# Development and Validation of Methods for Impurity Profiling of Amino Acids

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Raphael Kühnreich

aus Wertheim

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“It ain't what you don't know that gets you into trouble. It's what you know for sure that just ain't so.”

**Mark Twain (1835 – 1910)**

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**DANKE!**

*Meinen Eltern*

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# I Introduction

# 1. Amino Acid Analysis

## 1.1. General

Amino acids (AAs) are the building blocks of proteins, participate in metabolic processes and are fundamental for living organism. They have to be supplemented if the demand is not fully covered by food intake, which may be the case for certain illnesses and if parenteral nutrition is required. AAs are also used as drugs and are raw material for the synthesis of them. Thus, they have to be treated as substances for pharmaceutical use and have to be monographed in Pharmacopoeias accordingly (e.g. European Pharmacopoeia [1-3]).

For the production of AAs, various methods are available. By hydrolysation of proteins and separation by means of ion exchange chromatography, practically all proteinogenic AAs can be obtained at once. In this case, the individual AAs may be contaminated by other AAs. With fermentation, they can be obtained with biotechnological processes with the help of bacteria (e.g. *E. coli*). Mainly one AAs is produced at a time, but due to the extraction that is necessary, other AAs and non-AA impurities (e.g. originating from the bacteria's natural physiological pathways) can be present. Other options include the full synthesis of AAs with methods like the strecker amino acid synthesis or individual synthesis routes have been established (e.g. for methionine and cysteine).

In the European Pharmacopoeia [1], AAs have been traditionally analyzed by means of thin-layer chromatography and the subsequent treatment with ninhydrin spray reagent with the test for ninhydrin-positive substances [4]. Primary amines like AAs react with ninhydrin to Ruhemann's purple, a compound with a purple color (see Figure 1.1-1). Proline as a secondary AA does reacts differently and an imine with a yellowish color is formed [5].

However, this analysis technique offers a rather poor sensitivity and specificity and, therefore, does often not provide the performance needed for nowadays quality control of AAs. The limit of quantification for this method is about 0.5 %. This is not sufficient for the low amount of impurities that can be present in AAs and it is not in accordance to the ICH guidelines, which requires a reporting threshold for impurities of 0.05 % for active substances

with a maximum daily dose of less or equal 2 g/day or 0.03 % in case of a daily intake of more than 2 g [6]. In the recent decades, several more powerful analysis methods for AAs have been developed. Due to the high significance of AAs in life science and the technical progress in analysis, the number of new publications that deal with this topic area is still high. Growing attention is paid to the chiral separation of AAs, since D-AAs can have different physiological effects on living organism [7-11] which requires the development of chiral analytical methods.

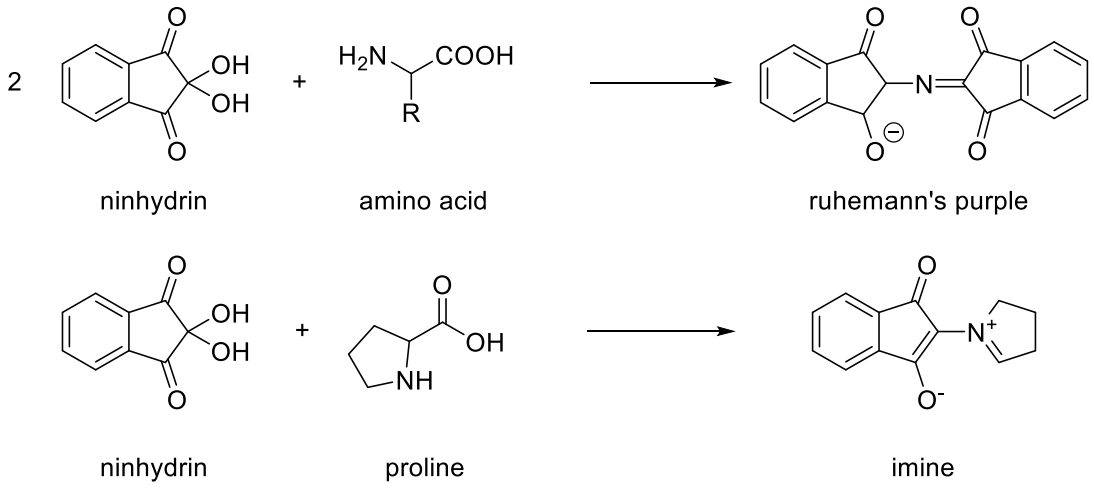



**Figure 1.1-1:** Schematic reaction of ninhydrin with primary AAs and proline [5].

## 1.2. Amino Acid Analyzer

The European Pharmacopoeia utilizes amino acid analysis (AAA) [12] for impurity profiling of AAs and protein hydrolysates in newer revisions of the monographs of AAs. It replaces the method for ninhydrin-positive substances by means of thin-layer chromatography [13] used before. An amino acid analyzer is basically a HPLC system that is specifically optimized for AAA. It exhibits special instrumentation for pre- or postcolumn derivatization. The instrument is particularly intended for AA analysis and therefore cannot be used for other purposes.

The intent of such an instrument is to provide a reliable, robust, accurate, versatile and fully automated system to analyze the AA composition in various samples. Different approaches are possible for the analysis of AA. For postcolumn derivatization (see Figure

1.2-1), the AAs are separated on a cation ion exchange column and afterwards the derivatization solution is mixed into the eluent flowing out of the column. The purpose of the derivatization is to make UV or fluorescent spectrophotometric detection possible. Since the derivatization occurs after the separation, the sample matrix does not interfere with the reaction and the reproducibility is high. The range of reagents is limited, because only reagents that react fast enough under the typical chromatographic conditions (pH-value, temperature) are suitable for this process. The instrumentation needed for postcolumn derivatization is more complex and expensive in comparison to precolumn derivatization. This is due to the capabilities it has to provide (e.g. heating for a faster reaction). Moreover, this technique requires a continuous flow of the derivatization solution to the eluent, which excludes expensive reagents. Derivatization reagents used for postcolumn derivatization of AAs in the European Pharmacopoeia are therefore only ninhydrin and *ortho*-phthalaldehyde.

If precolumn derivatization is applied (see Figure 1.2-1), the choice of derivatization reagents is much higher. Also, sample preparation can consist of additional steps e.g. extraction of the derivatives and can be done without special instrumentation outside the HPLC system. However, if an automated precolumn derivatization process with a modern autosampler or another derivatization device is necessary, the use of many reagents is not possible either. Beside to the introduction of a chromophore or fluorophore, the purpose of precolumn derivatization is the feasibility of modifying the retention behavior and therefore enabling other types than ion exchange chromatography (e.g. direct and indirect enantioseparation). Since, precolumn derivatization leaves more freedoms when it comes to the choice of reagents and the control of reaction conditions, the sensitivity is in general higher. However, as it takes some time until detection, the stability of the derivatives can be an issue. Furthermore, the degree of derivatization may be influenced by the sample matrix which is problematic when samples with different matrixes are analyzed.

With regards to AAs, the European Pharmacopoeia utilizes postcolumn derivatization with ninhydrin. With this approach, ninhydrin-positive impurities can be controlled with sufficient sensitivity but other methods for the control of compounds that do not react with ninhydrin may be necessary. In addition, the method is achiral which means that the separation of the enantiomer is not feasible and a chiral method has to be employed.

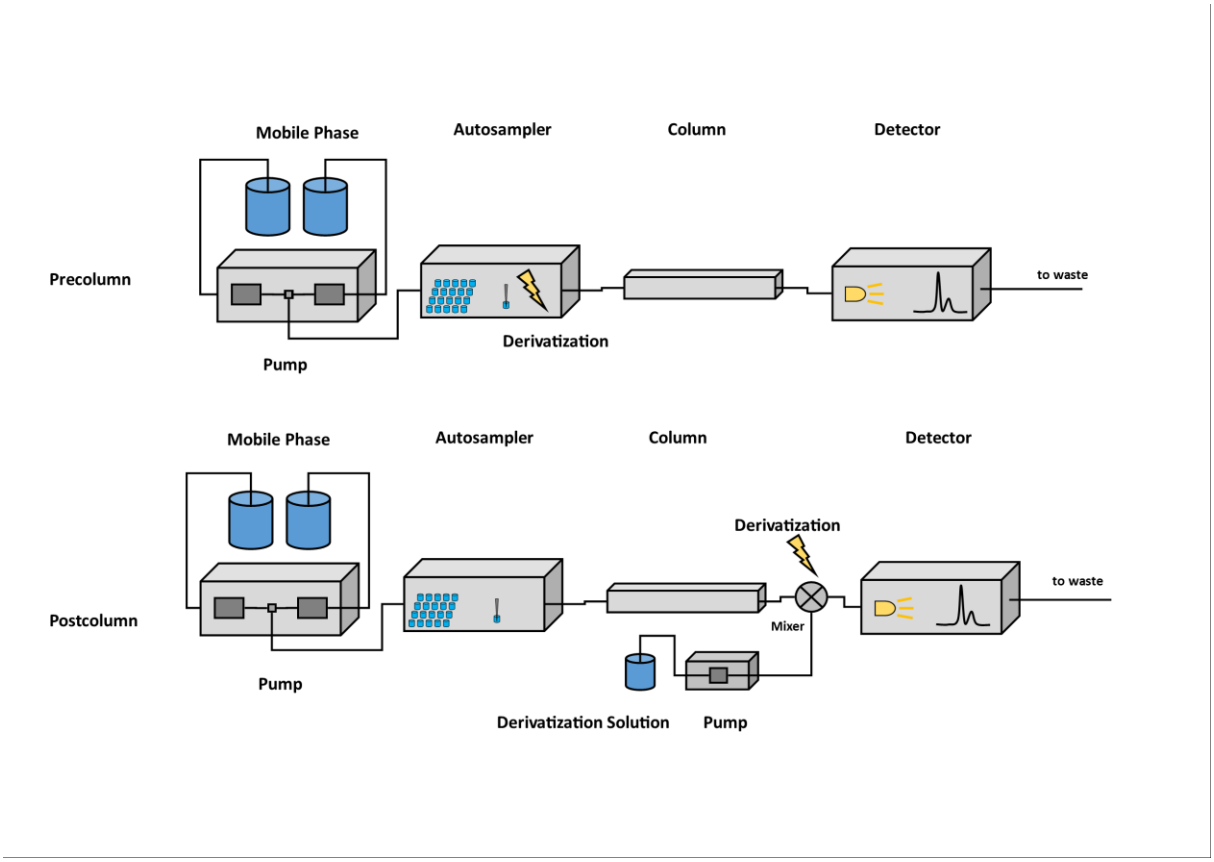



**Figure 1.2-1:** Schematic view of pre- and postcolumn derivatization with an amino acid analyzer. The precolumn derivatization takes place prior separation within the autosampler or is done outside the system. For postcolumn derivatization, the eluent is mixed with the derivatization solution afterwards. *Scheme by author*

