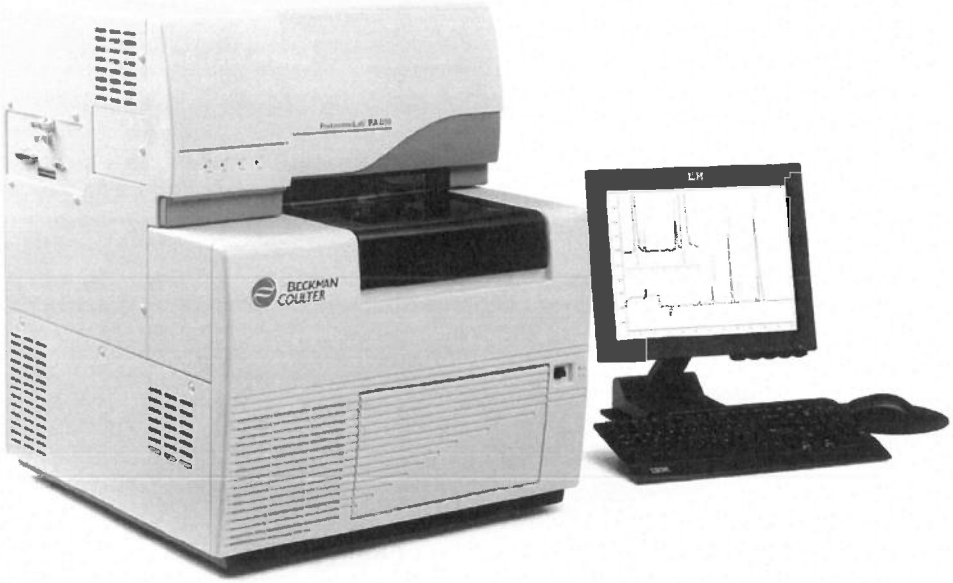

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2015

Method development for the chiral separation of amino acids through micellar electrokinetic chromatography and in-line derivatization with 9-fluoroenylmethyl chloroformate

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Samenvatting

Aminozuren vormen de bouwstenen voor eiwitten en het is algemeen bekend dat deze een belangrijke functie hebben in vele biologische functies. Er is daarom veel belang bij de mogelijkheid om aminozuren snel en efficiënt te kunnen analyseren. De meeste amino zuren hebben een chiraal centrum en daardoor twee vormen, een L- en D-enantiomeer. In voedingssupplementen worden uitsluitend de L-enantiomeren toegevoegd en uit meerdere studies is gebleken dat de D-enatiomeren belangrijke biologische functies vervullen in mensen en andere organismen. Er is daarom belang voor de ontwikkeling van snelle en efficiënte methodes om amino zuren chiraal van elkaar te scheiden. Des te sneller en simpeler de amino zuren gescheiden en gedetecteerd kunnen worden, des te meer tijd en geld dat bespaard voor bedrijven.
Het doel van dit onderzoek was het ontwikkelen van een efficiënte en gevoelige methode om aminozuren chiraal van elkaar te scheiden met in-line derivatisatie met behulp van 9-fluoroenylmethyl chloroformate (FMOC-Cl). Door in-line derivatisatie toe te passen wordt de monstervoorbwerking verminderd wat tijd bespaard. Door het gebruik van β-cyclodextrin als chirale selector en micellaire elektrokinetische chromatografie kunnen amino zuren chiraal van elkaar gescheiden worden. De bufferoplossing bestond uit 40 mM natrium tetraboraat (pH 9.4), 25 mM natriumdodecylsulfaat (SDS), 17% isopropanol en 30 mM β-cyclodextrine. Dit onderzoek is het begin van de ontwikkeling van een methode voor het chiraal scheiden en detecteren van amino zuren, wat in de toekomst kan leiden tot een methode dat wordt toegepast door bedrijven.

Nadat een mix van 5 aminozuren (methionine, isoleucine, fenylalanine, glutaminezuur en asparaginezuur) succesvol chiraal van elkaar werden gescheiden met in-line derivatisatie en fluorescentie detectie werden de mogelijkheiden onderzocht om de gevoeligheid van de methode te verhogen. Door de interne diameter van de capillair te verhogen van 50μm naar 75μm werd er een verbetering van 1000% waargenomen in de gevoeligheid van de methode.
Nadat alle amino zuren waren getest bleek tyrosine succesvol toegevoegd te kunnen worden aan de mix van aminozuren, deze mix bestond nu uit methionine, isoleucine, fenylalanine, glutaminezuur, asparaginezuur en tyrosine. De methode was ook in staat om valine, leucine, glycine, proline, alanine en tryptofaan chiraal te scheiden, maar de enantiomeren overlappen met andere pieken als deze in de eerder genoemde mix van amino zuren werden toegevoegd. In de hoop om meer aminozuren toe te kunnen voegen zijn er experimenten gedaan met verschillende temperaturen en SDS concentraties om de migratietijden van de enatiomeren te beïnvloeden. Uit het resultaat bleek dat het veranderen van deze parameters geen mogelijkheden gaf om nieuwe amino zuren toe te kunnen voegen aan de mix van methionine, isoleucine, fenylalanine, glutaminezuur, asparaginezuur en tyrosine.

Om de methode te optimaliseren werden de volgende parameters getest: de FMOC-Cl concentratie en injectietijd, aminozuur injectietijd, buffer injectietijd en de mix voltages en tijdsduur.
Nadat alle parameters waren geoptimaliseerd werd de herhaalbaarheid en lineariteit van de methode getest. Het laatste experiment was het analyseren van een urine monster, maar geen van alle pieken kon geïdentificeerd worden.

De ontwikkelde methode was in staat tyrosine, methionine, isoleucine, fenylalanine, glutaminezuur en asparaginezuur in één mengsel chiraal van elkaar te scheiden met een herhaalbaarheid van 3 - 9 %RSD in een lineair bereik van 41.7 tot 2.1 μM per enantiomeer. Behalve tyrosine, die had een bereik van 41.7 tot 8.3 μM per enantiomeer.

Index

1 Abbreviations

AA	Amino Acid
ACN	Acetonitrile
Ala	Alanine
Arg	Arginine
Asn	Asparagine
Asp	Aspartic acid
β-CD	Bèta-cyclodextrin
BGE	Background electrolyte
CCD	Charge-coupled device
CE	Capillary electrophoresis
CMC	Critical micelle concentration
Cys	Cysteine
EKC	Electrokinetic chromatography
EOF	Electroosmotic flow
Flu	Fluorescence
FMOC	9-fluoroenylmethyl chloroformate
FMOC-Cl	9-fluoroenylmethyl chloroformate-chloride
Gln	Glutamine
Glu	Glutamic acid
Gly	Glycine
His	Histidine
ID	Internal diameter
Ile	Isoleucine
IPA	Isopropanol
Leu	Leucine
LOD	Limit of detection
Lys	Lysine
MEKC	Micellar electrokinetic chromatography
Met	Methionine
MT	Migration time
NA	Not applicable
NaOH	Sodium hydroxide
Phe	Phenylalanine
Pro	Proline
R_s	Resolution
SDS	Sodium dodecyl sulfate
S/N	Signal to noise
Ser	Serine
STD	Standard deviation
%STD	Relative standard deviation
Thr	Threonine
Trp	Tryptophan
Tyr	Tyrosine
UV	Ultraviolet
Val	Valine

2 Introduction

It is well known that amino acids are very important compounds in nature. They are the building blocks of proteins and play a role in many biological functions. Over 500 different amino acids are found in nature, but the human genetic code only directly codes for 20 of those amino acids. Most amino acids have stereoisomers, a D-form and a L-form, caused by the chiral center on the α carbon, except for glycine. The structure is characterized by a carboxylic group, an amine group and a side group (see Figure 1).

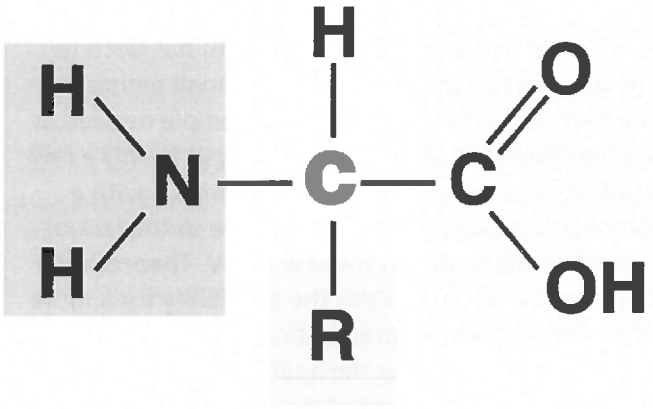

Figure 1: basic structure of an amino acid
Source: edited from website http://cmapspublic3.ihmc.us/rid=1181483020655_87116810_4691/Macromoleculesv2

Natural proteins are exclusively built from L-amino acids [1] and for a long time it was hypothesized that only L-forms are present in higher animals. It was thought that the D-forms were only utilized in lower species such as bacteria and microorganisms [2]. Due to technological and analytical advances studies have shown that D-amino acids are present in mammals [3]. It was assumed that the D-form did not serve a specific biological function and in this context the concentrations of D-aspartate in rat tissue and human blood were surprisingly high [4] Subsequent studies confirmed that some D-amino acids exist in mammalian tissues in high concentrations and sometimes even exceeding the concentration of its L-enantiomer, for example D-asparate and D-serine [5].

These D-amino acids fulfill specific biological functions. D-aspartic acid plays an important role in endocrine regulation and D-serine with neurotransmission [4]. Multiple relationships between pathophysiological processes (Alzheimer's disease, Parkinson's disease, schizophrenia and renal disease) and the amounts of D-serine and D-aspartic acid are observed [3]. Increased concentrations of D-alanine were observed in the brains of Alzheimer's patients, 20.8±5.3 nmol/ g for Alzheimer brains and 9.5±2.9 nmol/g for normal brains [6]. An increase of D-proline, D-serine and D-alanine concentration was found with patients of renal disease [7].
Amino acids have become a popular nutritional supplement marketed to athletes. Commercial supplements containing valine, isoleucine and leucine are available on the market and are widely used to improve performances. The amino acids may be racemized to their mirror image configuration during food processing or could be synthesized by microorganisms [1]. Although technical advances make single enantiomer production on a commercial scale possible, analytical methods are still necessary to determine the enantiomeric purity.
It is clear that chiral analysis of amino acids is of great interest and importance in many fields. Chiral separation of amino acids has extensively been studied using chiral stationary phases in gas chromatography and liquid chromatography [2, 7]. Currently established chiral chromatographic separation methods are able to separate most of the amino acids [8, 9], but there is still a need for a fast and sensitive method for the analysis of amino acid in complex matrices.

Enantiomers which are not resolved by high performance liquid chromatography may often be resolved by a simple capillary electrophoresis method (CE) [10]. The CE technique is a separation method based on the electrokinetic separation of samples and was first introduced by Mikkers et al [11], Jorgenson and Lukacs [12] and Hjertén [13]. The CE technique can offer very high separation efficiency and separation power and is nowadays considered as an important technique complementary to chromatographic separation techniques [10]. CE separates charged analytes which is a limitation that was resolved by the development of electrokinetic chromatography (EKC). EKC is a branch of CE based on chromatographic separation principles using a solution that contains an ionic carrier [14]. Several modes are available in EKC of which micellar EKC (MEKC) has become the most popular. MEKC uses micellar solutions of ionic surfactants for separating small neutral molecules [14]. The advantage of CE over HPLC for example is the low amount of sample needed, a few microliters is often enough. Also the volume of buffers used after a day of analysis is only a few centiliters which is a lot less compared to the amounts used by an HPLC. By combining CE with a chiral selector and UV detection, chiral separation of amino acids is possible [8]. Due to the lack of chromophore groups the amino acids need to be derivatized to detect them with UV. Theoretically fluorescence detection is more sensitive than UV detection which enables the possibility for a more sensitive method. The derivatization process can be done as sample preparation before the analysis (off-line) or the derivatization can be performed in the capillary during the analysis (in-line) as was shown by Fradi et al [8]. By using in-line derivatization the overall speed of the analysis is increased while also reducing the amount of manual labor. The goal of this study was to develop an efficient method to chirally separate amino acids using β-cyclodextrin as chiral selector with MEKC and fluorescence detection with in-line derivatization. For a company a relatively simpler and faster method, compared to other methods, is often cheaper in the long run. This study is the beginning of the development for a method to chirally separate and detect amino acids which in the future might lead to a method used by companies.