# 2. Derivatization Reagents for Amino Acids

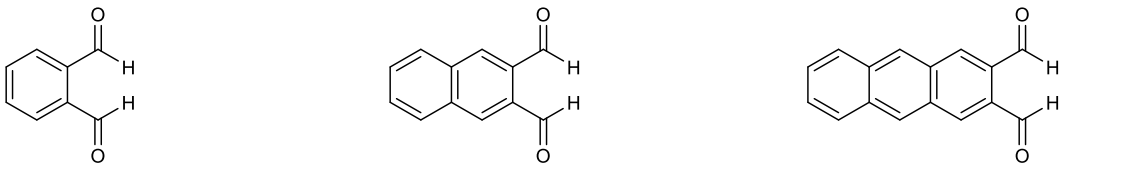
There is a broad variety of derivatization reagents available for AAs and amines. Among other things, they differ in reaction kinetic, applicability for primary and secondary AAs, detection capabilities, stability of the derivatives, and the possibility of an indirect enantioseparation of the products with reversed phase liquid chromatography. Therefore, depending on the analytical task that has to be solved, an appropriate reagent has to be chosen. Sometimes it is unavoidable that compromises have to be made.

However, not all derivatization reagents are suitable for the analysis of AAs with liquid chromatography or capillary electrophoresis. The following chapter is providing an overview over the ones that play a significant role in publications over the last decades. In addition to

that, the synthesis of new reagents especially for chiral separation purposes is an ongoing development. Therefore, new substances are being published regularly but the use of those is often limited to specific applications.

## 2.1. Dialdehydes

Dialdehydes are aromatic compounds with two aldehyde moieties in ortho position (see Figure 2.1-1). In contrast to most other derivatization reagents, they provide short reaction times with high yields. The compounds itself exhibits practically no fluorescence and does hence not interfere with the detection of the derivatives if fluorescence detection is used. On the downside, dialdehydes do only react with primary AAs. However, by the previous treatment of secondary AAs with hypochloric acid, derivatization with dialdehydes is also possible [14]. Cysteine is another AA that yields poor results. Response of the derivative is low and additional peaks can be present in the chromatogram. Here, the alkylation of the sulfur atom with iodoacetic acid can be advantageous [15, 16]. Especially when *ortho*-phthalaldehyde (OPA) is used, the poor stability of the derivatives is a major drawback.




*ortho*-phthalaldehyde (OPA) 2,3-naphthalenedicarboxaldehyde (NDA) anthracene-2,3-dialdehyde (ADA)

**Figure 2.1-1:** Overview of the dialdehydes used for the derivatization of AAs and amines.

### 2.1.1. *Ortho*-Phthalaldehyde

The most important compound of the dialdehyde group and one of the most used reagents for the derivatization of AAs in general is *ortho*-phthalaldehyde (OPA). Since it was first described in literature [17], it has found great application in analysis of amines and AAs. OPA shows high reliability and is easy to use. It reacts with primary AAs in a borate buffer and thiol compound within a minute or less to a fluorescent isoindole derivative (see Figure 2.1-2). An excess of the reagent is advisable to ensure a fast and quantitative conversion of the AAs.

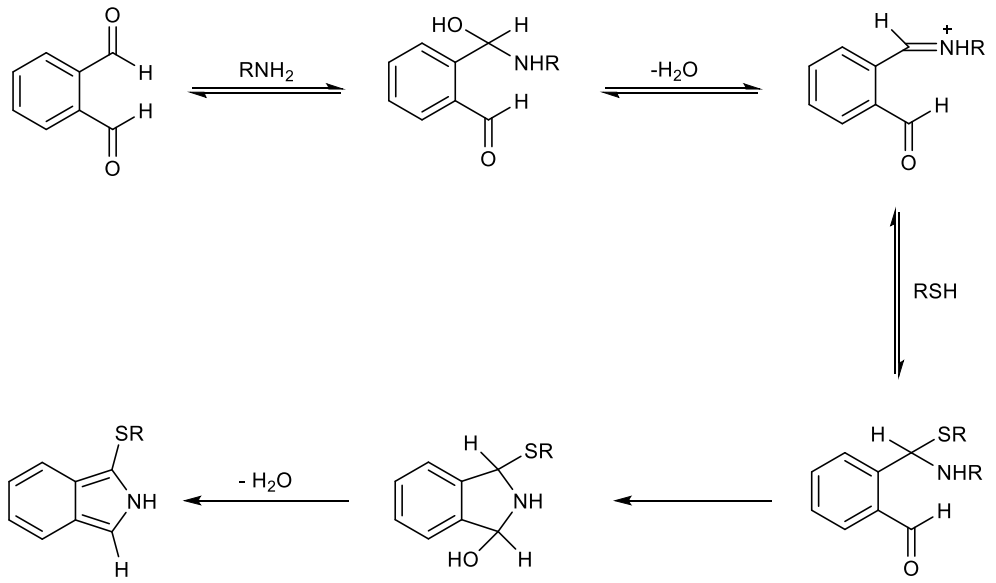



**Figure 2.1-2**: Reaction mechanism of OPA with AAs [18]. The reaction is performed in a borate buffer (pH ≈ 8.0 for amines and ≈ 10.0 for AAs).

A thiol is needed for the reaction to take place. 2-Mercaptoethanol (ME) has been used in the beginning [17] but has often been replaced by different thiols that provide better characteristics in terms of separation performance and stability for the derivatives (see Figure 2.1-3). With the use of chiral thiols, diastereomeric derivatives are formed, which make an indirect enantioseparation of chiral amines and AAs on common reversed phase columns feasible. This is a cost-effective alternative to the use of chiral columns, which may exhibit a smaller plate number. The derivatives obtained with *N*-acetyl-cysteine (NAC) [19, 20] and *N*-isobutyryl-L-cysteine (NIBLC) [21, 22] offer enhanced selectivity with regard to the ones obtained with ME and 3-mercaptopropionic acid (MPA), in particular when indirect enantioseparation is applied.

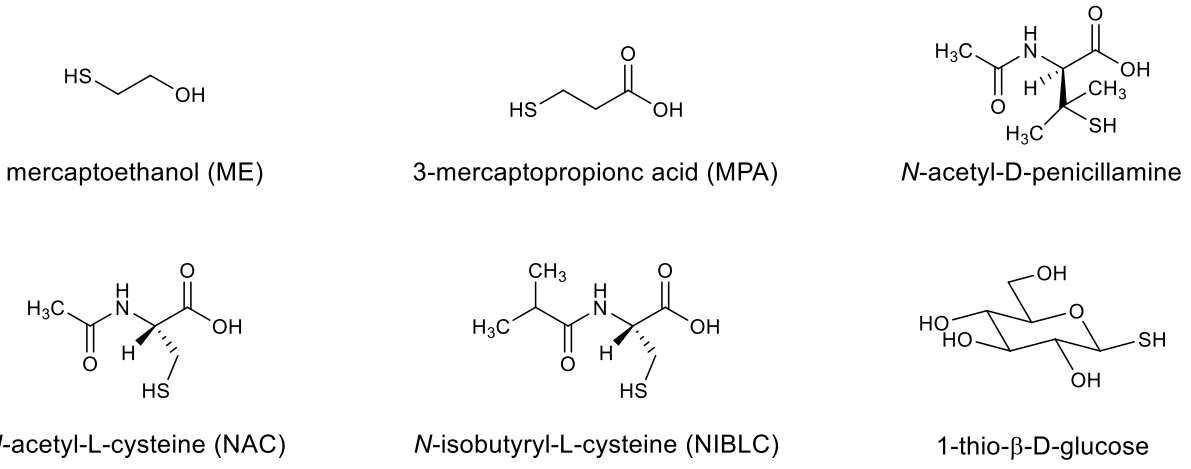



**Figure 2.1-3:** Thiols suitable for the derivatization of AAs with OPA.

The poor stability of the OPA-derivatives has been the major drawback in the use of this reagent since the beginning. Due to the otherwise simple and effective handling of OPA, many attempts have been made to investigate this issue and to improve it either [23-27]. The results show that the stability of the derivatives is dependent on several factors. The size of the substituents at the sulfur and the nitrogen atom of the isoindoles derivatives is crucial. Using thiol reagents that have a more bulky rest at the sulfur atom than the ethanol rest in ME leads to derivatives that are more stable [24]. This explains the improved stability of derivatives with NAC or NIBLC over ME. It could also be shown, that bulky groups at the nitrogen atom of the isoindoles slows down the decay of fluorescence [28], so that derivatives of comparatively large AAs are likely more stable. The ratio of OPA and thiol in the derivatization solution has an effect too. OPA/MPA or OPA/NAC in the ratio of 1/3 or 1/50 results in a higher stability of the isoindoles within the derivatization solution [29]. In general, the derivatives obtained with NAC are more stable than the ones with MPA as thiol [30]. The thiol should be added to the derivatization solution with OPA before the reaction with the AA starts [25] and since the excess of OPA does facilitate the degradation of the isoindoles, extraction can increase the stability [28]. Alternatively, the addition of hydroxypropyl-β-cyclodextrin has a similar effect [31].

Taken together, the instability of the isoindoles is somehow related to the reagents and buffers used for the reaction. An automated derivatization procedure would not only reduce the time the isoindoles have to be stable, it would also separate them quickly from the derivatization solution.