3 Experimental design

3.1 CE and MEKC

CE is a separation method based on the electrokinetic separation of samples in a silica capillary. The capillary is filled with a running buffer called the background electrolyte (BGE) with the ends of the capillary placed in a vial with the BGE and the anode and cathode. By applying an electrical field between the two vials the analytes will migrate through the capillary depending on their charge. With bare-fused silica capillaries the silanol groups are negatively charged which attracts cations. This creates a so called electrical double layer which moves through the capillary due to the applied voltage. Because of this movement, the bulk of the buffer is dragged along which creates the electroosmotic flow (EOF). Because of the EOF it is possible to migrate both positive, negative and neutral analytes to the same direction in a capillary. [15]

MEKC is one of the modes of operation that can be used with CE and was used for this study. It is a combination of electrophoresis and chromatography. Its strength is that it is the only electrophoretic technique that can be used for the separation of both neutral and charged analytes.
The separation of the neutral analytes with MEKC is accomplished by the use of surfactants in the running buffer. When the concentration of the surfactant is above the critical micelle concentration (CMC), micelles are formed. The spherical micelles have a charged side which is focused to the outside and the hydrophobic tails are oriented towards the center.
The micelles migrate either with or against the EOF depending on the charge. Even when the micelles

migrate against the EOF, the EOF is generally faster so the net movement of the micelles are in the direction of the EOF. [15]
Other studies have used MEKC with sodium dodecyl sulfate (SDS) as surfactant combined with a chiral selector for the separation of amino acids [8, 9]. SDS has a critical micelle concentration of 8 to 9 mM in water and it is an anionic surfactant. This means that SDS micelles will migrate towards the anode, which is the opposite direction of the EOF. The SDS micelles act as a pseudo-stationary phase and the solutes (amino acids) will partition in and out of the micelles. The stronger the amino acid interacts with the SDS micelle the longer its migration time since the micelle carries it against the EOF (see Figure 2). When the amino acid is not in contact with the micelle it is carried with the EOF. The more hydrophobic the amino acid is, the more strongly it interacts with the SDS micelles, thus will be retained longer. For this study MEKC with SDS as surfactant and β-cyclodextrin as chiral selector was used to develop a method for the enantioseparation of amino acids with in-line derivatization and fluorescence detection.

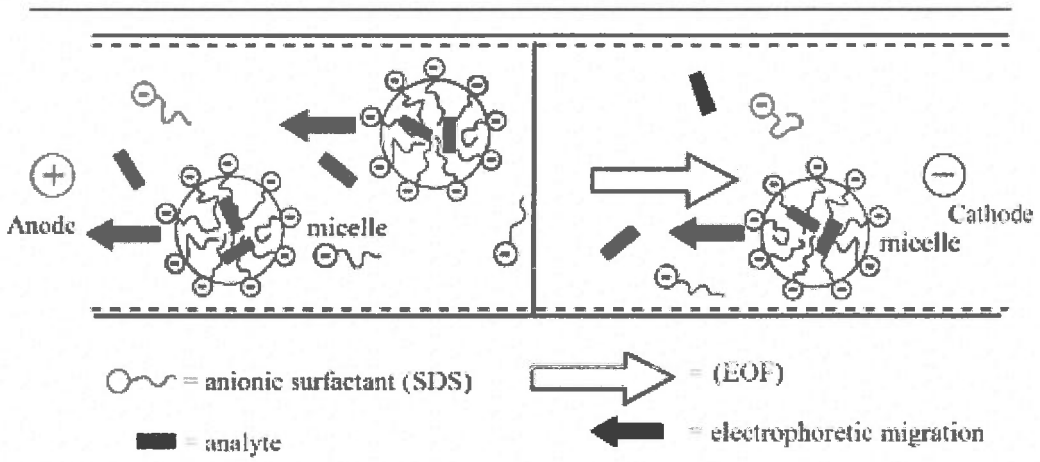

Figure 2: principle of MEKC with SDS
Source: edited from [27]

3.2 Fluorescence detection

UV detection is a very common form of detection with capillary electrophoresis and almost all CE devices are equipped with a built-in UV detector. The UV detector measures the UV absorption of the analytes migrating through the capillary through a small window which is made in the capillary. This window is usually made by simply burning of the exterior coating of the capillary, creating a small clear window. Unfortunately the internal diameter of the capillary is small resulting in a relatively short path length compared to liquid chromatography. As a result the sensitivity of UV detection is limited with CE. Theoretically fluorescence detection is more sensitive than UV detection, this makes a fluorescence a logical alternative for more sensitivity and was therefore used for this study. The challenge with fluorescence detection is that the molecules need to be excited at a given wavelength while the emitted light has to be efficiently captured without interference of scatter and excitation light.
The fluorescence setup used for this study was based on the setup described by Kort et al [16]. The capillary window was placed through a detector cell outside the CE device. Inside the cell the capillary was placed through an optical cone where only a small part of the capillary window was inside the cone. The excitation light was focused on the capillary through a ball lens which excites the analytes. The fluorescent emission light was then trapped in the capillary due to reflection because the refractive index of fused silica is higher than that of the running buffer solution and air. A small layer of glycerol was placed on the section of the window inside the cone, because of the refractive index of glycerol, which is slightly higher than that of fused silica, the emission light could enter the cone. The emission light was transferred through the cone into the optical cable to the detector. By

using this setup the noise caused by the excitation light was reduced because the light from the source could only reach the detector through the capillary and cone (see Figure 3).
The emission light is detected in a large wavelength range by a charge-coupled device (CCD), this setup is called wavelength-resolved fluorescence.

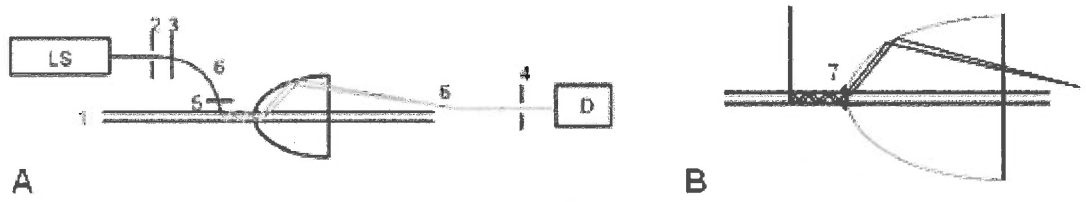

Figure 3: Schematic representation of the detector cell. The flow direction in the capillary is from left to right. LS=light source, D=detector, 1=capillary, 2=pinhole, 3=excitation filter, 4=slit, 5=lens, 6=optic cable and 7=glycerol
Source: edited from [16]

It is possible to extract an electropherogram from a specific wavelength from the range of measured wavelengths. Usually this specific wavelength is the maximum emission wavelength. The signal to noise ratio can be improved by averaging a range of wavelengths around the maximum emission wavelength [17]. This was possible due to the broad emission spectra of the FMOC-amino acids and was done through a program written manually within the software. For this study the program was used to average the signal of the chosen wavelength 330 ± 10 nm, this wavelength was chosen based on the optimum of the FMOC emission spectrum measured with the same fluorescence detector that was used for this study. With the averaging of the spectra the obtained electropherogram had reduced noise with a small loss of signal compared to the electropherogram of a single wavelength. Overall this resulted in improved signal to noise ratio's because the program removes additional background noise.

3.3 Derivatization and separation of amino acids

All of the amino acids, besides 3 (tryptophan, phenylalanine and tyrosine), cannot be detected with UV or fluorescence detection due to lack of chromophore groups. An efficient way to solve this problem is derivatization. Derivatization is a technique where a compound is transformed through a reaction with another compound. There are many reagents which can be used for the derivatization, some of the derivatization reagents that has been used in other studies are dansyl chloride [18], 3-(2-furoyl)quinoline-2-carboxaldehyde [19], 6-aminoquinolyl-N-hydrosuccinimidyl carbamate [20], napthalene-2,3-dicarboxaldehyde [21], ortho-phthalaldehyde/N-acetyl l-cysteine [22], 1,2-naphtoquinone-4-sulfonate [23], 9-fluoroenylmethyl chloroformate [24] and 1-(9-fluorenyl)-ethyl chloroformate [9].

For this study 9-fluoroenylmethyl chloroformate (FMOC) was chosen because of its fast reaction and stable products [25]. FMOC is a derivatization reagent which reacts with primary and secondary amine groups (see Figure 4), and can be used to derivatize multiple amino acids at the same time in one mixture [10, 25].

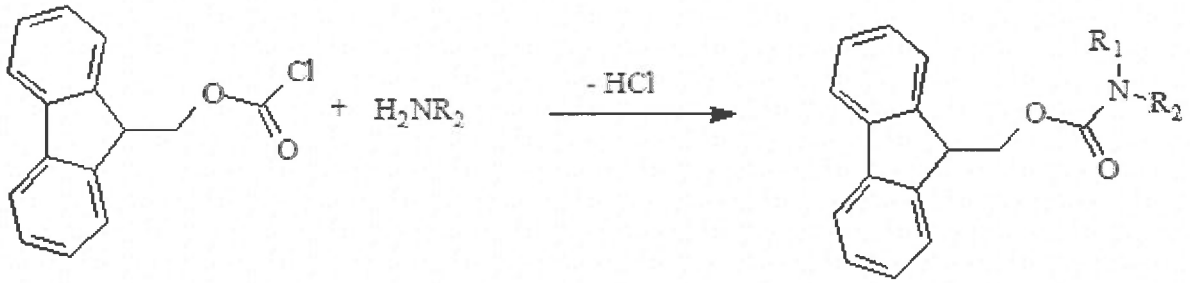

Figure 4: FMOC derivatization reaction of an amino acid, with the FMOC and an amino acid on the left side, and the derivatized amino acid on the right side

The in-line derivatization was performed by injecting the amino acid sample, a borate buffer plug and the FMOC solution subsequently. By applying a voltage the amino acid plug moves through the buffer plug and the FMOC plug. The high pH borate buffer plug deprotonates the amino acids which promotes the derivatization of the amino acids with the FMOC.

For the chiral separation of the amino acids a chiral selector must be used. For this purpose cyclodextrins were used because they can be dissolved in the background electrolyte. Cyclodextrins are a family of cyclic oligosaccharides with glucopyranose subunits [26]. The three different types of cyclodextrins (α-cyclodextrin, β-cyclodextrin and γ-cyclodextrin) are determined by their amount of glucopyranose subunits. α-cyclodextrin has 6 glucopyranose subunits, β-cyclodextrin has 7 and γ-cyclodextrin has 8 (see Figure 5).

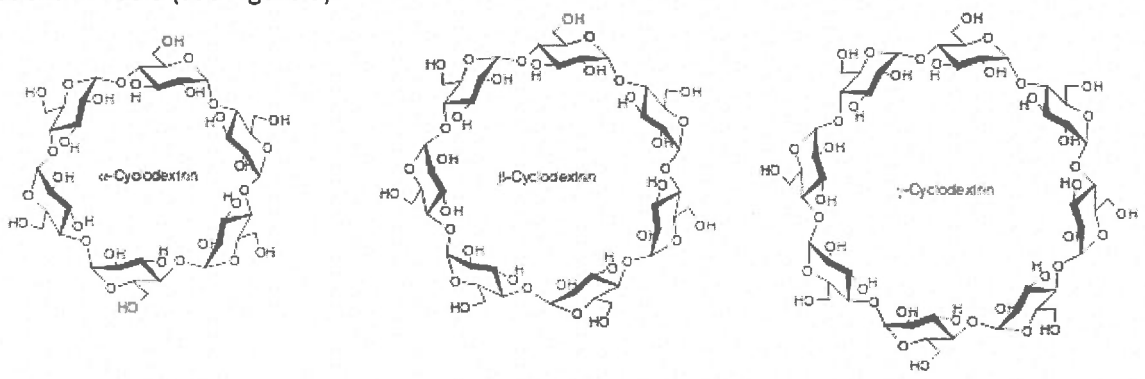

Figure 5: From left to right respectively α-, β- and γ-cyclodextrin
Source: edited from [28]

The exterior of a cyclodextrin is hydrophilic and its interior is a lot less hydrophilic but not hydrophobic. The cyclodextrin molecule is able to host other molecules which results in a host-guest complex. The formation of such a complex influences the physical and chemical properties of the guest molecule which allows discrimination between enantiomers and makes enantioseparation possible. For this study β-cyclodextrin was chosen as chiral selector based on the results from experiments done by a previous student. These results revealed that the β-cyclodextrin had the best resolution compared to γ-cyclodextrin, α-cyclodextrin was not tested. Additional advantages of the β-cyclodextrin was its availability and relatively low price.

3.4 Outline of research

The main goal of this study was to develop an efficient method to chirally separate amino acids using β-cyclodextrin as chiral selector with MEKC and fluorescence detection with in-line derivatization. This study is the beginning of the development for a method to chirally separate and detect amino acids which in the future might lead to a method used by companies and further research.

To achieve the main goal the following subgoals were made:

1. Successfully transition to in-line derivatization instead of off-line derivatization
2. To obtain better sensitivity by transitioning to fluorescence detection
3. Baseline separation with as many amino acid enantiomers possible in a single mixture without overlap
4. Optimize the sensitivity of the method as much as possible
5. Determine the repeatability and the linearity of the method
6. Test the final method on a real sample

The outline of the research is shown in Figure 6. The research is divided in five sections: Development, Testing, Optimization, Validation and Real sample.

Development

In this section five experiments are performed to start the development and to achieve subgoals 1 and 2. With the first three experiments the transition to in-line derivatization was made and it was hypothesized that Trp would be successfully enantioseparated without overlap with both the off-line and the in-line derivatization. This hypothesis was based on the results of a similar experiment by a previous student.

With the last two experiments the transition to fluorescence detection was made. It was hypothesized that this transition would increase the sensitivity by circa 100 times. This hypothesis was based on the theory that fluorescence detection is generally circa 100 times more sensitive than UV detection.

Testing

In this section two experiments are performed, in the first experiment 20 amino acids are measured individually. The second experiment is a preliminary experiment to create a mixture with as many amino acids as possible which are all enantioseparated without overlap. Both these experiments work towards achieving subgoal 3. The results of the 20 amino acids will determine which of the amino acids can already be observed and enantioseparated with the current method. The migration time is also an indication for which amino acids might be able to be measured in a mixture and baseline separated without overlap. These amino acid are measured together in the second experiment to determine if they can indeed be baseline separated without overlap.

Optimization

In this section 10 experiments are performed. These experiments were done to optimize the method by improving the sensitivity as much as possible and to expand the amino acid mixture as much as possible. These experiments work towards achieving subgoals 3 and 4.

With the '50µm ID capillary VS 75µm ID capillary' experiment it was hypothesized that the 75µm ID capillary would result in a 25% increase in sensitivity. This was believed because more light from the excitation source would be able to go through the 75µm ID capillary compared to the 50µm ID capillary.

With the 'Amino acid mixture' experiment it was believed that one or more from the following amino acids could be added to the amino acid mixture: Val, Leu, Ala, Gly, Pro and Tyr. This was based on the previously acquired migration times of these amino acids from the section 'Testing'.

The 'Temperature' experiment was done because it was hypothesized that changing the temperature would change the migration times and thus the migration window. It was believed that by changing the migration window it might be possible to add more amino acids to the mixture.

With the 'SDS concentration' experiment it was known that by changing the SDS concentration the migration window could be influenced. It was believed that by increasing the SDS concentration, the migration times would increase as well. And that this would enable the possibility to add more amino acids to the mixture.

The 'FMOC peak width' experiment was performed because it was considered that some of the amino acids which overlap with the FMOC impurities would not overlap if the FMOC peak width would be reduced. It was believed that reducing the FMOC concentration or FMOC injection would reduce the FMOC peak width.

The 'FMOC concentration and injection time' experiment was done because no optimal FMOC concentration and injection time was found in the previous experiment. The previous experiment did conclude that decreasing the concentration and injection time reduce the FMOC peak width, but the data was insufficient to determine which concentration and injection time were best. This experiment was used to determine what FMOC concentration and injection time results in the best sensitivity while reducing the FMOC peak width.

With the 'Amino acid injection time' experiment it was thought that the amino acid injection time could influence the sensitivity. It was believed that the size of the amino acid injection plug had impact on the derivatization yield. For example, if the injection time was increased the amino acid plug would also increase and therefore the amount of amino acids that could be derivatized.

With the 'Buffer injection time' experiment it was considered that the buffer plug is unnecessary for the derivatization. It was believed that the buffer plug created a high pH environment for the derivatization of the amino acids, but it was hypothesized that this was unnecessary due to the high pH of the BGE.

The 'mixing voltage duration and ramp times' experiment was performed because it was thought that the function of the mixing voltage could be replaced by the use of ramp time. The mixing voltage was used to mix the injected plugs together for the in-line derivatization. It was believed this voltage could also be achieved by using a ramp time for the separation voltage.

The 'Final method' experiment was done to test if one of the potential amino acids (Val, Leu, Ala, Gln, Pro and Thr) could be added to the mixture after all the optimizations. It was hypothesized that after all these optimizations Thr could be added to the amino acid mixture because of the reduced FMOC peak. This also finishes subgoals 3 and 4.

Validation

In this section 2 experiments are performed to determine the repeatability and the linearity of the method, and to achieve subgoal 5. The repeatability and linearity are important if the method will be used for quantitative purposes, and can be used for future studies.
It was hypothesized that for the repeatability of the method the RSD% of the corrected area would be below 10% and that for the linearity the R^2 would be between 0.999 and 0.970.

Real sample

In this section one experiment was performed to measure a urine sample spiked with an amino acid mixture to determine if the amino acids can be observed and enantioseparated in a complex matrix with the developed method. The measurement of real samples is more difficult due to matrix effects, but necessary since most companies analyze complex samples. It was hypothesized that the complex matrix of the urine sample interfered with the derivatization of the amino acids, and that only 2 or 3 amino acid peaks would be observed. Through this experiment subgoal 6 was achieved, this was the final subgoal of this study.

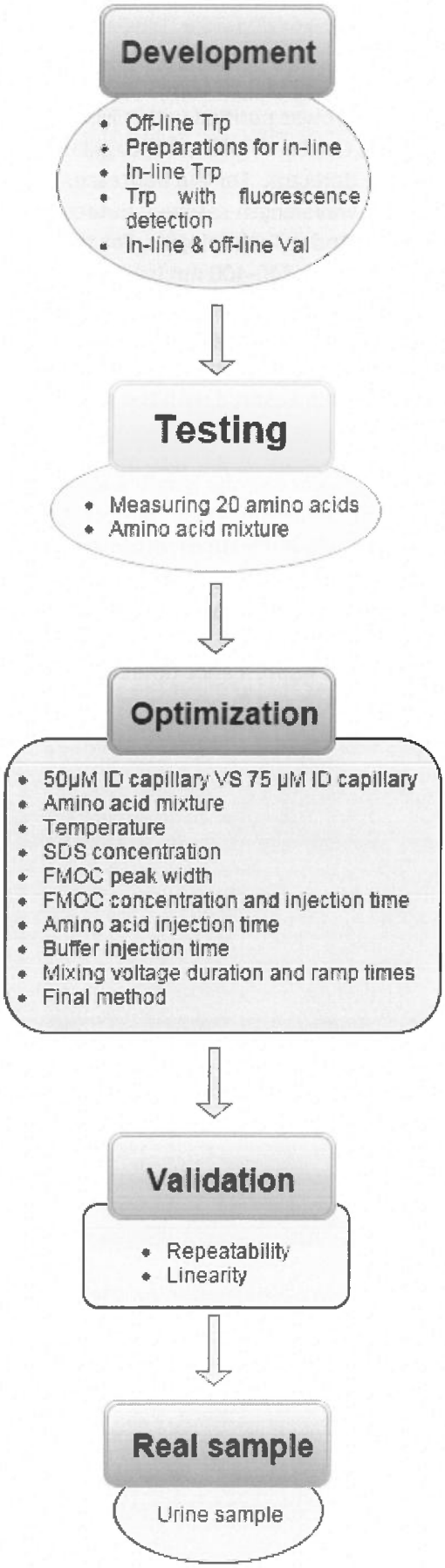

Figure 6: Outline of the research

4 Experimental methods

4.1 Materials & equipment

All the chemicals that were used are described in Table 1 and water was purified with a Milli-Q purification system prior to use. The device used was a Beckman Coulter P/ACE MDQ Capillary Electrophoresis system equipped with a diode array absorbance detector. For the fluorescence detection an Argos 250B Flu cell was used, this was coupled to a wavelength-resolved detector with a SR-163 spectrograph equipped with a DV420A CCD camera from Andor Technologies. For the excitation light a combination of the following three filters were used: 240-400 nm bandpass, a <300 nm shortpass and a <325 nm shortpass. For the acquisition a slit width of 50 μm was used with an exposure time of 3 seconds and a vertical shift speed of 16,25 μs. The UV absorbance was measured at 214, 257 and 280 nm. The fluorescence emission was measured at 330±10 nm, this wavelength was chosen based on the optimum of the FMOC emission spectrum measured with the same fluorescence detector that was used for this study.

The capillaries that were used had an internal diameter of 50 or 75 μm and the length was changed when the fluorescence detector was used. Since the fluorescence detector was set up outside the CE system, the total length of the capillary needed to be longer than when the UV detector was used, which was build within the CE system itself. This meant that the first few analysis with the UV detector the capillary length was shorter than with the rest of the measurements of this study. When using fluorescence detection the total length of the capillary was 79 cm with 65 cm to the window. The first few measurements using UV detection, until the change to fluorescence detection, the total length of the capillary was 43,9 cm with 34 cm tot the window. All UV and fluorescence measurements after that used the same capillary length as is described for fluorescence detection.

Table 1: Overview of all the chemicals that were used

Description	Supplier	CAS No.
Acetonitrile 99,9%+	Fluka	75-05-8
DL-Alanine >99%	Sigma Aldrich	302-72-7
DL-Arginine >97%	Sigma Aldrich	7200-25-1
DL-Asparagine monohydrate >99%	Sigma Aldrich	3130-87-8
DL-Aspartic acid >99%	Sigma Aldrich	617-45-8
β-Cyclodextrin	Sigma Aldrich	7585-39-9
DL-Cysteine	Sigma Aldrich	3374-22-9
FMOC-Cl	Sigma Aldrich	28920-43-6
FMOC-tryptophan-OH	Sigma Aldrich	35737-15-6
DL-Glutamic acid monohydrate >98%	Sigma Aldrich	19285-83-7
D-Glutamine >98%	Sigma Aldrich	5959-95-5
L-Glutamine >99%	Sigma Aldrich	56-85-9
Glycine >99%	Sigma Aldrich	56-40-6
DL-Histidine >99%	Sigma aldrich	4998-57-6
DL-Isoleucine 99%	Sigma Aldrich	443-79-8
DL-Leucine >99%	Sigma Aldrich	328-39-2
DL-Lysine monohydrochloride >98%	Sigma Aldrich	70-53-1
DL-Methionine >99%	Fluka	59-51-8
N-pentane >99%	Sigma Aldrich	109-66-0
DL-Phenylalanine >99%	Sigma Aldrich	150-30-1
DL-Proline >99%	Sigma Aldrich	609-36-9
2-Propanol 99,9%+	Fluka	67-63-0
DL-Serine >98%	Sigma Aldrich	302-84-1
Sodium dodecyl sulfate 99%	Sigma Aldrich	151-21-3
Sodium tetraborate decahydrate 99.5%	Sigma Aldrich	1303-96-4
D-Threonine >98%	Sigma Aldrich	632-20-2
L-Threonine >98%	Sigma Aldrich	72-19-5
DL-Tryptophan >99%	Sigma Aldrich	54-12-6
DL-Tyrosine >99%	Fluka	556-03-6
DL-Valine >97%	Sigma Aldrich	516-06-3

4.2 Background electrolyte preparation

The BGE was made daily and consists of 40 mM sodium tetraborate + isopropanol (IPA), SDS and β-CD.
The additives were always added in the same order: IPA > SDS > β-CD.
The 40 mM sodium tetraborate solution (pH 9.4) was always made in quantities of 500 ml and stored at room temperature for daily use. The exact concentrations of the additives vary between experiments and will be specified in section 4.6. The BGE was filtered with a 0,45 μm disposable filter before use.

4.3 Amino Acid preparation

The amino acid samples were prepared by dissolving it in 40 mM sodium tetra borate solution and put in a sonication bath for at least 10 minutes. For every amino acid a stock was made of 5 mM and from this stock the samples were made in the concentrations that are specified for each experiment in section 4.6

4.4 FMOC preparation

The FMOC solution was made daily by dissolving FMOC-Cl in acetonitrile (ACN). The FMOC concentration that was used is specified in section 4.6 for each experiment and was made from a stock solution. The concentration of the stock solution depended on the final concentration needed for the experiment and ranged from 50 mM to 2.5 mM.

4.5 Derivatization procedure

The off-line derivatization was done by taking 1 ml of amino acid sample with a total amino acid concentration of 5 mM and adding 1 ml of 50 mM FMOC. After mixing the 2 solutions it was incubated for 10 minutes and then 1 ml of pentane was added. After shaking vigorously 2 separate layers were visible, the aqueous layer was pipetted from the solution, this layer was the derivatized product.

The in-line derivatization was done by subsequently injecting the amino acid solution, 40 mM sodium tetraborate solution and FMOC solution. Then a mixing voltage was applied followed by the separation voltage, the applied voltage is specified in section 4.6.

4.6 CE method

Off-line derivatization

When measuring off-line derivatized amino acids the following CE method was used for each individual run. First the capillary was rinsed with 1M NaOH followed by water, both for 3 minutes. Then the capillary was rinsed with the BGE for 2 minutes, all the rinsing was done at 30 psi. After the rinsing the sample was injected and the voltage was applied, the exact parameters are specified in section 4.6.

In-line derivatization

When measuring in-line derivatized amino acids the following CE method (based on Fradi et al. [10]) was used for each individual run. First the capillary was rinsed with water for 1 minute, followed by acetonitrile for 2 minutes. Then the capillary was rinsed with water again for 1 minute and then rinsed with 1M NaOH for 2 minutes. After that the capillary was rinsed with BGE for 2 minutes, all the rinsing was done at 30 psi. After the rinsing the sample, buffer solution and FMOC solution were injected respectively and the mixing voltage was applied. After a pause the separation voltage was applied. The exact parameters are specified with each experiment.

Parameters

The exact parameters used for the experiments described in the sections Development, Testing, Optimalization, Validation and Real sample are given in the following tables respectively: Table 2, Table 3, Table 4, Table 5, Table 6 and Table 7.