### 2.1.2. Naphthalene-2,3-Dicarboxaldehyde

Naphthalene-2,3-dicarboxaldehyde (NDA) is another dialdehyde for the derivatization of primary amines. Unlike with OPA, cyanide is used instead of a thiol to form fluorescent *N*-2-substituted-1-cyanobenz-[f]-isoindoles (CBI). These derivatives show higher chemical stability in comparison to its OPA counterparts [32] and improved sensitivity when fluorescence detection is applied. A strong excitation maximum at 250 nm (with weaker ones at 420 and 440 nm) and a maximum of fluorescence emission at 490 nm is present [33]. Furthermore, amperometric detection is also feasible [34].

### 2.1.3. Anthracene-2,3-Dialdehyde

Anthracene-2,3-dialdehyde (ADA) is a dialdehyde which leads to derivatives with further improved fluorescent sensitivity and increased liphophilicity in comparison to the ones obtained with OPA and NDA. Higher resolutions on reversed phase columns could also be achieved [35]. However, only in very few papers this compound has been described. The exact emission and excitation wavelength are not specified but are higher than with OPA and NDA [36]. For a baclofen derivative, $\lambda_{ex}$ was determined to be within 500 – 550 nm and $\lambda_{em}$ to approximately 580 nm [37].

### 2.1.4. 3-(4-Carboxybenzoyl)-2-Quinolinecarboxaldehyde

A derivatization reagent that is often used in conjunction with capillary electrophoresis is 3-(4-carboxybenzoyl)-2-quinolinecarboxaldehyde (CBQCA) [38-41]. In a phosphate buffer (pH 7.0) the AAs react with CBQCA in a 50-fold molar excess after addition of potassium cyanide in at least one to several hours protected from sunlight (see Figure 2.1-4) [41, 42]. The detection is done by means of laser-induced fluorescence ($\lambda_{ex}$ 488 nm; $\lambda_{em}$ 520 nm). With the help of cyclodextrins, an indirect enantioseparation is possible [43, 44].

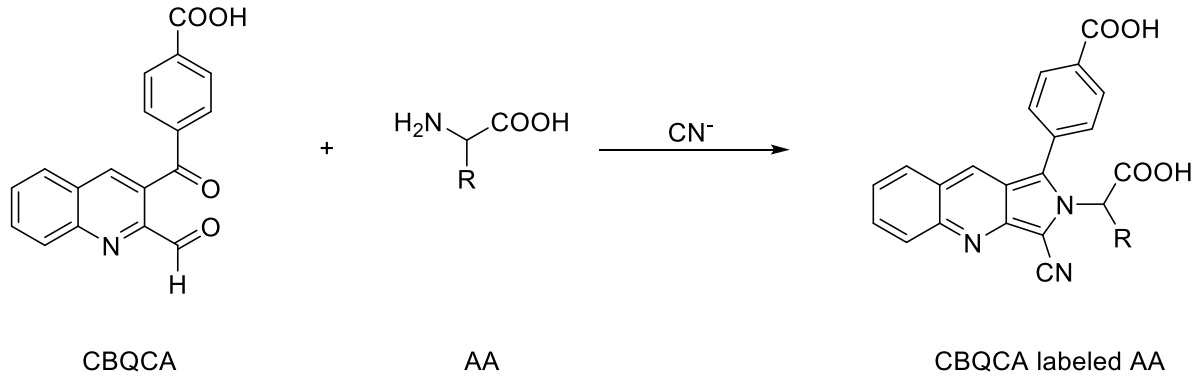



**Figure 2.1-4:** Reaction scheme of CBQCA with AAs.

## 2.2. Fluorenylmethyloxycarbonyl Chloride

A reagent that can be used for the precolumn derivatization of primary and secondary amines is fluorenylmethyloxycarbonyl chloride (FMOC-Cl) [45]. The reaction with AAs takes place in a mixture of borate buffer (pH 8-10) and an organic solvent such as acetone or acetonitrile in a few minutes only [46]. The derivatives are fluorescent and can be detected at

315 nm with excitation at 265 nm. Due to the excess, necessary for a quantitative conversion of the AAs, the unused FMOC-Cl is hydrolyzed to FMOC-OH with the time. FMOC-OH does interfere with the separation due to its broad peak shape on reversed phase columns. To avoid this, FMOC-OH has to be extracted with hydrocarbons like pentane or hexane. However, FMOC-OH cannot be fully extracted and the content of FMOC-AAs in the sample might be reduced by the extraction process. Alternatively, 1-aminoadamantan (ADAM) can be utilized (see Figure 2.2-1) [47, 48]. ADAM serves as a scavenger for the excess of FMOC-Cl which is reacted to a FMOC-ADAM complex [47]. This compound is very hydrophobic and elutes after all other FMOC-AA-derivatives at the end of the chromatogram. Hence, interferences with FMOC-AAs do not occur. The ADAM reagent has to be added to the derivatization solution immediately after the reaction of FMOC-Cl with the AAs has finished in order to keep the FMOC-OH peak and thus its interference with other peaks as small as possible.

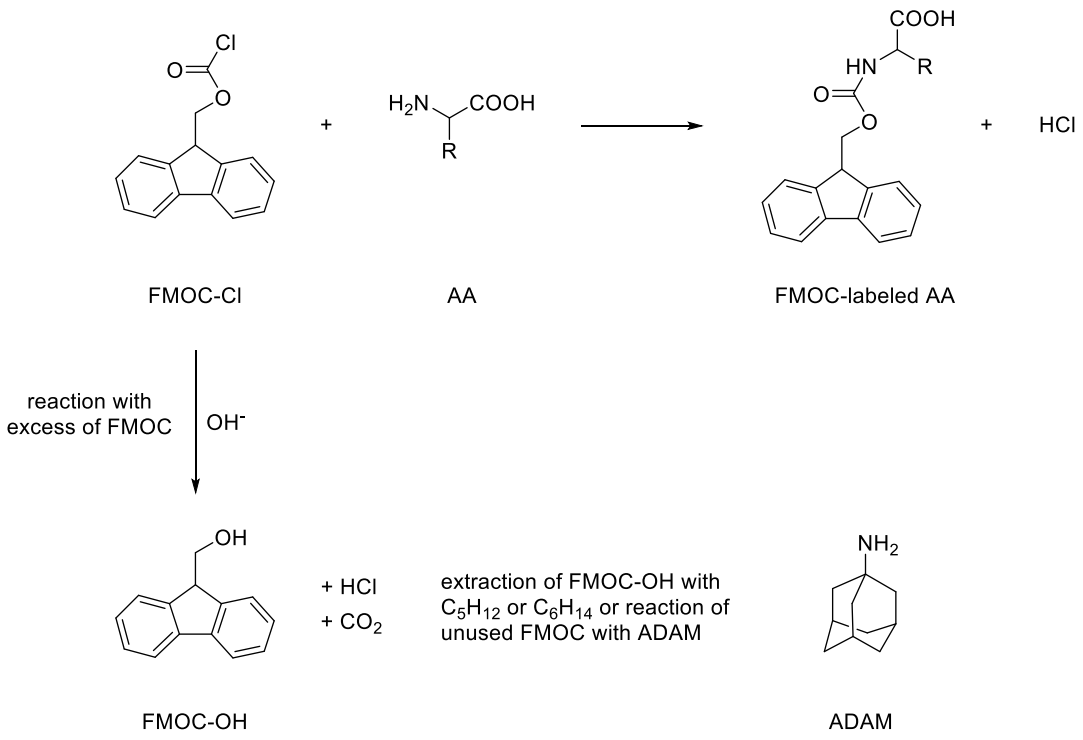



**Figure 2.2-1:** Reaction of FMOC-Cl with AA and side reactions.

Because of its short reaction time, it can be used for automated precolumn derivatization. It is also utilized in addition to the derivatization of AAs with OPA, to convert the remaining secondary AAs [49, 50]. FMOC-Cl is achiral and hence not suitable for an indirect enantioseparation of AAs.

## 2.3. Isothiocyanates

Phenylisothiocyanate (PITC) is also known as Edman's reagent and used for the Edman degradation [51]. It enables protein sequencing by continuous cleavage of the N-terminus of proteins. It is also suitable for direct derivatization of primary and secondary AAs and subsequent analysis with HPLC [52-54]. After the preparation of the samples, they have to be dried under vacuum. The residue is afterwards dissolved in a solution of ethanol : water : triethylamine : PITC (7:1:1:1). The reaction (see Figure 2.3-1) takes about 20 min at room temperature and the excess of the reagent has to be removed by vacuum evaporation [55]. First, phenylthiocarbamoyl (PTC) compounds are formed but they should be converted to the more stable cyclic 3-phenyl-2-thiohydantoin (PTH) derivatives in acid conditions [54]. The products are non-fluorescent but UV-detection at 254 nm can be applied. The chromophore formed with primary and secondary AAs is the same [54].