Table 2: Overview of all the parameters used for the development of the method

Parameters					
	Off-line Trp	**Preparation for in-line**	**In-line Trp**	**Trp with fluorescence**	**In-line & off-line Val**
Derivatization	Off-line		in-line		In-line & off-line
Capillary	50 µm ID, 43.9 cm total length (34 cm to window)			50 µm ID, 79 cm total length (65 cm to window)	
Detector	UV (214nm)			Fluorescence (emission at 330±10 nm)	
Temperature	20 °C	25 °C			
Voltage	13.7 kV (0.17 minutes ramp time)	18.1 kV (0.17 minutes ramp time)		30 kV (0.17 minutes ramp time)	
Amino acid injection	13 seconds (0.5 psi)	8 seconds (0.3 psi)			
Borate buffer injection	NA	16 seconds (0.3 psi)			
FMOC injection	NA	24 seconds (0.3 psi)			
Mixing voltage	NA	72 seconds (3 kV)			
Waiting time after mixing	NA	60 seconds			
FMOC concentration	NA		5 mM		5 mM, 2.5 mM & 0 mM
BGE					
Concentration sodium tetraborate	40 mM				
Concentration iso-propanol	15%	17%			
Concentration SDS	0 mM / 30 mM	25 mM			
Concentration β-CD	30 mM	30 mM			
Amino acids					
Trp concentration	25 µM D, 25 µM L (50 µM total)		250 µM D, 250 µM L (500 µM total)		NA
Trp concentration with L spiked	17 µM D, 33 µM L (50 µM total)	NA	NA	NA	NA
L-Trp-FMOC concentration	NA	250 µM L	NA	NA	NA
Val concentration	NA	NA	NA	NA	250 µM D, 250 µM L (500 µM total)

Table 3: Overview of all the parameters used for the testing of the method

Parameters		
	20 amino acids	**Mixture**
Derivatization	in-line	
Capillary	50 µm ID, 79 cm total length (65 cm to window)	
Detector	Fluorescence (emission at 330±10 nm)	
Temperature	25 °C	
Voltage	30 kV (0.17 minutes ramp time)	
Amino acid injection	8 seconds (0.3 psi)	
Borate buffer injection	16 seconds (0.3 psi)	
FMOC injection	24 seconds (0.3 psi)	
Mixing voltage	72 seconds (3 kV)	
Waiting time after mixing	60 seconds	
FMOC concentration	2.5 mM	
BGE		
Concentration sodium tetraborate	40 mM	
Concentration iso-propanol	17%	
Concentration SDS	25 mM	
Concentration β-CD	30 mM	
Amino acids		
Val & Phe	125 µM D, 125 µM L (for both) 500 µM total	NA
Val & Leu	125 µM D, 125 µM L (for both) 500 µM total	NA
Asp	125 µM D, 125 µM L (for both) 500 µM total	NA
Val & Glu	125 µM D, 125 µM L (for both) 500 µM total	NA
Val & Thr	125 µM D, 125 µM L (for both) 500 µM total	NA
Val & Ile	125 µM D, 125 µM L (for both) 500 µM total	NA
Met & Ile	125 µM D, 125 µM L (for both) 500 µM total	125 µM D, 125 µM L (for both) 500 µM total
Ala & Ile	125 µM D, 125 µM L (for both) 500 µM total	NA
His & Ile	125 µM D, 125 µM L (for both) 500 µM total	NA
Trp & Ile	125 µM D, 125 µM L (for both) 500 µM total	NA
Pro & Ile	125 µM D, 125 µM L (for both) 500 µM total	NA
Arg & Ile	125 µM D, 125 µM L (for both) 500 µM total	NA
Tyr & Ile	125 µM D, 125 µM L (for both) 500 µM total	NA
Asn & Ile	125 µM D, 125 µM L (for both) 500 µM total	NA
Gln & Ile	125 µM D, 125 µM L (for both) 500 µM total	NA
Gly & Ile	125 µM D, 125 µM L (for both) 500 µM total	NA
Ser & Ile	125 µM D, 125 µM L (for both) 500 µM total	NA
Cys & Ile	125 µM D, 125 µM L (for both) 500 µM total	NA
Lys & Ile	125 µM D, 125 µM L (for both) 500 µM total	NA
Met, Ile & Glu	NA	83.3 µM D, 83.3 µM L (for all) 500 µM total
Met, Ile & Asp	NA	83.3 µM D, 83.3 µM L (for all) 500 µM total
Met, Ile, Glu & Asp	NA	62.5 µM D, 62.5 µM L (for all) 500 µM total
Met, Ile, Glu, Asp & Phe	NA	50 µM D, 50 µM L (for all) 500 µM total

Table 4: Overview of all the parameters used for the optimization of the method, part 1 of 3

Parameters				
	Capillary change	**Mixture**	**Temperature**	**SDS**
Derivatization	in-line			
Capillary	50 & 75 µm ID, 79 cm total length (65 cm to window)	75 µm ID, 79 cm total length (65 cm to window)		
Detector	UV (214nm) & Fluorescence (emission at 330±10 nm)	Fluorescence (emission at 330±10 nm)		
Temperature	25 °C		20 - 22 - 25 - 27 - 30 °C	25 °C
Voltage	30 kV (0.17 minutes ramp time)			
Amino acid injection	8 seconds (0.3 psi)			
Borate buffer injection	16 seconds (0.3 psi)			
FMOC injection	24 seconds (0.3 psi)			
Mixing voltage	72 seconds (3 kV)			
Waiting time after mixing	60 seconds			
FMOC concentration	2.5 mM			
BGE				
Concentration sodium tetraborate	40 mM			
Concentration iso-propanol	17%			
Concentration SDS	25 mM			25 - 30 - 35 mM
Concentration β-CD	30 mM			
Amino acids				
Met & Phe	125 µM D, 125 µM L (for both) 500 µM total	NA	NA	NA
Ile	NA	250 µM D, 250 µM L (500 µM total)	NA	NA
Phe & Ile	NA	125 µM D, 125 µM L (for both) 500 µM total	NA	NA
Met & Ile	NA	125 µM D, 125 µM L (for both) 500 µM total	NA	NA
Met, Ile & Phe	NA	83.3 µM D, 83.3 µM L (for all) 500 µM total	NA	NA
Met, Ile, Phe & Asp	NA	62.5 µM D, 62.5 µM L (for all) 500 µM total	NA	NA
Met, Ile, Phe, Asp & Glu	NA	50 µM D, 50 µM L (for all) 500 µM total	NA	NA
Met, Ile, Phe, Asp, Glu & Leu	NA	41.7 µM D, 41.7 µM L (for all) 500 µM total	NA	NA
Met, Ile, Phe, Asp, Glu & Val	NA	41.7 µM D, 41.7 µM L (for all) 500 µM total	NA	NA
Met, Ile, Phe, Asp, Glu & Ala	NA	41.7 µM D, 41.7 µM L (for all) 500 µM total	NA	NA
Met, Ile, Phe, Asp, Glu & Gly	NA	41.7 µM D, 41.7 µM L (for all), 83.4 µM Gly, 500 µM total	NA	NA
Met, Ile, Phe, Asp, Glu & Pro	NA	41.7 µM D, 41.7 µM L (for all) 500 µM total	NA	NA
Met, Ile, Phe, Asp, Glu & Tyr	NA	41.7 µM D, 41.7 µM L (for all) 500 µM total	41.7 µM D, 41.7 µM L (for all) 500 µM total	41.7 µM D, 41.7 µM L (for all) 500 µM total

Table 5: Overview of all the parameters used for the optimization of the method, part 2 of 3

Parameters					
	FMOC peak width	**FMOC conc.**	**AA injection**	**Buffer injection**	**Mixing & ramp time**
Derivatization	in-line				
Capillary	75 µm ID, 79 cm total length (65 cm to window)				
Detector	Fluorescence (emission at 330±10 nm)				
Temperature	25 °C				
Voltage	30 kV (0.17 minutes ramp time)				30 kV (5 - 2 - 1 - 0.5 - 0.17 minutes ramp time)
Amino acid injection	8 seconds (0.3 psi)		12 - 10 - 8 - 6 seconds (0.3 psi)	10 seconds (0.3 psi)	
Borate buffer injection	16 seconds (0.3 psi)			28 - 24 - 20 - 16 - 12 - 8 - 4 - 0 seconds (0.3 psi)	NA
FMOC injection	24 - 20 - 16 - 12 - 8 - 6 - 4 seconds (0.3 psi)	8 seconds (0.3 psi)			
Mixing voltage	72 seconds (3 kV)				NA
Waiting time after mixing	60 seconds				NA
FMOC concentration	2.5 - 2.0 - 1.5 - 1.0 - 0.5 mM	5.0 - 7.5 - 10 - 15 mM	10 mM		
BGE					
Concentration sodium tetraborate	40 mM				
Concentration iso-propanol	17%				
Concentration SDS	25 mM				
Concentration β-CD	30 mM				
Amino acids					
Met, Ile, Phe, Asp, Glu & Tyr	41.7 µM D, 41.7 µM L (for all) 500 µM total	41.7 µM D, 41.7 µM L (for all) 500 µM total	41.7 µM D, 41.7 µM L (for all) 500 µM total	41.7 µM D, 41.7 µM L (for all) 500 µM total	41.7 µM D, 41.7 µM L (for all) 500 µM total

Table 6: Overview of all the parameters used for the optimization of the method, part 3 of 3

Parameters	
	Final result
Derivatization	in-line
Capillary	75 µm ID, 79 cm total length (65 cm to window)
Detector	Fluorescence (emission at 330±10 nm)
Temperature	25 °C
Voltage	30 kV (2 minutes ramp time)
Amino acid injection	10 seconds (0.3 psi)
Borate buffer injection	**NA**
FMOC injection	8 seconds (0.3 psi)
Mixing voltage	**NA**
Waiting time after mixing	**NA**
FMOC concentration	**10 mM**
BGE	
Concentration sodium tetraborate	**40 mM**
Concentration iso-propanol	**17%**
Concentration SDS	25 mM
Concentration β-CD	30 mM
Amino acids	
Met, Ile, Phe, Asp, Glu & Tyr	41.7 µM D, 41.7 µM L (for all) 500 µM total
Ala	125 µM D, 125 µM L (250 µM total)
Pro	125 µM D, 125 µM L (250 µM total)
Gln	125 µM D, 125 µM L (250 µM total)
Thr	125 µM D, 125 µM L (250 µM total)
His	125 µM D, 125 µM L (250 µM total)
Arg	125 µM D, 125 µM L (250 µM total)
Asn	125 µM D, 125 µM L (250 µM total)
Ser	125 µM D, 125 µM L (250 µM total)
Cys	125 µM D, 125 µM L (250 µM total)
Lys	125 µM D, 125 µM L (250 µM total)
Met, Ile, Phe, Asp, Glu, Tyr & Leu	35.7 µM D, 35.7 µM L (for all) 500 µM total
Met, Ile, Phe, Asp, Glu, Tyr & Val	35.7 µM D, 35.7 µM L (for all) 500 µM total

Table 7: Overview of all the parameters used for the validation of the method and the real sample

Parameters			
	Repeatability	**Linearity**	**Urine sample**
Derivatization	in-line		
Capillary	75 µm ID, 79 cm total length (65 cm to window)		
Detector	Fluorescence (emission at 330±10 nm)		
Temperature	25 °C		
Voltage	30 kV (2 minutes ramp time)		
Amino acid injection	10 seconds (0.3 psi)		
FMOC injection	8 seconds (0.3 psi)		
FMOC concentration	10 mM		
BGE			
Concentration sodium tetraborate	40 mM		
Concentration iso-propanol	17%		
Concentration SDS	25 mM		
Concentration β-CD	30 mM		
Amino acids			
Met, Ile, Phe, Asp, Glu & Tyr	41.7 µM D, 41.7 µM L (for all) 500 µM total	41.7 µM D, 41.7 µM L (for all) 500 µM total	41.7 µM D, 41.7 µM L (for all) 500 µM total
Met, Ile, Phe, Asp, Glu & Tyr	NA	33.3 µM D, 33.3 µM L (for all) 400 µM total	NA
Met, Ile, Phe, Asp, Glu & Tyr	NA	25 µM D, 25 µM L (for all) 300 µM total	NA
Met, Ile, Phe, Asp, Glu & Tyr	NA	16.7 µM D, 16.7 µM L (for all) 200 µM total	NA
Met, Ile, Phe, Asp, Glu & Tyr	NA	10.4 µM D, 10.4 µM L (for all) 125 µM total	NA
Met, Ile, Phe, Asp, Glu & Tyr	NA	8.3 µM D, 8.3 µM L (for all) 100 µM total	NA
Met, Ile, Phe, Asp, Glu & Tyr	NA	6.3 µM D, 6.3 µM L (for all) 75 µM total	NA
Met, Ile, Phe, Asp, Glu & Tyr	NA	4.2 µM D, 4.2 µM L (for all) 50 µM total	NA
Met, Ile, Phe, Asp, Glu & Tyr	NA	2.1 µM D, 2.1 µM L (for all) 25 µM total	NA

4.7 Rinsing procedure of the capillaries

At the start of every day the capillary was rinsed with NaOH 1M, water and the BGE for 10 minutes each respectively at a pressure of 30 psi. When a new capillary was made, before it was used, it was rinsed the same as at the start of the day.
At the end of the day the capillary was rinsed with NaOH 1M and water for 10 minutes each.

4.8 Validation

Resolution

The resolution was calculated with the following formula:

$$Rs = \frac{MT2 - MT1}{0.5 * (w1 + w2)}$$

For example

$$\frac{1756 - 1714}{0.5 * (27 + 26)} = 1.6$$

For the first peak with a migration time of 1716 seconds and a width of 27 seconds, and the second peak with a migration time of 1756 seconds and a width of 26 seconds, the resolution would be 1.6.

Corrected area

The corrected area of a peak was calculated with the following formula:

$$\frac{Length\ to\ capillary\ window\ (m) * Area}{migration\ time\ (s)} = Corrected\ Area$$

For example:

$$\frac{0.65 * 11879}{33.05 * 60} = 3.89$$

For a capillary with a length of 65cm to the window, and for a peak that was detected at 33.05 minutes with an area of 11879, the corrected area would be 3.89.

Mobility

The mobility of a peak was calculated with the following formula:

$$Apparent\ mobility - Mobility\ EOF = Mobility$$

$$\frac{Length\ \ to\ capillary\ window\ * length\ capillary}{applied\ voltage * migration\ time\ (s)} - \frac{Length\ to\ capillary\ window * length\ capillary}{applied\ voltage * migration\ time\ EOF\ (s)} = Mobility$$

For example

$$\frac{0.65\ * 0.79}{30000 * 33.05 * 60} - \frac{0.65\ * 0.79}{30000 * 15.7 * 60} = -9.54 * 10^{-9}$$

For a capillary with a length of 79cm with 65cm to the window, and for a peak that was detected at 33.05 minutes with an EOF migration time of 15.7 minutes and 30k applied voltage, the mobility would be $-9.54*10^{-9}$.

Repeatability

The repeatability of the method was deemed successful if the %STD of the corrected area was 9% or lower and the %STD of the mobility was 0.8% or lower. These requirements were discussed and accepted within the study group.

Linearity

The linearity of the method was deemed successful if the R^2 of the corrected area was 0.98 or higher. These requirements were discussed and accepted within the study group.

4.9 Statistics

All measurements were done once (n=1), except for the repeatability which was measured five times (n=5). Therefore the only statistical calculations were performed with the results of the repeatability experiment.

Paired T-test

Because the same measurement system was used and the only difference was the sample treatment the paired T-test must be used to determine if the two sets of data were significantly different.

$$t = \frac{|\pm\Delta| * \sqrt{n\Delta}}{S\Delta} = t_{cal}$$

For example:

$$t = \frac{1.305 * \sqrt{50}}{0.146} = 63.2$$

When the difference between the average of the two data sets is $\pm\Delta = 1.305$, the total number of different analytes is $n\Delta=50$ and the difference in standard deviation between the two data sets is $S\Delta=0.146$.

T (0.05, 49) = 2.01

$T_{cal} > T = 63.2 > 2.01$

F-test

To determine the variances between two data sets the F-test was used.

$$F = \frac{S_a^2}{S_b^2} = F_{cal}$$

Where $S_a > S_b$

For example:

$$F = \frac{0.33^2}{0.15^2} = 4.81$$

When the standard deviation from the first set is $S_a^2=0.33$ and the standard deviation from the second set is $S_a^2=0.15$ then $F_{cal}=4.81$

F(0.05, 4, 4) = 9.60

$F_{cal} < F = 4.81 < 9.60$

LOD

The LOD in nM was calculated with the following formula:

$$LOD = \frac{Enantiomer\ C}{Signal/_{Noise * 3}} * 1000$$

For example:

$$LOD = \frac{41.67}{572/_{2.8*3}} * 1000 = 612 \text{ nM}$$

When the enantiomer concentration is 41,67 μM, the signal is 572 and the noise is 2.8, the LOD will be 612 nM.

5 Results & discussion

5.1 Development

Off-line Trp

To start the development of the method Trp was measured using a BGE with and without SDS. This was done as a starting point for this study to observe the influence of SDS in the BGE.

The method used was based on a method which was previously developed by another student at the VU university. This method used a BGE containing 40 mM sodium tetraborate, 15% IPA, 30 mM SDS and 30 mM β-CD separated with 13.7 kV at 20°C. This method was used as a starting point for this study. Trp was measured with a BGE without SDS and with a BGE with SDS to determine the influence of SDS. Trp spiked with L-Trp was also measured with a BGE without SDS and with a BGE with SDS to determine the influence on the migration of the two enantiomers.

See Figure 7 and Figure 8 for the results.

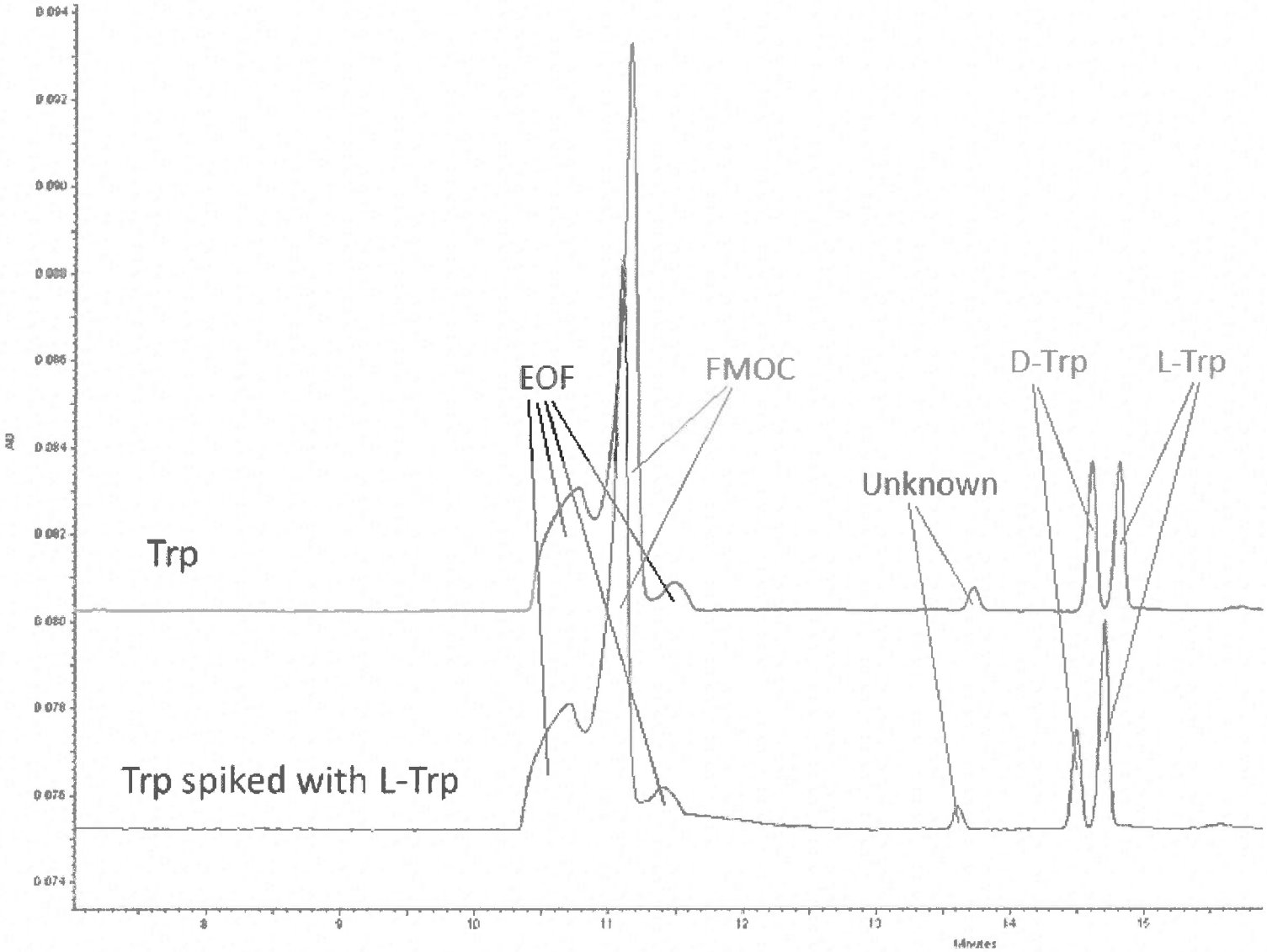

Figure 7: Electropherograms of Trp and Trp spiked with L-Trp from top to bottom respectively, using a BGE with 40mM sodium tetraborate (pH 9.4), 15% IPA and 30mM β-CD

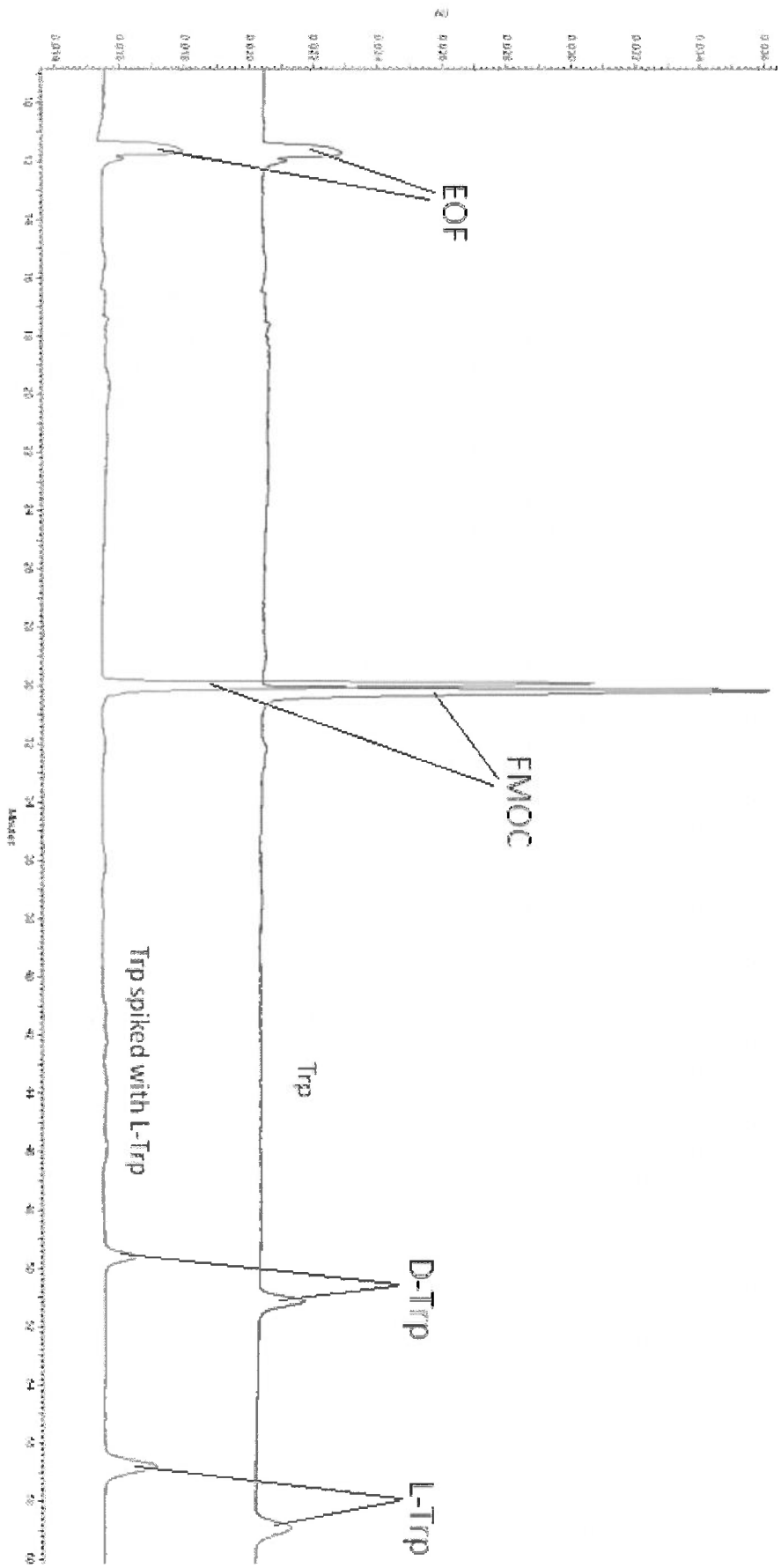

Figure 8: Electropherograms of TRP and TRP spiked with L-TRP from top to bottom respectively, using a BGE with 40mM sodium tetraborate (pH 9.4), 15% IPA, 30mM β-CD and 30mM SDS

In Figure 7 there were 6 peaks observed in both electropherograms. In Figure 8 there were 4 peaks observed in both electropherograms. The two electropherograms in each figure seem identical to each other except for the last two peaks of D- and L-Trp. The peak intensity of the D- and L-Trp was different in the spiked run compared to the normal run. The unknown peaks in Figure 7 were not observed in Figure 8. In both figures the EOF and FMOC was observed in the electropherograms.

In both electropherograms in Figure 7 an unknown peak was observed and the EOF and FMOC peak overlapped with each other. In both electropherograms in Figure 8 the unknown peak was not observed and the EOF and FMOC did not overlap, also the overall migration time was longer (60 minutes compared to 15 minutes).

In Figure 7 the first three peaks in both electropherograms were hypothesized to be due to the EOF and the remaining FMOC in the sample. The so called EOF peak was caused by the injection plug that was carried with the EOF which is called mobility zero. It was hypothesized that the FMOC had no interaction with the BGE and was carried with the EOF and therefore overlapped with the EOF peak. The peak at t=13.7 minutes was unknown and was most likely an impurity. It was hypothesized that the last two peaks at t=14.6 and t=14.8 minutes were most likely D- and L-Trp respectively. The two peaks seem identical in peak intensity which was in accordance with the identical concentration of the injected amino acid. This hypothesis corresponds with the results of the spiked sample where the injected amino acid had different concentrations. D-Trp had a lower concentration and L-Trp had a higher concentration which indicated that the left peak at t=14.5 minutes was D-Trp and the peak at t=14.7 minutes was L-Trp.