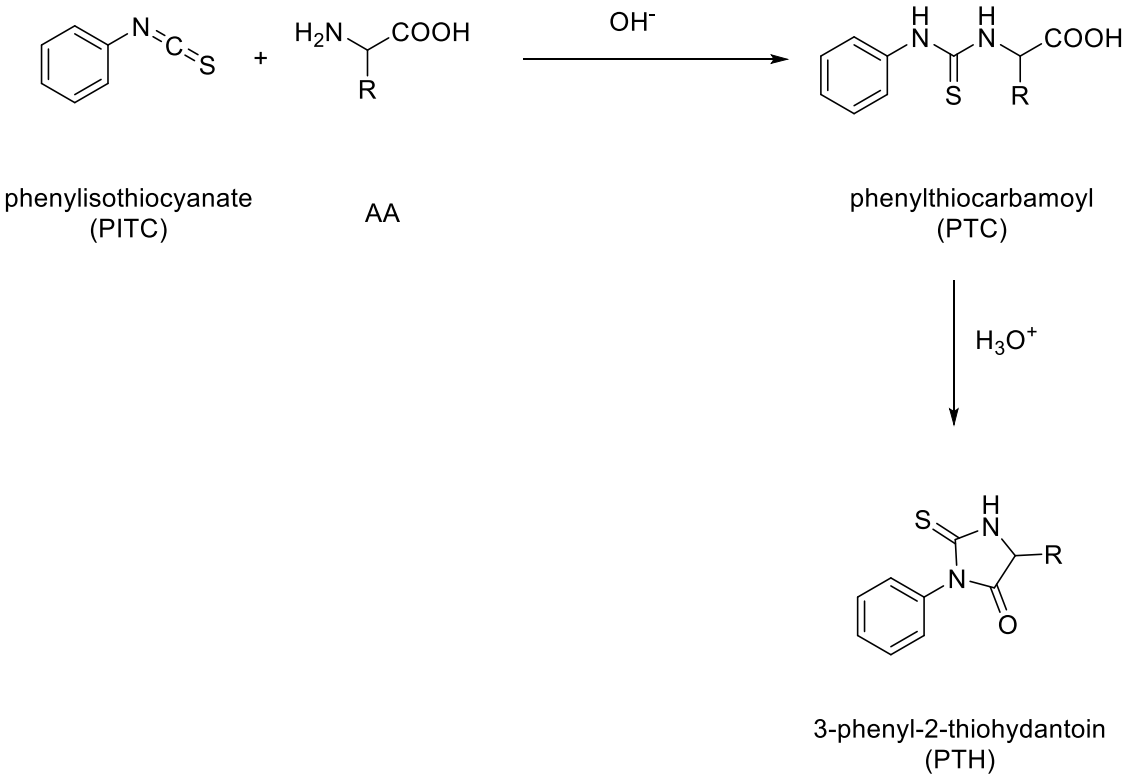



**Figure 2.3-1:** Reaction scheme of PICT with AAs

## 2.4. Dabsyl-Cl

4-(4-Dimethylaminophenylazo)benzenesulfonyl chloride (Dabsyl-Cl) is a derivatization reagent for primary and secondary amines and AAs [56-58]. Its reaction with AAs is carried out in acetonitrile as solvent and heating to 70 °C for 15 – 30 min [59, 60]. A longer time or a different pH was found to be negligible [59]. The obtained derivatives (see Figure 2.4-1) are stable and show a maximum absorption in the range 448 – 468 nm. Temperatures lower than 70 °C can lead to incomplete derivatization of some AAs, whereas a higher temperature causes lower peak intensity [59]. This might be explained with an instability of the derivatives at higher temperatures. The number of recent papers describing the application of Dabsyl-Cl is rather low [61]. This might be related to the fact, that fluorescence detection is not possible and the required heating prevents the automatization of the derivatization process.

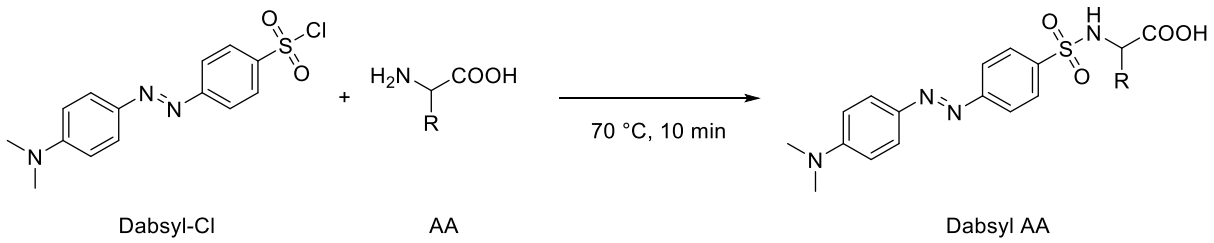



**Figure 2.4-1:** Scheme of the derivatization of AAs with Dabsyl-Cl

## 2.5. Dansyl-Cl

A derivatization reagent similar to Dabsyl-Cl, but enabling fluorescence detection is 5-(dimethylamino)naphthalene-1-sulfonyl chloride (Dansyl-Cl). It reacts with primary and secondary amines, amino acids as well as alcohols and produces derivatives that are stable enough for analysis [62, 63]. The reaction can be performed in a water bath at 80 °C for 30 minutes in the dark with a pH of 9.8 [63]. The excess is precipitated by reaction with ammonia [62] to prevent the hydrolysis of Dansyl-Cl, which could lead to broad peaks that interfere with the detection of the Dansyl-AAs. Alternatively, an AA that is of no interest in the sample is used as a scavenger [64] for Dansyl-Cl. Fluorescence spectrophotometric detection

is normally used ($\lambda_{ex}$: 360 nm and $\lambda_{em}$: 470 nm) but UV spectrophotometric detection at 250 nm is also feasible [65].

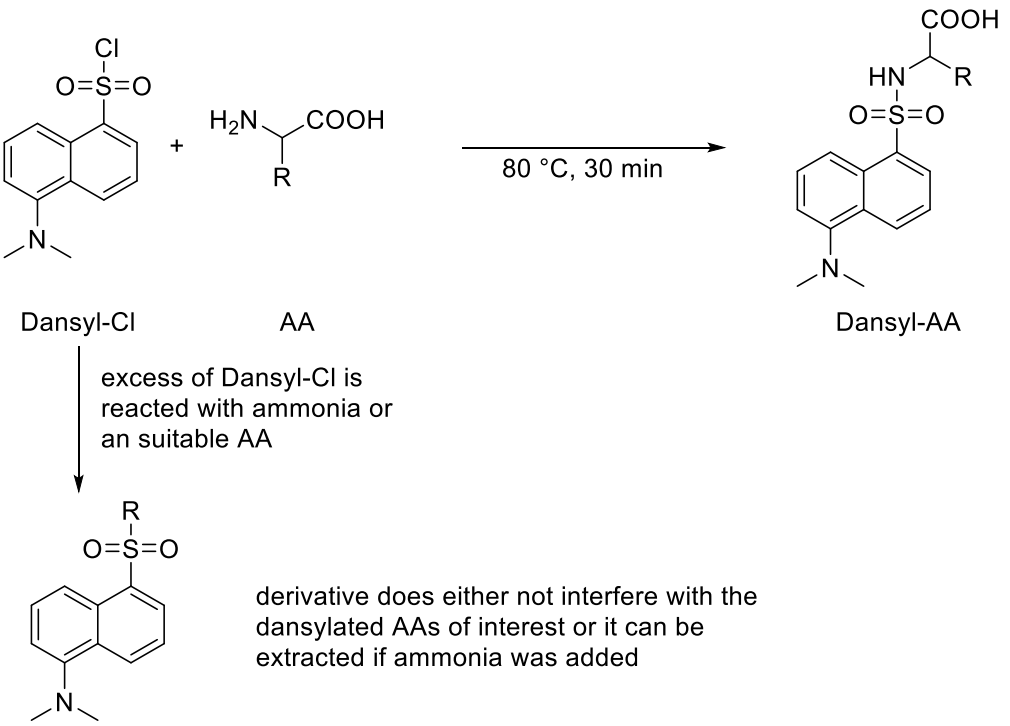



**Figure 2.5-1**: The reaction of Dansyl-Cl with AAs.

## 2.6. Marfey‘s Reagent

A newer derivatization reagent that is particularly suitable for the chiral analysis of AAs is 1-fluoro-2-4-dinitrophenyl-5-L-alanine amide (FDAA, Marfey’s reagent) [66, 67]. It reacts within 60 min at 40 °C with all common AAs. The derivatives are stable for 48 hours and can be detected by means of UV spectroscopy at 340 nm in the pmol range [68, 69]. Diastereomers are formed due to the stereogenic center that is present in the reagent. This makes an indirect enantioseparation of AAs by means of reversed phase liquid chromatography feasible. The derivative of the L-AAs is eluting before the D-derivative on reversed phase columns. This is explained by a stronger intramolecular H-bonding of D-derivatives which leads to more hydrophobic molecules [66] and thus showing a stronger affinity for the stationary phase on reversed phase columns. Structural variants of the L-alanine amide side chain can be utilized to further improve the separation [69]. As with many derivatization reagent, an excess is necessary for a quantitative conversion. This is especially true for AAs like lysine and ornithine having two amino groups. These have to be derivatized twice in order to give only one peak

in the chromatogram. However, the excess cannot be removed and does appear in the same region as derivatized AAs. Hence, a fully automated analysis is not possible.

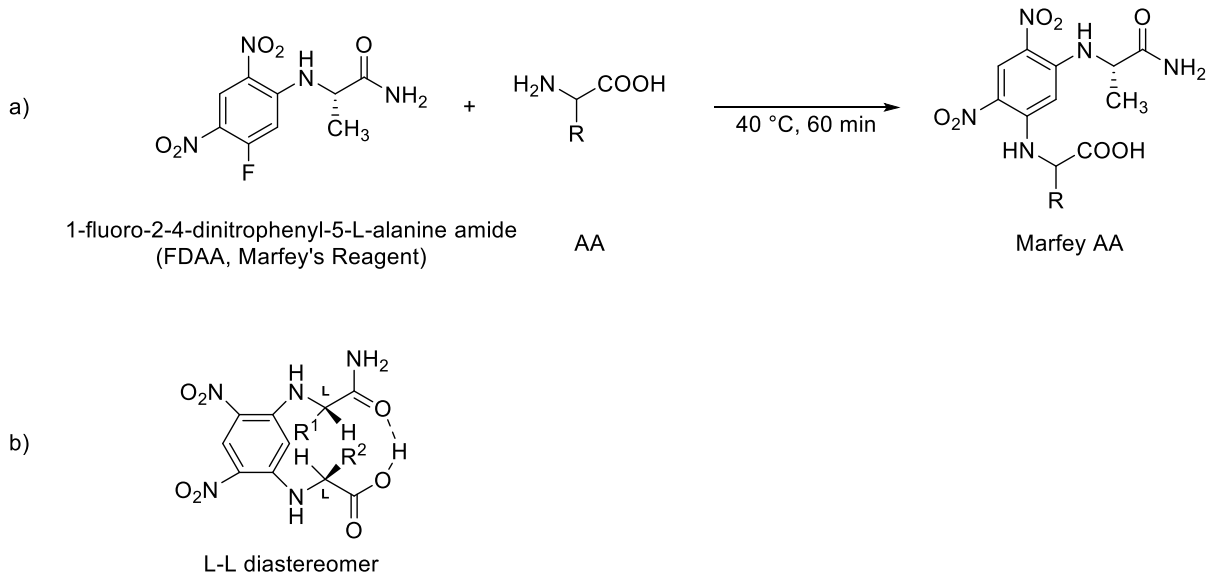



**Figure 2.6-1:** a) reaction Marfey's reagent with AAs; b) intramolecular H-bonding of derivatives [66]

# 3. Chiral Separation of Amino Acids

The chiral separation of AAs with HPLC and CE has been subject to many studies [70-73]. There are two basic principles for chiral separation of AAs by means of HPLC. In the case of indirect enantioseparation, the substance is first modified, mostly by derivatization with a chiral derivatization reagent (CDA), to facilitate a chiral separation indirectly. The actual separation is not chiral at all. Usually, the enantiomeric compounds are converted to diastereomeric compounds, which can easily be separated with liquid chromatography on achiral reversed phase columns. The direct enantioseparation on the other hand does not modify the enantiomers in any way. Hence, separation is only possible with chiral stationary phases (CSP) or with an achiral stationary phase and a chiral selector included in the mobile phase. Diastereomers are temporarily formed by interaction of the enantiomers with the CSP or the selector. Indirect enantioseparation is always performed with a precolumn derivatization whereas a direct enantioseparation can have an additional postcolumn derivatization step if necessary. A special case is the derivatization with an achiral

derivatization reagent (e.g. FMOC) and the subsequent separation with a CSP. This can sometimes be inevitable, when an achiral reagent offers the best results for the derivatization.