In Figure 8 the first peak in both electropherograms was most likely the EOF peak, with the peak at t=30.4 minutes coming from the FMOC. The unknown peak that was observed in Figure 7 was not observed in these electropherograms which indicates it was most likely an impurity in the sample because these runs were made on different day with newly made samples. The ratio of signal intensity between the hypothesized D- and L-enantiomers were identical with the results from Figure 7. This corresponds with the hypothesis of the identification of the D- and L-Trp.

Most noticeable was the difference in migration time between Figure 7 and Figure 8. In Figure 8 the FMOC peak did not overlap with the EOF peak, which indicates it interacts with the SDS micelles in the BGE. The migration time of D- and L-Trp in Figure 8 was more than three times longer than in Figure 7, which also indicates that Trp interact with the SDS micelles. In both figures the Trp was enantioseparated but the resolution was better in Figure 8 (R_s=4.5) compared to Figure 7 (R_s=1.4).

Preparation for in-line

The next step was to try the method for the in-line derivatization by Fradi et al. [10]. By measuring the off-line derivatized Trp with the parameters used by Fradi et al. the changes could be observed compared to the previous results in preparation for the step to in-line derivatization.

The method used was based on the in-line method from Fradi et al. [10] in preparation for the transition to in-line derivatization. This method used a BGE containing 40 mM sodium tetraborate, 17% IPA, 25 mM SDS and 30 mM β-CD measured with 18.1 kV at 25°C. For this method an amino acid plug was injected, followed by a borate buffer plug and then an ACN plug. Mixing voltage was then applied followed by a waiting time so the derivatization reaction could take place.

To determine the influence of these plugs on the separation of Trp, 3 runs were made.
Trp was off-line derivatized and separated with the off-line CE method as a reference.
Trp was off-line derivatized and separated with the in-line CE method.
A standard solution of L-Trp-FMOC was separated with the in-line CE method and was measured to verify the migration order with the in-line CE method.

See Figure 9 for the results.

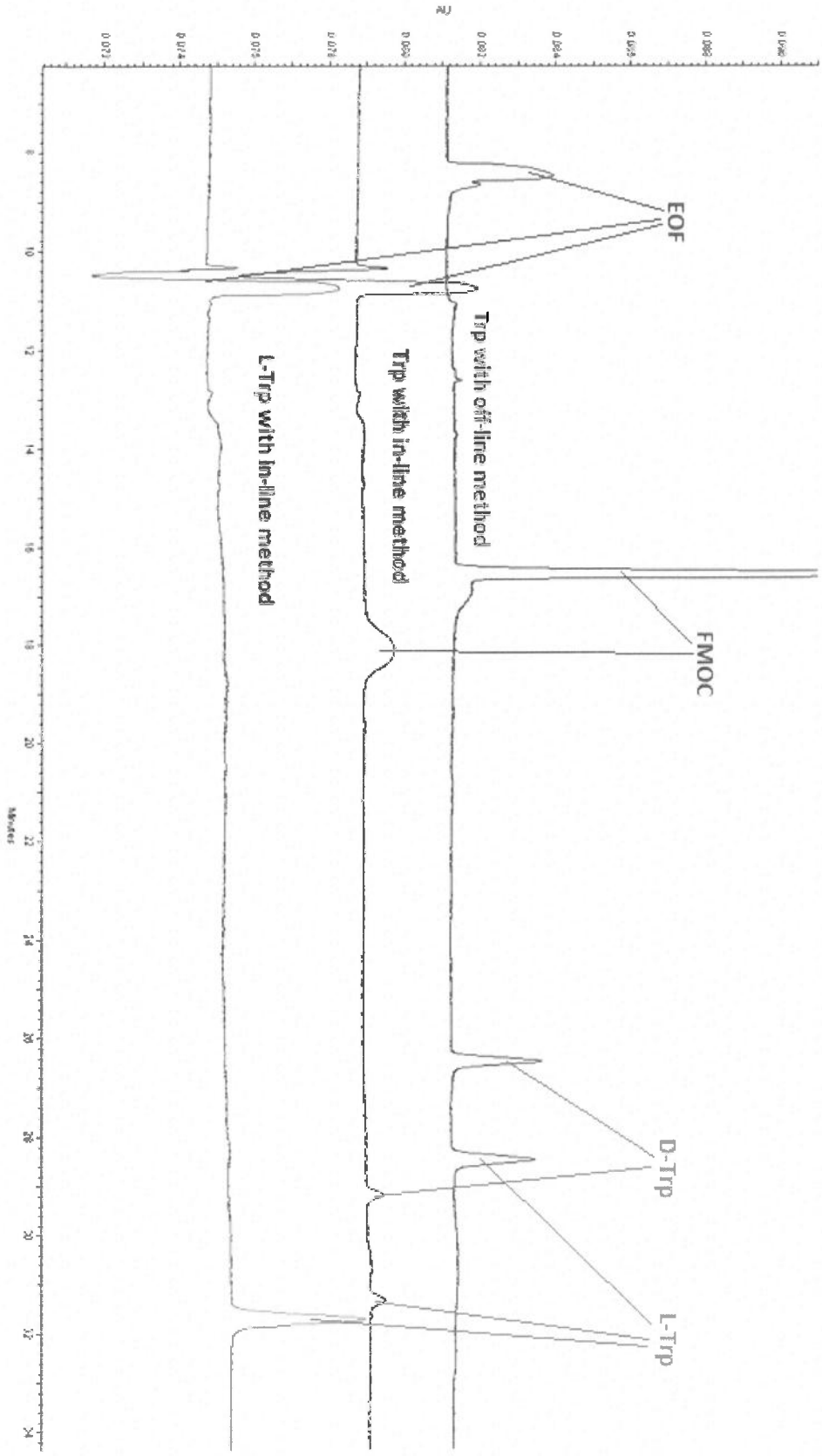

Figure 9: Electropherograms of Trp analyzed with off-line method, Trp analyzed with in-line method and L-Trp analyzed with in-line method with parameters according to Fradi et al.

The reference run of Trp analyzed with the off-line method in Figure 9 appeared to be identical to the run in Figure 8, except for the total migration time and the peak height of the D- and L-Trp. The EOF peak in the other runs with in-line method in Figure 9 had a different shape compared to the EOF peak of the reference run. The run with Trp analyzed with the in-line method also had 4 peaks just as the reference run, but the peak intensities appeared to be lower. The run with L-Trp analyzed with in-line method only had 2 peaks. The migration times of both runs which were analyzed with the in-line method appear to have shifted by circa 2 minutes.

The run with Trp analyzed with the off-line method in Figure 9 appeared to be identical to the run in Figure 8, the shorter migration time was most likely due to the lower concentration of SDS in the BGE, which was 25 mM, compared to the previous experiment which used 30 mM. A lower concentration of SDS results in less micelles to interact with which would explain the shorter migration time. The shorter migration time most likely resulted in less diffusion and thus less peak broadening, which would explain why the final two peaks also had a greater peak height compared to Figure 8.
The EOF peak of the two runs analyzed with the in-line method, appeared to be different than all previous runs. This was most likely caused by the different injection sequence of those two runs. The other peaks of the run Trp analyzed with the in-line method appeared to be relatively smaller compared to the reference run analyzed with the off-line method. It is unknown why this happened even though the concentration of the amino acid injection was identical. The shift in migration time of the two runs with the in-line method was due to the different injection sequence compared to the off-line method. It was hypothesized that the peak at t=29.6 minutes was D-Trp and the peak at t=31.8 minutes was L-Trp for the runs analyzed with the in-line method. The last run with L-Trp analyzed with the in-line method only had 2 peaks. This run did not have the FMOC peak and D-Trp peak as was observed in the other two runs which was expected since there should be no residual FMOC nor D-Trp in the L-Trp-FMOC standard. The relatively high intensity of the L-Trp peak in the run with L-Trp analyzed with the in-line method corresponds with its higher sample concentration of 250 μM instead of 25 μM. The migration time of L-Trp appeared to be similar in both runs analyzed with the in-line. This corresponds with the previous established hypothesis that the last peak at t=32 minutes was indeed L-Trp.

In-line Trp

The next step would be to perform the in-line derivatization with a higher sample concentration to compensate for the lower signal intensity. The in-line derivatization method was used to analyze three runs. The runs were Trp analyzed with in-line method with FMOC, borate buffer analyzed with in-line method with FMOC and borate buffer analyzed with in-line method without FMOC . The two runs with borate buffer were blanks and were measured to determine the influence of the amino acid and FMOC with the in-line method.

See Figure 10 for the results.

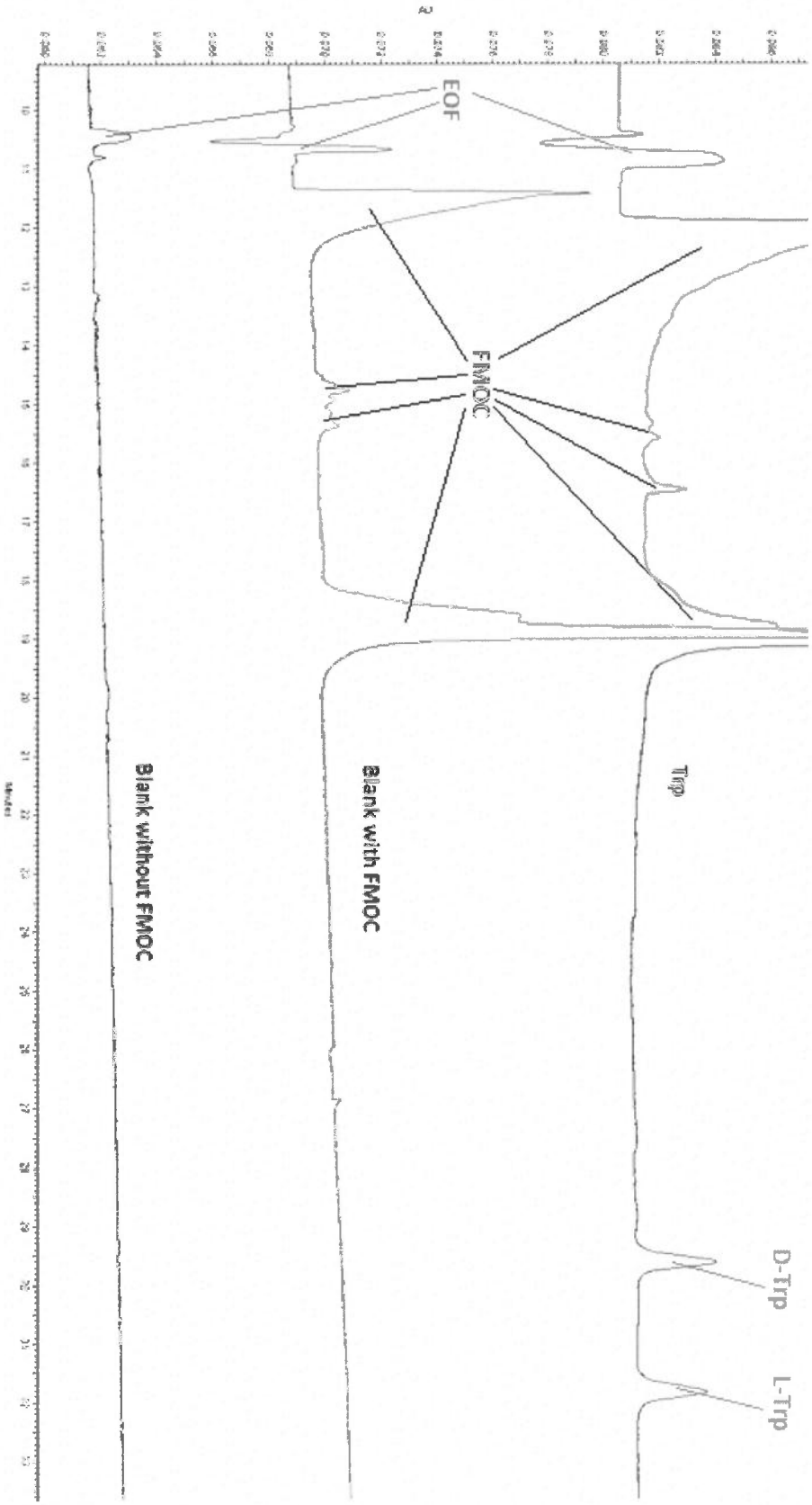

Figure 10: Electropherograms of in-line derivatized Trp, blank with FMOC and blank without FMOC

In all three runs an EOF peak was observed. The first run with in-line derivatized Trp appeared to have 7 peaks in total and Trp was enantioseparated. The second run with the blank with FMOC appeared to have 5 peaks in total. The third run with the blank without FMOC had only 1 peak.

The peaks from t=12 to t=21 minutes of the runs with in-line derivatized Trp and buffer with FMOC were similar, these peaks were most like caused by the FMOC. The lack of those peaks in the run with the blank without FMOC corresponds with that hypothesis since no FMOC was injected with that run. The last two peaks at t=29.5 and t=32 minutes from the in-line derivatized Trp run were most like D- and L-Trp respectively. This hypothesis corresponds with the lack of those peaks in the other 2 runs since no Trp was injected at those runs.

Trp with fluorescence detection

Since Trp was successfully enantioseparated using in-line derivatization, the next step would be to make the transition to fluorescence detection. It was believed that this transition should be done as soon as possible because it was believed that the results obtained with UV detection are not identical to the results obtained with fluorescence detection. The change to fluorescence detection was done to improve the sensitivity of the method and this experiment was done to determine the results of the in-line method with fluorescence detection compared to UV detection.

Similar to the previous experiment, the in-line derivatization method was used to analyze three runs. The runs were Trp analyzed with in-line method with FMOC, borate buffer analyzed with in-line method with FMOC and borate buffer analyzed with in-line method without FMOC. The two runs with borate buffer were blanks and were measured to determine the influence of the amino acid and FMOC with the in-line method with fluorescence detection.

See Figure 11 for the results.

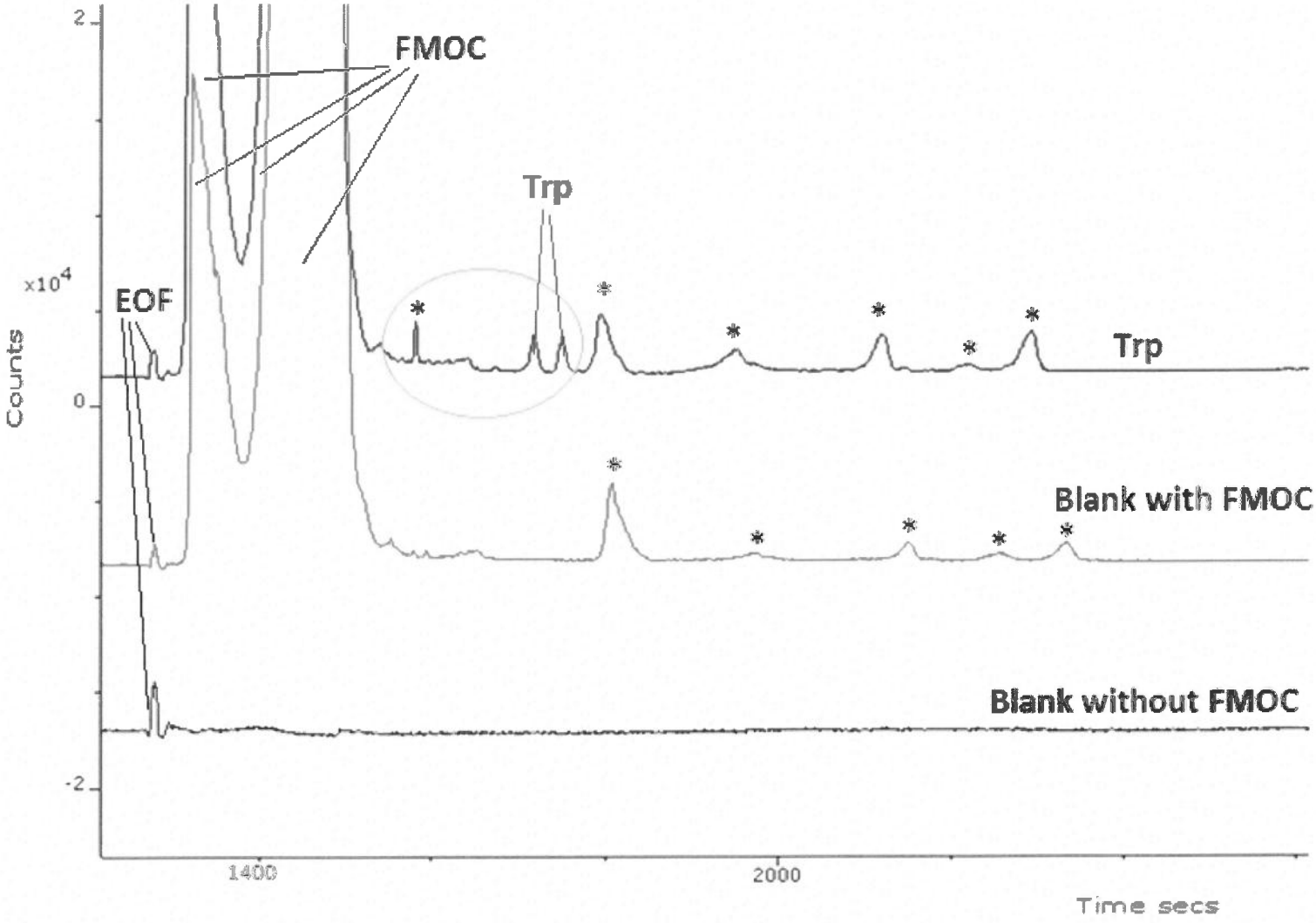

Figure 11: Electropherograms of in-line derivatized Trp, blank with FMOC and blank without FMOC. Peaks marked with a * were impurities

In all three runs an EOF peak was observed. The first run with in-line derivatized Trp appeared to have 10 more peaks besides the EOF peak. The second run which was the blank with FMOC appeared to have 7 more peaks besides the EOF peak. The last run which was the blank without FMOC had no peaks in addition to the EOF peak. The overall migration times are different compared to the previous results at Figure 10.

The differences in peaks between the two blank runs, one with FMOC and the other without FMOC, most likely indicated the influence of FMOC. This would have meant that all the peaks that were not observed with the blank without FMOC (all the peaks marked with a * and the two peaks at t=1370 and t=1450 seconds), in comparison with the blank with FMOC, were caused by the FMOC. This hypothesis corresponds with the observed peaks in the run with in-line derivatized Trp since the same peaks were observed compared to the blank with FMOC. In addition to those peaks, three new peaks were observed as well which were not seen with the blanks at t=1590, t=1720 and t=1760 seconds. Those three peaks are encircled in Figure 11. The peak at t=1590 seconds was most likely an impurity. It was hypothesized that the next two peaks at t=1720 and t=1760 seconds were D- and L-Trp. The change in migration time could have been caused by the different capillary length compared to the previous experiments. Due to the fluorescence detector, a longer capillary was necessary. This

means that the sample had to travel a longer distance through the capillary, thus increasing the amount of time it takes for the amino acids to reach the detection window. A longer capillary also results in more interaction of the amino acids with SDS and β-CD compared to the previous experiments, this would increase the overall migration as was observed in Figure 11.
The intensity of the Trp peaks was lower than was expected. Theoretically fluorescence detection is more sensitive than UV detection, and based on the results in Figure 10 it was expected that the peak intensities would have been higher. It was unknown if those peaks were indeed Trp and why the signal intensities were low.

In-line & off-line Val

The previous experiment was repeated but with a different amino acid to determine if the low signal intensity was because of the Trp. Val was measured with both in-line and off-line methods. Four runs were performed: Val analyzed with off-line method, Val analyzed with in-line method with 5 mM FMOC, Val analyzed with in-line method with 2.5 mM FMOC and Val analyzed with in-line method with ACN (0 mM FMOC).
See Figure 12 for the results.

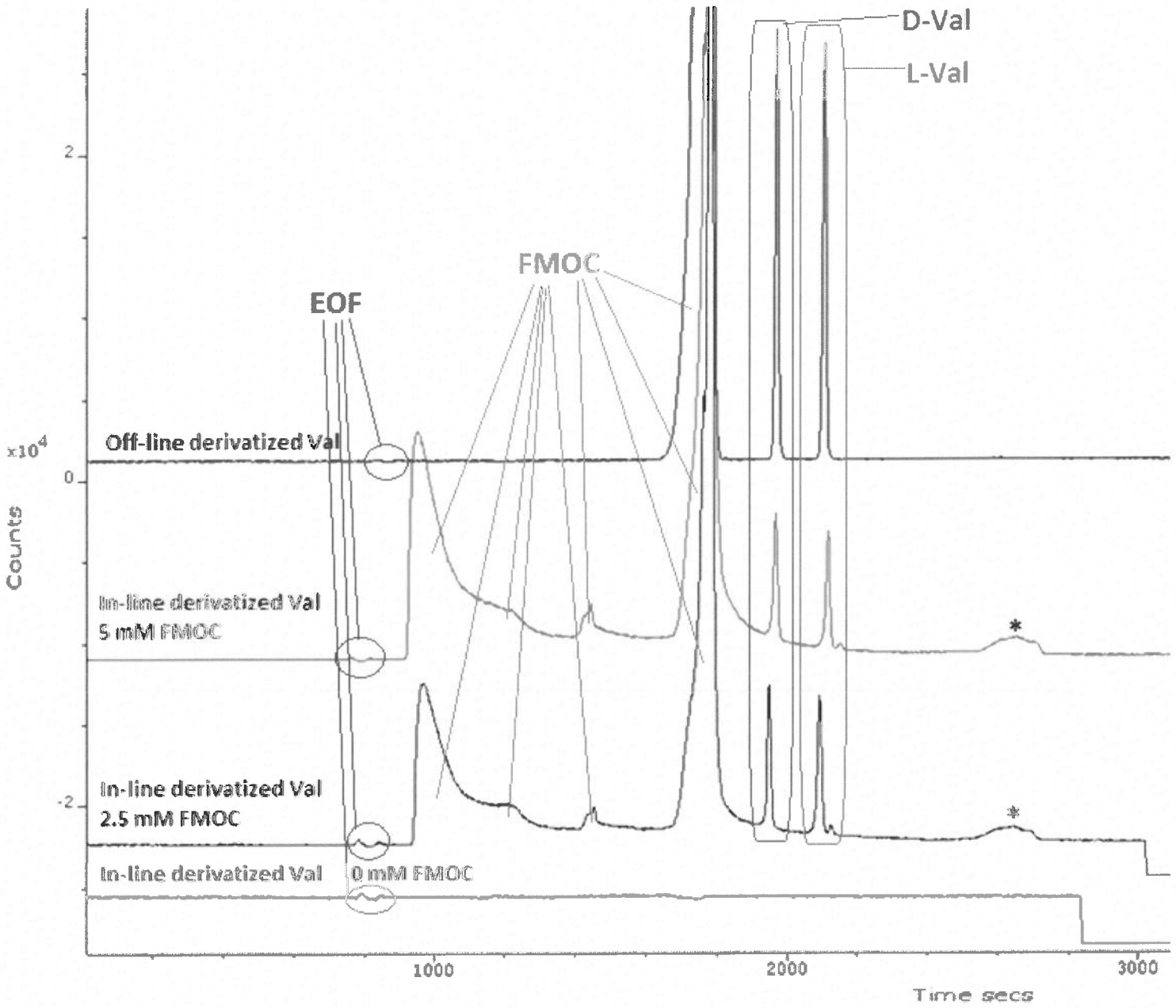

Figure 12: Electropherograms of off-line derivatized Val and in-line derivatized Val with different concentrations of FMOC Peaks marked with a * were impurities

The EOF peak could be observed at all four runs around t=800 seconds. Three peaks were observed with the off-line derivatized Val. The two runs with in-line derivatized Val with 2.5 and 5 mM FMOC seven peaks were observed in each run. The migration times of those peaks appeared to be similar to each other. No peaks were observed with the last run besides the EOF peak.

The off-line derivatized Val had 3 peaks as was expected. Based on the results in Figure 9 the first peak was most likely the FMOC peak which would have meant that the last 2 peaks were valine. This hypothesis corresponded with the similar peak intensity of the last two peaks, since the same concentration of both enantiomers were injected. The in-line derivated Val with 5 and 2.5 mM FMOC had a similar peak formation as was observed with the in-line derivatization of Trp (Figure 10). This indicated that the first four peaks originated from the FMOC and that the following two peaks were the Val enantiomers. This hypothesis corresponded with the observations done with off-line derivatized Val because the migration times of the hypothesized FMOC and Val peaks are similar to

the migration times of the last three peaks of the in-line derivatized Val (not counting the impurity peak marked with a *).
There were no peaks observed with the in-line derivatized Val with 0 mM FMOC as was expected since no FMOC was injected to derivatize the amino acids.
The peaks marked with a * which were observed with the in-line derivatized Val were most likely an impurity. The absence of this peak in the off-line derivatized and in-line derivatized Val with 0 mM FMOC indicated it did not originate from the underivatized or derivatized valine. Due to this it was hypothesized that the impurity most likely originated from the FMOC.

There were no substantial differences observed between the in-line derivatized Val 2.5 mM and 5.0 mM FMOC. The hypothesized Val enantiomers appeared to have a circa 5% higher peak intensity with 2.5 mM FMOC than the 5 mM FMOC. It was uncertain if that was due to the difference in FMOC concentration or variances between runs.

5.2 Testing

Measuring 20 amino acids

To test the method all 20 amino acids were measured to determine if they can be enantioseparated with the current method using 2.5 mM FMOC. The CE method for in-line derivatization was used to enantioseparate all 20 amino acids. Every amino acid was measured in a mixture with another, previously enantioseparated, amino acid as a positive control. This was done to be able to determine if the run itself was successfully finished to avoid observations based on faulty runs. If a run had a similar migration time and peak intensity combined with similar FMOC peaks as previously enantioseparated amino acids, the run was deemed successful.

See Figure 13, Figure 14 and Figure 15 for the results.