## 3.1. Indirect Enantioseparation with HPLC

The additional steps required for derivatization brings along some serious issues, that must carefully be evaluated during method development and validation. First of all, the process does take extra time and the automation of the process is not always possible. The selection of the derivatization reagent must be well thought-out, since it determines and limits the capability of the method. The derivatization always requires an excess of the CDA which can interfere with the separation and detection of the derivatives. It has to be extracted prior analysis or it has to be ensured that no interference is occurring. Matrix effects have to be considered as they can alter the behavior of the reaction of CDA and analyte. Racemization of the analyte during the reaction and kinetic resolution is another very important point that can distort the results. Checking this is far from being simple and does often require the use of orthogonal methods. Furthermore, there are usually differences in the response factors of the produced diastereomers. This has to be taken into account and makes the indirect approach even more complex. As the analytes get modified, indirect enantioseparation cannot be used for preparative purposes. Lastly, the chiral purity of the CDA does ultimately determine the limit of quantification of the method. The L- and D-AA does react with an enantiomeric impurity of the CDA. The resulting derivatives are enantiomeric to the ones obtained with the CDA (see Figure 3.1-1) and therefore interfere with the quantification due to coelution on achiral stationary phases. Relatively pure CDAs do have a chiral impurity of about 0.01 %. Since the exact content is not known, a higher limit of quantification has to be chosen in order to determine the content of the chiral impurity of the analyte with sufficient precision and accuracy.

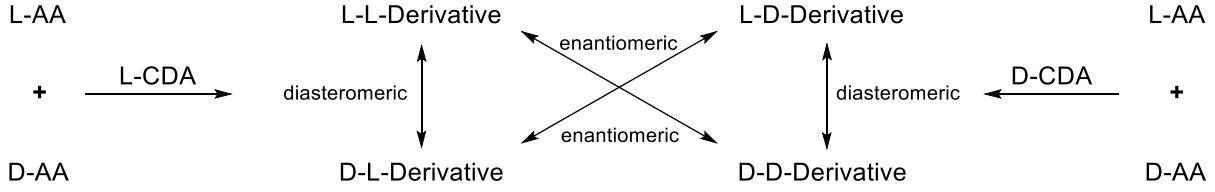



**Figure 3.1-1:** Diastereomeric and enantiomeric relationships between the individual derivatives

Nevertheless, indirect enantioseparation does also have some important advantages. By the introduction of strong fluorophores and chromophores, highly sensitive methods for substances that are otherwise difficult to detect, is easily feasible. The separation is done with common reasonably priced achiral columns. These provide a much better chromatographic performance than chiral columns [74]. The superior separation efficiency enables the evaluation of complex mixtures of substances. The elution sequence of the derivatives of two enantiomers can be reversed by using the enantiomer of a given CDA for derivatization. The method development is simple, more predictable and robustness is high. Due to the progress in direct enantioseparation, the indirect approach has lost some of its importance in recent decades [70], since the direct approach is becoming favored nowadays. However, it is still frequently applied [74-78].

## 3.2. Direct Enantioseparation with HPLC

In principle, the situation for the direct enantioseparation is the reverse as for the indirect one. Sample preparation is comparatively simple. The chiral purity of the CDA, kinetic resolution, racemization, non-quantitative derivatization and limitations of a CDA is no issue. The challenging task is the development of the chromatographic method. An expensive chiral column is required and the room for optimization is limited. Chiral columns do not allow the use of a variety of buffers and organics for the mobile phase as is the case for reversed phase columns. Gradients cannot be utilized due to the long equilibration times and the effect of changes of chromatographic parameters is difficult to predict. Therefore, the method development follows the trial-and-error principle and is time consuming, especially for complex mixtures of substances. In comparison with reversed phase columns, the peaks obtained with chiral columns are typically broader and smaller and thus, sensitivity and separation is inferior. However, since the first CSPs which were introduced by Pirkle [79], the development of new stationary phases has been going strong [80-82] and they are nowadays available for various applications. Another disadvantage is the comparatively short lifetime of these columns. For substances without chromophore, special detectors are required which further increases the costs. Alternatively, pre- or postcolumn derivatization can be done with an achiral derivatization reagent to enable UV or fluorescence spectrophotometric detection.

Issues with a chiral impurity does not apply in this case, but racemization and kinetic resolution still have to be considered. The opposite is the case, if a chiral selector is utilized instead of an CSP. Direct enantioseparation of AAs with HPLC is a growing field of interest in HPLC [83], especially for β-AAs [84, 85].

## 3.3. Enantioseparation with Capillary Electrophoresis.

Chiral separation by means of capillary electrophoresis (CE) is a proven technique [86] This also applies for the enantioseparation of AAs [73, 87, 88]. Over the past three decades, several approaches for enantioseparation with capillary migration techniques have evolved. Due to the increasing importance of chiral separations in the 1980s, the demand for suitable methods was also growing. CSPs for HPLC were already available at the time but the use was limited and the costs were high [86]. CE enabled very early the enantioseparation of compound with relatively low costs. This is still true today and a clear advantage over HPLC.

AAs can be derivatized (indirect enantioseparation) or not (direct enantioseparation) prior analysis. However, both approaches require the need for a selector in the background electrolyte, since diastereomers are not directly separated by CZE due to the same charge-to-mass ratio (z/m). Smaller analytes like AAs can be too small for an interaction with the selector (e.g. with cyclodextrins). In this case, a derivatization is necessary. Apart from the separation itself, the advantages and disadvantages of a direct and indirect approach are very similar for both, CE and HPLC.

### 3.3.1. Cyclodextrins

The most common selectors used with CE are cyclodextrins (CDs) [89, 90]. These compounds are basically cyclic oligosaccharides consisting of α-D-glucopyranoside monomers as building blocks. CDs with 6, 7 and 8 units are common and known as α-, β- and γ-CD respectively (see Figure 3.3-1). The three-dimensional structure of the CDs is conical. They have one primary and two secondary free hydroxy groups which are orientated to the exterior and make the CDs hydrophilic and water soluble [89]. The cavity is less hydrophilic and can interact with hydrophobic analytes. However, the nature of the chiral recognition is more

complex and includes hydrogen bonds and dipole-dipole interactions with the hydroxyl groups of the CD [91]. The free hydroxy groups are often substituted (e.g. with methyl-, sulfate- and acetyl-groups) to optimize its characteristics for analytical use, e.g. water solubility and affinity to the analyte. The introduction of a negative or positive charge greatly extend its applicability for different types of analytes and is a way to improve resolution. One issue with randomly substituted CDs is the poor reproducibility [92], when CDs from different manufactures or even batches from the same manufacture are utilized. The degree and position of the substitution may change, thus changing its properties. CDs with a defined substitution pattern are available, but the cost are vastly increased in comparison to the otherwise mainly reasonable priced CDs. The choice of a suitable CDs for a specific application is mostly done by trial and error. Due to the ease of use and the applicability for different types of chiral separations, they have become the first choice as selector for CE and been successfully applied for the direct and indirect enantioseparation of AAs [73, 93-95].

### 3.3.2. Crown Ethers

Typically, analytes that do interact with CDs have to be hydrophobic to some extent, thus most AAs have to be derivatized prior analysis. Crown ethers are cyclic polyethers, consisting of 4 to 6 -$CH_2$-$CH_2$-O- units (see Figure 3.3-1). The oxygen atoms define more or less a plane. Unlike the CD, the cavity of crown ethers is hydrophilic. They do interact with the analyte by complexation of cations by the oxygen atoms of the selector. Crown ethers can bind various types of cations e.g. alkali metals, alkaline earth metals and also potassium and ammonium cations like these in zwitterionic AAs. The complex is formed in the case of ammonium cations in a tripode arrangement with the three hydrogens of the nitrogen atom and three oxygens atoms of the crown ether. The axis of the nitrogen carbon bond of the AA is vertical to the plane of the oxygen atoms of the crown ether [91]. Furthermore, ionic- and dipole-dipole interactions as well as hydrogen bonds do contribute to the selectivity of crown ethers. An diastereomeric complex is formed whose structure is dependent on the absolute configuration of the analyte. The chiral separation of AAs with crown ethers was one of the first applications with this selector [96]. 18-Crown-6-tetracarboxylic acid is a common used selector for the analysis of AAs [97, 98]. The migration of both enantiomers can be reversed by choosing a selector with the opposite chirality [99].