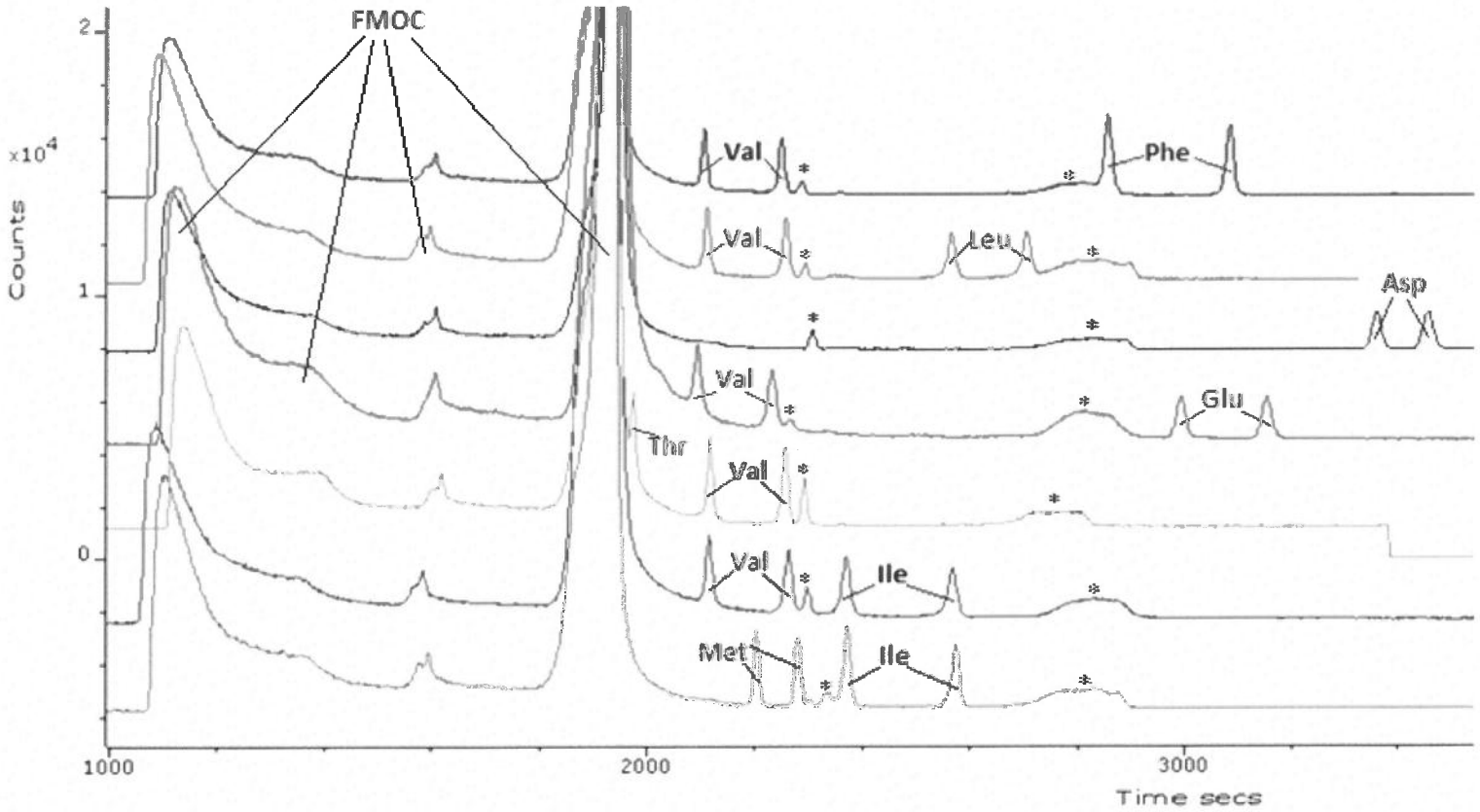

Figure 13: Electropherograms of in-line derivatized Val, Phe, Leu, Asp, Glu, Thr, Ile and Met. All runs had the same FMOC peaks but not all FMOC peaks were marked to maintain visibility.
Peaks marked with a * were impurities

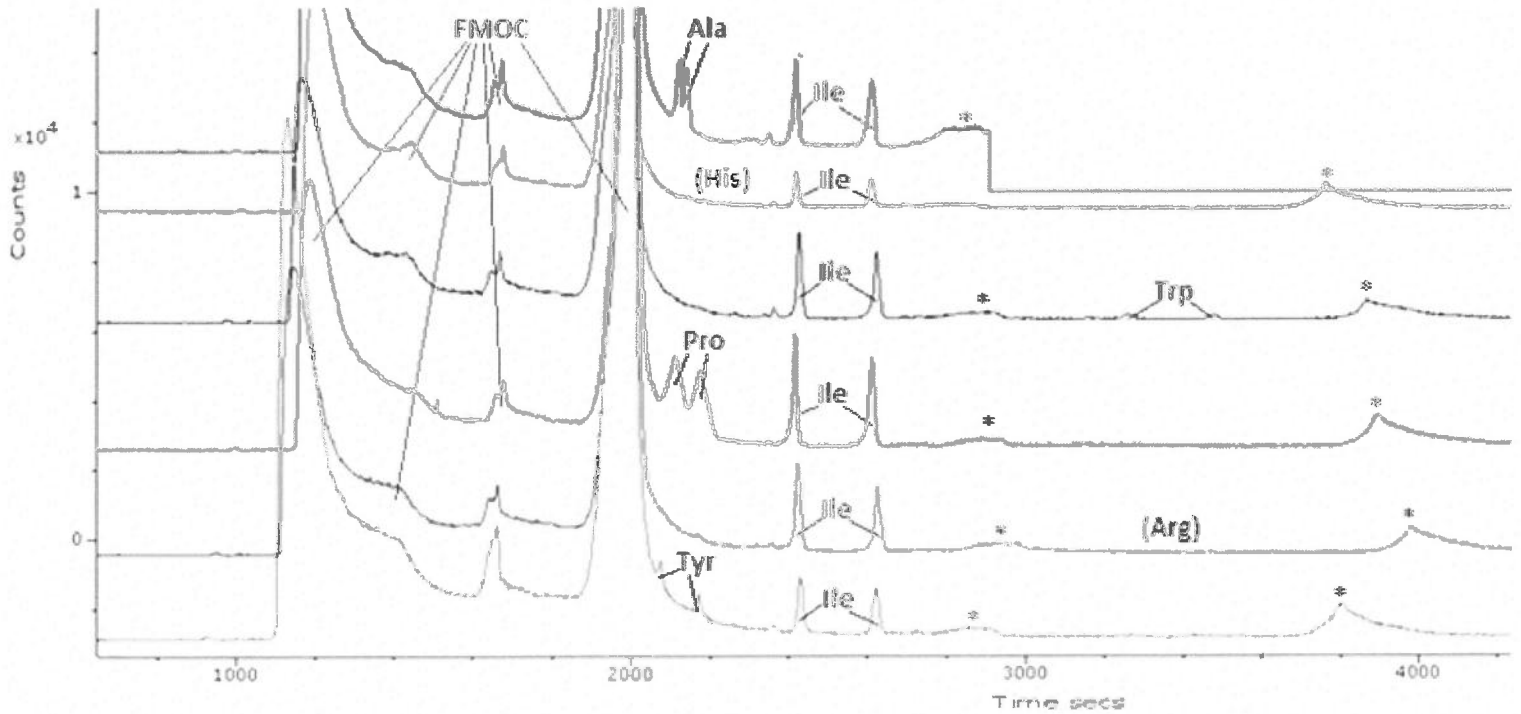

Figure 14: Electropherograms of in-line derivatized Ala, Ile, Trp, Pro, Tyr, (His) and (Arg). Amino acids between brackets () were unobserved. All runs had the same FMOC peaks but not all FMOC peaks were marked to maintain visibility.
Peaks marked with a * were impurities

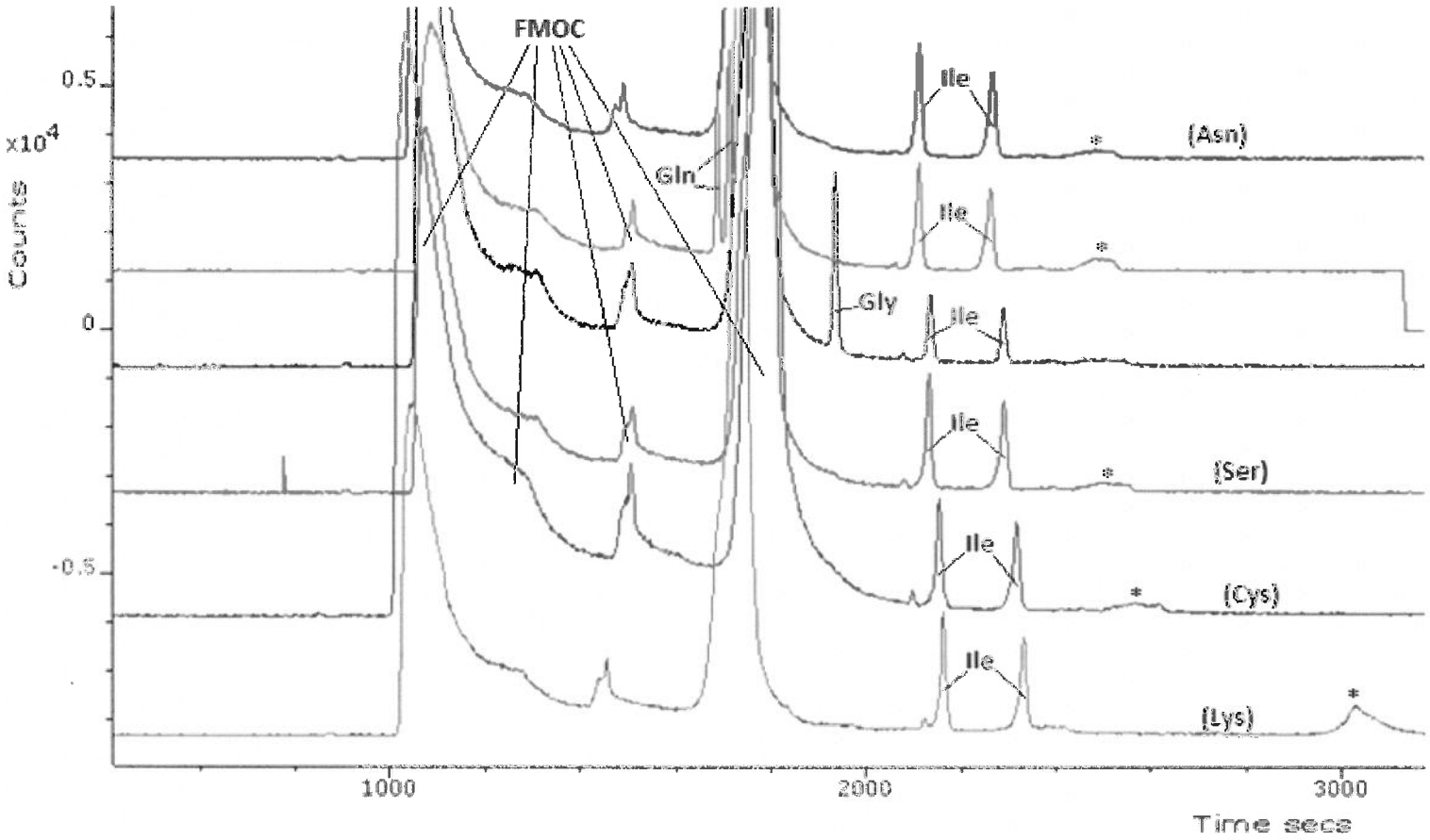

Figure 15: Electropherograms of in-line derivatized Ile, Gln, Gly , (Asn), (Ser), (Cys) and (Lys). Amino acids between brackets () were unobserved. All runs had the same FMOC peaks but not all FMOC peaks were marked to maintain visibility.
Peaks marked with a * were impurities

All runs had a similar FMOC peak formation and were deemed successful according to the criteria stated in the beginning of this experiment. The amino acids Val, Phe, Leu, Asp, Glu, Ile, Met, Trp and Tyr were observed and enantioseparated. The amino acids, Ala, Pro and Gln were also observed but not baseline separated. Only one peak of the amino acids Gly and Thr were observed and His, Arg, Asn, Ser, Cys and Lys were not observed. There were also some reoccurring impurities observed.

All observed amino acids were identified through spiking and all D-enantiomers migrated before the L-enantiomer of its respective amino acid.
All amino acid peaks appeared to have lower signal intensity compared to the results in Figure 12 which was expected because a total amino acid concentration of 500 μM was maintained for all injected samples. This meant that each enantiomer was injected with a concentration of 125 μM which was half of what was injected per enantiomer with the results in Figure 12 (250 μM). After Val a small peak was observed in some runs with irregular signal intensities (marked with a *), see Figure 13 at circa t=2300 seconds. Due to its irregular signal intensities it was believed this peak was an impurity because these runs were done on different days and different samples. The samples and BGE were freshly made on each day.

The peaks marked with an * at circa t=2600-2800 seconds were most likely an impurity and were also observed in Figure 12 at the same migration time. Therefore it was believed those peaks originated from the FMOC.
The peaks marked with an * at circa t=3900-4000 seconds in Figure 14 were also most likely an impurity. It was uncertain where the peak originated from but it was hypothesized it also originated from the FMOC because the peak was observed in all runs which were 4000 seconds or longer. Since