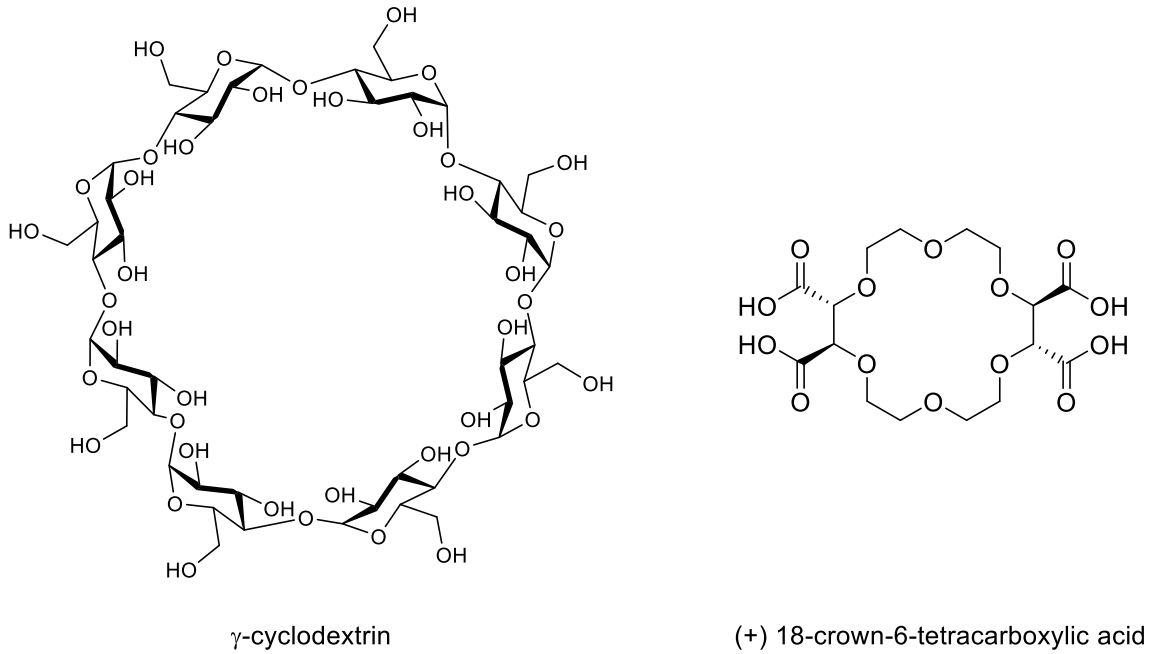



**Figure 3.3-1:** Structure of γ-cyclodextrin and 18-crown-6-tetracarboylic acid

### 3.3.3. Ligand-Exchange

Another principle of the enantioseparation of compounds by means of CE is ligand exchange (LE). It is based on the formation of ternary metal complexes by a chiral selector and the analyte. Usually, the metal ion is copper(II) and the chiral selectors are L-AAs. Besides this the usage of cobalt(II), nickel(II) and zinc(II) combined with sugar acids is also possible [100]. One of the ligands is exchanged by the analyte during analysis (see Figure 3.3-2). A necessity is the ability to form chelate complexes and a difference in stability of the complexes with the R and the S enantiomer. When this conditions are met, an enantioseparation is possible. One of the issues is the lack of suitable chiral ligands. However, LE has been proven to be very suitable for the enantioseparation of AAs [101]. Novel promising chiral ligands are ionic liquids amino acids [102, 103].

### 3.3.4. Other Chiral Selectors

Several other chiral selectors are available. Surfactants like sodium dodecyl sulfate have been utilized in micellar electrokinetic chromatography (MEKC) to form micelles at a concentration above the critical micelle concentration. These allow the separation of compounds due to their differential partitioning to the hydrophobic inside (pseudostationary

phase) and the hydrophilic exterior. Chiral surfactants like bile salts and saponines enable a chiral separation [104, 105].

a)

$$[Cu(L\text{-}CS)_2] + L\text{-}AA \underset{}{\overset{k_{i,L}}{\rightleftharpoons}} [Cu(L\text{-}CS)(L\text{-}AA)] + L\text{-}CS$$

$$[Cu(L\text{-}CS)_2] + D\text{-}AA \underset{}{\overset{k_{i\text{-}D}}{\rightleftharpoons}} [Cu(L\text{-}CS)(D\text{-}AA)] + L\text{-}CS$$

b)

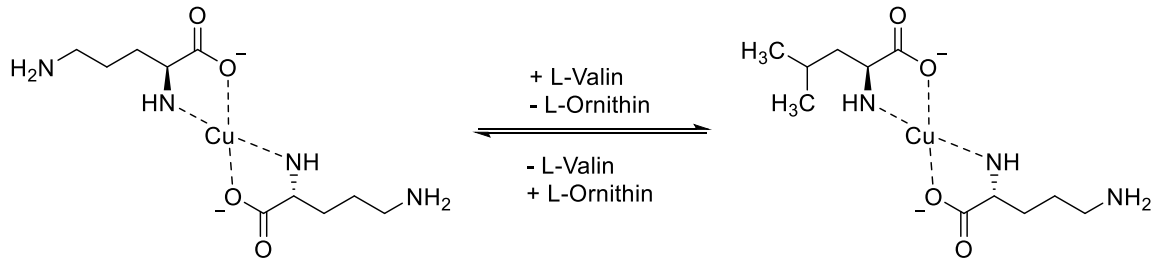



**Figure 3.3-2:** a) Equilibria of the ligand exchange complex with a chiral selector (L-CS) and an L- or D-AA; b) Structure of the ternary metal complex formed with L-ornithine, L-leucine and copper(II).

Macrocyclic antibiotics are another example of chiral selectors [106, 107]. They enable enantioseparation by various recognition mechanism e.g. hydrophobic, ionic, dipole-dipole and π-π interaction [99]. A major drawback is the strong UV absorbance of these compounds under 250 nm. Four classes of these selectors are known: Ansamycins, glycopeptides, aminoglycosides and polypeptides [99]. Vancomycin [108], Teicoplanin [109] (both glycopeptides) and Erythromycin [110] (an ansamycin) are some of the more widely used ones in CE.

# 4. Detection of Amino Acids

When dealing with the analysis of AAs, a suitable method for the detection of these must be selected. If the AAs have been derivatized, UV or fluorescence spectrophotometric detection is usually applied. If that is not the case, the selection becomes more difficult and requires the use of special detectors.

## 4.1. UV/Vis Detector

The UV/Vis detector is the most used detector for HPLC. It offers decent sensitivity for most applications in impurity profiling. The Beer-Lambert law is valid over a wide concentration range, which is important for quantification. The use of gradients is not a problem and the detector is simple and robust with overall small total costs of ownership. With the use of diode array detectors, analytes with different UV/Vis maxima can be detected at the same time. By the recording of spectra, the evaluation of peak purity is possible and spectral libraries can be searched for matches. The system is a concentration-depended detector.

The disadvantages of this detector are the varying response for different analytes, the need for a compatible mobile phase with solvents and additives with low UV cutoffs and the need for a chromophore. Since this is usually not a problem when it comes to impurity profiling of active pharmaceutical ingredients, the detector is by far the most used one in the European Pharmacopoeia. However, only a few AAs do have a strong chromophore and do qualify for a detection by UV/Vis spectrophotometric detection. However, most of them can be detected a low UV wavelength due to their carboxylic function [111], but with a weak sensitivity of the detector for the AAs. If UV/Vis spectrophotometric detection for all AAs with a more adequate sensitivity is desired, derivatization is applied.

## 4.2. Fluorescence Detector

The fluorescence detector is not as popular as the UV/Vis detector, but in comparison to other detectors widely used. It requires the presence of a fluorophore in a molecule or the introduction of it with a suitable derivation reagent. A direct detection of AAs is only feasible with phenylalanine, tyrosine and tryptophan [112]. If possible, a fluorescence detector offers excellent sensitivity and specificity for the analytes. Besides the need for a fluorophore, the downsides are the noticeably higher total cost of ownership, the possibility of quenching effects caused by the mobile phase or the coelution of the sample matrix, and the limited

concentration range where the Beer-Lambert law is valid. Since AAs are often derivatized with OPA or FMOC, the fluorescence detector finds great application in this field.

## 4.3. ELSD and CNLSD

A modern type of detector for liquid chromatography that does not require a chromophore is the evaporative light scattering detector (ELSD). The principle of the ELSD is to nebulize the eluent with an inert carrier gas to form droplets. These are afterwards evaporated in a heated drift tube and particles are left behind. The extend of light scattering of these particles is detected. Hence, this only works for non-volatile compounds. The mobile phase, on the other hand, has to be volatile for a low background noise and to prevent damages of the device. The signal is proportional to the analytes concentration, but linear in a narrow range only. The sensitivity to the temperature and the flow rate is rather low, but the response is dependent on the composition of the mobile phase [113]. However, the use of the ELSD in impurity profiling is limited due to the typical low sensitivity. Higher concentrated samples can be used but spike peaks do appear after the main peak. These spike peaks are not reproducible and prevent the evaluation of impurity profiles [114]. Nevertheless, the ELSD has been successfully applied for the analysis of underivatized amino acids [115-117].

The condensation nucleation light scattering detector (CNLSD) is a further development addressing the sensitivity issue of the ELSD. After evaporation, the size of the particle is increased by the condensation with a saturated stream. The sensitivity of the CNLSD is hereby increased by the factor 10-100 and the linear range is wider [113, 118].

## 4.4. Charged Aerosol Detector

A promising new principle for similar applications like the ones for ELSD and CNLSD is charged aerosol detection (CAD) [119]. It is intended to overcome the shortcomings of the other detectors and qualify as a true universal detector. The technique that is used by the CAD is very similar to that of the ELSD. The mobile phase is first nebulized with a nitrogen flow and

the droplets are then evaporated in a drift tube. But unlike the ELSD, a second stream of nitrogen is positively charged by a high-voltage platinum wire. This charge is transferred to the particles in a collision chamber and finally measured by an electrometer [119, 120].

As with the ELSD, the analytes do have to be considerably less volatile than the mobile phase. This is the virtually the only limitation for the analytes. When choosing the stationary phase, the extent of column bleeding has to be taken into account also to achieve a low background noise [120, 121]. The CAD is a mass-dependent detector and as with all aerosol based detectors, no additional data like spectra is acquired.

For isocratic elution, the response that is given by the CAD is nearly universal for all analytes [122], since it does not rely on spectral of physiochemical properties [119]. Signal intensity increases with the amount of organic modifier present in the mobile phase. This effect can be used to improve the sensitivity of a method by the postcolumn addition of organic solvent to the eluent. For a gradient elution, this is disadvantageous and does limit its use. However, this can be solved with the postcolumn mixture of an inverse gradient, which is the exact opposite (in terms of the aqueous to organic ratio) of the gradient of the analytical system at a given time [123, 124]. Unfortunately, a second gradient pump is required for this which increases the costs. The concentration of analyte and the signal intensity is proportional but not linear. A linear fit can be made in a narrow concentration range, but a linear curve can also be obtained by means of a log-log plot of the peak area against the analyte quantity [125].

The CAD has been successfully applied to the analysis of AAs [126-128] and the evaluation of enantiomer ratios of compounds without a chromophore [129].

## 4.5. Chemiluminescent Nitrogen Detector

Another detector that is very suitable for the analysis of AAs and provides high sensitivity is the chemiluminescent detector (CLND) [130]. The CLND is a detector that is specific for nitrogen containing compounds, which applies naturally to all AAs. The compounds are incinerated at a temperature of 1050 °C in a furnace. This results in the formation of water, nitric oxide, and other dioxides. Excited nitric oxide is then produced by reaction with ozone and photons are emitted when returning back to ground state [131]. The signal intensity is

therefore directly proportional to the amount of nitrogen that is present in the analyte. However, this does not work with molecular nitrogen and molecules with N=N moieties. AA quantification can be done with just on calibration curve [130]. This also applies for other analytes if the molar mass is known and does eliminate the need for reference standards [132]. In conjunction with a UV/Vis detector, the relative response factors can be calculated.

A wide distribution of the CLND is impeded by several severe disadvantages. Besides the limitation to nitrogen containing compounds, the costs of this device are reasonably high and requires more maintenance. As with evaporative detectors, the mobile phase has to be volatile. Acetonitrile which is widely used in HPLC contains nitrogen and is therefore not compatible with CNLD.

## 4.6. Mass Spectrometer

Mass spectrometry (MS) is with no doubt one of the most powerful detection methods for HPLC and CE. It allows the analysis of underivatized AAs with very high sensitivity and selectivity. The devices can be coupled to an HPLC or CE instrument by an interface such as electrospray ionization (ESI) or atmospheric pressure chemical ionization (APCI). In the last decades, various types of mass spectrometers have been developed. Triple quadrupole (QqQ) mass spectrometers are especially suitable for quantification. The special feature to these devices is that a precursor ion with a specific mass is isolated in the first quadrupole and then fragmented in the second quadrupole. One of the fragmented product ions is isolated in the third quadrupole and finally detected. This principle allows the specific detection of analytes in complex matrices with very high sensitivity. Time-of-flight (TOF) mass spectrometers determine the mass of analytes by measuring how long it takes for these to reach the detector after they are accelerated with the same amount of kinetic energy. The time is then converted to a m/z ratio. This allows for the recording of mass spectra with an unlimited mass range. In ion trap MS, an analyte of interest is trapped by an electromagnetic field. The analyte can then be fragmented or ejected and the emerging product ions are available for further steps. On modern ion traps, this can be repeated infinitely often. Ion traps are therefore well suited for the structural elucidation in proteomics and metabolomic analysis.

However, due to the high costs of total ownership and the limited availability in laboratories, MS is only used for purposes where it is really necessary. The use of MS for impurity profiling of AAs is unreasonably, since other cheaper detectors offer sufficient performance to do so. Nevertheless, MS is widely used for the qualification and quantification of AAs in biological fluids like blood [133-135], plasma [136], urine [135, 137] or food [138] and even paintings [139]. Here, the samples are far more complex and the required selectivity and sensitivity is achieved with tandem mass spectrometry. A related research field is proteomics, where MS finds great application [140-142].

## 4.7. Other

Contactless Conductivity Detection (CCD) is a detection method that is mainly used in CE [143] and has been used for the analysis of AAs [144]. Two metal tubings, which act as electrodes, are placed anywhere along the capillary. As ions migrate inside the gap between the two electrodes, a change in electric conductivity is caused [143]. This is measured and further processed to obtain a chromatogram. CCD can also be used in HPLC [145] and has already successfully been applied for the analysis of AAs as well [146, 147].

The refractive index detector (RID), or differential refractometer (DRI) is a universal detector for HPLC which is measuring the changes that is caused in the refractive index (RI) of the mobile phase by the analyte. It does so by comparison of the RI of the eluent with the one of a reference cell. As long as the RI of the analyte differs from the RI of the eluent, all analytes can be detected. Due to the relative nature of this process, positive and negative peaks are possible. The detector is very sensitive to changes in temperature, flow rate and pressure. A gradient elution is not possible at all. It has been used for the analysis of AAs [148], but the aforementioned problems and the poor sensitivity is the reason for the virtually nonexistent usage of this device in impurity profiling.

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# II Aim of the Work

The purity of substances for pharmaceutical use is controlled by the European Pharmacopoeia by means of various instrumental methods like liquid chromatography, capillary electrophoresis, thin-layer chromatography, and optical rotation. Most of the typical drugs that are in use nowadays have already been monographed. However, some of these methods used in the monographs are outdated and do not make use of the technical progress in instrumental analytics that has been taken place. To satisfy the increasing requirements in purity control of drugs, older monographs have to be revised. The new methods have to provide a significant improvement over the old one. However, since methods that are published in the European Pharmacopoeia have to be applicable in most laboratories, the use of modern instrumentation is limited. Therefore, only common equipment is used to ensure its broad applicability. Enantioseparation, a field with growing importance is also part of this work.

Methionine is a substance with lipophilic and hydrophilic impurities, so that a satisfying separation with reversed phase liquid chromatography only is not possible. A mixed mode column with reversed phase and cationic exchange is utilized. A method for the separation of the putative impurities of methionine has to be developed and validated.

The enantiomeric purity of amino acids is controlled by means of optical rotation in the European Pharmacopoeia, which provides only low sensitively and specificity. Due to the lack of a chromophore and the sensitivity needed, a suitable derivatization technique has to be utilized and indirect enantioseparation for liquid chromatography and capillary electrophoresis are subject for development and validation. A similar method that can be used as an alternative for the impurity profiling with the amino acid analyzer that is employed by the European Pharmacopoeia has to be investigated.

An impurity profiling method for glyceryl trinitrate solution shall be developed. The substance and its impurities can be detected with common UV spectrophotometric detection at a low wavelength. Due to the differences in lipophilicity, a gradient with a common revered phase column is applied. The method should also enable an assay for glyceryl trinitrate.

# III Results

# 1. Impurity Profiling of L-Methionine by HPLC on a Mixed Mode Column

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**ABSTRACT**

Methionine is mostly produced synthetically. Thus, impurities are synthesis by-products in addition to oxidation and dimerization products. Here, a sensitive HPLC method for the determination of impurities in L-methionine was developed and validated using a SIELC® Primesep 100 column. Impurities were separated on the mixed mode column by reversed phase and cationic exchange mechanism. The limit of detection was in the range of 0.06 – 0.30 µg/mL (0.0004 – 0.002 %), limit of quantification in the range of 0.30 – 0.75 µg/mL (0.002 - 0.005 %) and linearity was shown in the range of 0.3 – 30.0 µg/mL (0.002 – 0.200 %). The method was found to be precise (intermediate precision RSD < 5 %; n=2) and accurate (recovery 96.0 - 121.4 %, n=3). The method is also suitable for the purity assessment of DL-methionine and D-methionine. The amount of impurities found in batches was very low. Only L-methionine-sulfoxide and *N*-acetyl-DL-methionine could be detected in levels less than 0.05 %.

*Abbreviations*: MetOx: L-methionine-sulfoxide; AcMet: *N*-acetyl-DL-methionine; AcMetMet 1: *N*-acetyl-L-methionyl-L-methionine and its enantiomer; AcMetMet 2: *N*-acetyl-L-methionyl-D-methionine and its enantiomer; N.D.: not detectable, N.Q.: not quantifiable

# 1. Introduction

The essential amino acid L-methionine plays an important role in the biosynthesis of proteins and as a methyl donor in the form of S-adenosyl methionine. Additionally, it is also used as a drug for acidifying urine in order to optimize the effect of antibiotics (e.g. ampicilline), it prevents the formation of phosphate stones, and inhibits the growth of bacteria in the urinary system. Furthermore, it is used in solutions for parenteral nutrition [1].

The purity assessment of amino acids has always been challenging due to the lack of a chromophore. In the European Pharmacopoeia (Ph.Eur.), the test for related substances has been done by thin-layer chromatography and treatment with ninhydrin for many years. However, sensitivity and performance of this method is poor. In the recent years, many monographs have been revised and an amino acid analysis with ion exchange utilizing a post-column ninhydrin derivatization for detection is now performed in most amino acids monographs of the Ph.Eur. [2]. This method can only detect and quantify other amino acids in an amino acid which is produced by fermentation. Methionine is produced synthetically and has therefore synthesis by-products instead of amino acids as impurities. Thus, this procedure of amino acid analysis is not suitable and a new method had to be developed.

Methionine is prepared synthetically from simple raw materials. First, methylmercaptan (**1**) is formed by hydrogen and sulfur and acrolein (**2**) by propylene and oxygen [3]. Afterwards, methylmercaptan (**1**) and acrolein (**2**) react in a Michael-Addition to 3-(methylthio)propanal (**3**). 2-Hydroxy-4-(methylthio)butanenitrile (**4**) is formed by conversion (**3**) with hydrocyanic acid and the hydantoin (**5**) is obtained by reacting (**4**) with ammonium carbonate. The product is then hydrolyzed with a basic potassium substance to give the racemic methionine (**6**) (see Fig. 1a)[3-8]. For resolution of the racemate, DL-methionine (**6**) is acetylated with acetic anhydride to achieve *N*-acetyl- DL-methionine (**7**). This is selectively hydrolyzed using the enzyme L-acylase to give L-methionine (**8**) (see Fig. 1b) [6], which can be separated from *N*-acetyl-D-methionine by ion exchange [6] or precipitation and filtration [9]. Afterwards, *N*-acetyl-D-methionine is transformed to *N*-acetyl-DL-methionine by racemization with high temperatures [9] and made available for further hydrolysis with L-acylase [9].

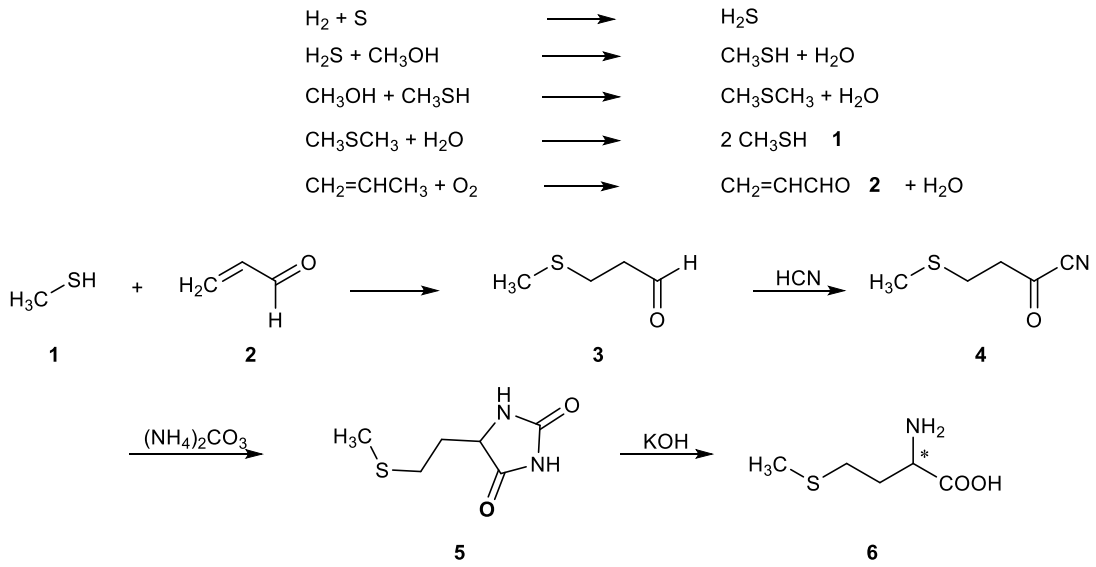


**Fig. 1a:** Synthesis of DL-methionine according to [3-8]

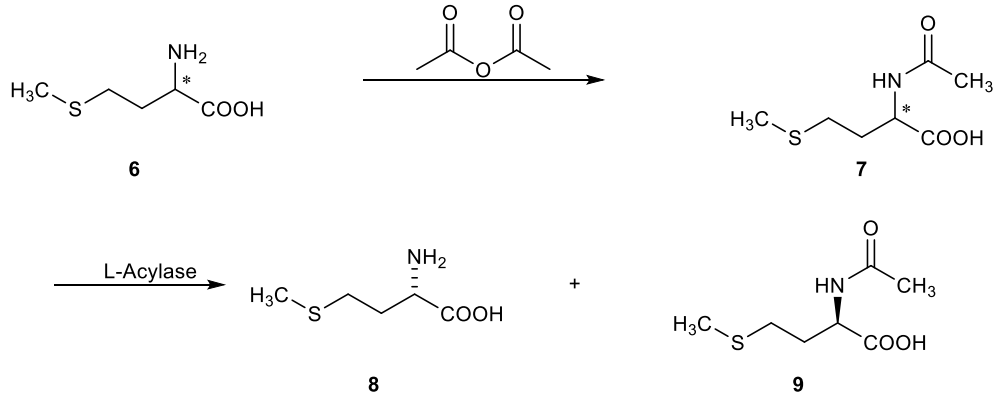


**Fig. 1b:** Chiral resolution of L-methionine with the impurity *N*-acetyl-DL-methionine (7) as intermediate according to [3, 6]

Fig. 2 displays the putative impurities: *N*-acetyl-DL-methionine is an intermediate of the synthesis and the diastereomers *N*-acetyl-L-methionyl-L-methionine, *N*-acetyl-L-methionyl-D-methionine and its enantiomers respectively are by-products of the chiral resolution, which arise from the racemization process. L-methionine-sulfoxide is formed through oxidation of the sulfur in L-methionine in the raw product.

Most analytical methods based on HPLC in the literature that deal with methionine, focus on its determination besides other compounds (e.g. amino acids) in samples like blood, human serum, food and its diverse role in physiological pathways of living organism [10-24].

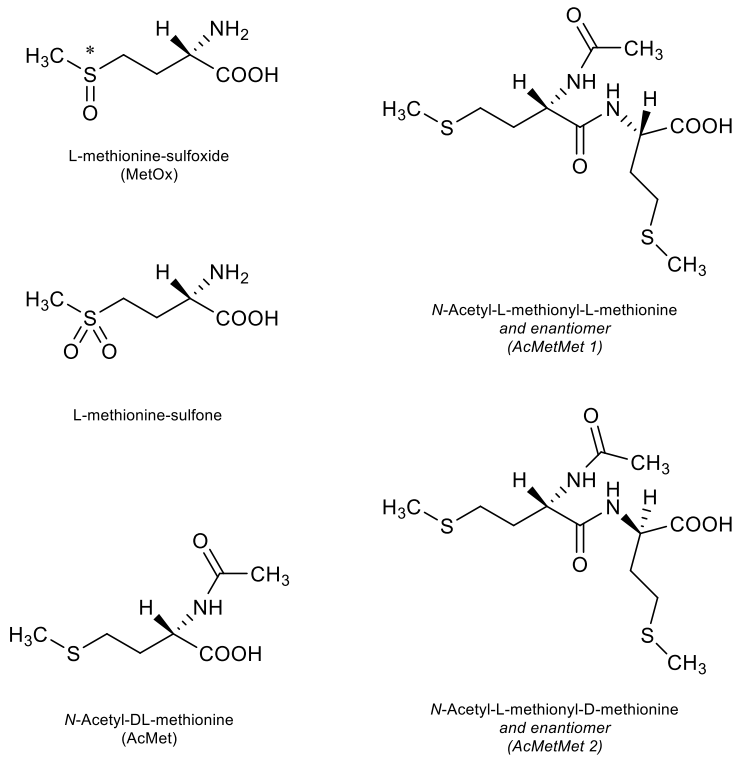


**Fig. 2:** Putative impurities of L-methionine [25]

To the best of our knowledge, there is no study focusing on impurity profiling of methionine in literature. However, the 8th edition 2016 (8.7) of the Ph.Eur. describes a monograph of L-methionine, that uses reversed phase liquid chromatography for the purity assessment [25]. This method uses a water/acetonitrile gradient and has a run time of 60 minutes. Impurities with a free amine moiety show poor retention. Therefore, the aim of the new method was to improve retention for these impurities by a simultaneous reduced overall run time.

## 2. Experimental

### *2.1. Chemicals and Reagents*

L-methionine was obtained from the EDQM (Straßbourg, France) and Sigma-Aldrich/Fluka (Schnelldorf, Germany). DL-methionine, D-methionine, L-methionine-sulfoxide, *N*-acetyl-DL-methionine, trifluoracetic acid and phosphoric acid 85% solution were purchased from Sigma-Aldrich Chemie GmbH (Schnelldorf, Germany), HPLC grade acetonitrile from VWR

International GmbH (Darmstadt, Germany). Water for HPLC was purified using the Milli-Q purification system by Merck Millipore (Schwalbach, Germany). The impurities AcMetMet 1 and AcMetMet 2 were synthesized in our lab (see section 2.5).

### *2.2. Apparatus*

The development and validation of the method was performed on a 1100 series from Agilent Technologies (Waldbronn, Germany) consisting of a vacuum degasser (G1379A), binary pump (G1312A), autosampler (G1313A), thermostated column department (G1316A) and a diode array detector (G1315B). Chromatographic data was acquired and evaluated with the Agilent ChemStation® Rev B.03.02 software. The separation of the diastereomers was performed on an 1100 series from Agilent consisting of two preparative pumps (G1361A), a preparative autosampler (G2260A), a multiple wavelength detector (G1365B) and a preparative fraction collector (G1364B). LC-MS data were acquired with LC/MSD Trap G2445D ion trap from Agilent with electro spray ionization (ESI) attached to a HPLC equipped with a vacuum degasser (G1379B), a binary pump (G1312A), an autosampler (G1329A) with a thermostat (G1330B), a thermostated column compartment (G1316A) and a diode array detector (G1315B). A Bruker AV 400 instrument by Bruker Biospin (Ettlingen, Germany) was used to record the $^{1}H$ (400.132 MHz) and $^{13}C$ (100.613 MHz) NMR spectra. As internal standard, the signals of the deuterated solvents were used (DMSO-$d_6$: $^{1}H$ 2.50 ppm, $^{13}C$ 39.51 ppm; MeOH-$d_4$: $^{1}H$ 3.31 ppm, $^{13}C$ 49.15 ppm). NMR data were evaluated with the Bruker TopSpin v3.0 software. IR spectra were measured with a Jasco FT/IR-6100 FT-IR spectrometer from Jasco Germany GmbH (Gross-Umstadt, Germany) equipped with the PIKE MIRacle single reflection ATR sampling accessory by PIKE Technologies (Madison WI, USA). For pH-measurements a Metrohm 744 pH-Meter from Deutsche METROHM GmbH & Co. KG (Filderstadt, Germany) was used.

### *2.3. Chromatographic Procedure*

For the analytical separation of L-methionine and its impurities, a mixed mode column SIELC Primesep® 100 (250x 4.6 mm; 5 µm practice size; 100 Å pore size) was used. Isocratic elution mode was chosen using a mobile phase consisting of a mixture of 80 % [V/V] 12.5 mM aqueous phosphoric acid and 20 % [V/V] acetonitrile. The flow rate was set to 1 mL/min and

the temperature to 30 °C. The detector was set to a detection wavelength of 210 nm with 8 nm bandwith. The injection volume was 50 µl. The preparative separation of the diastereomers was carried out using a Nucleodur Sphinx RP (125 x 10 mm; 5 µm particle size; 110 Å pore size) by Machery-Nagel (Düren, Germany). Isocratic elution using a mixture of 0.1 % formic acid in water and acetonitrile in a ratio of 85 : 15 [V%/V%] was applied. The flow was set to 2 mL/min, and the detector to 210 nm with 8 nm bandwith. The injection volume was 250 µl and the total runtime of the method was 25 minutes.

For the LC/MS measurements, an Agilent Zorbax SB-CN column (4.6 x 50 mm; 3.5 µm particle size) was used. The flow was set to 0.4 mL/min and the mobile phase was 0.1 % aqueous formic acid and 0.1 % formic acid in acetonitrile with a ratio of 50 : 50 [V%/V%]. The injection volume was 1 µl, the dry temperature was set to 350 °C, the nebulizer to 50 psi, the dry gas to 10 L/min, the ion polarity to positive and the capillary voltage to 3500 V. The total runtime of the method was set to 10 minutes.

### *2.4. Preparation of Solutions*

For the test solutions 150 mg of methionine were dissolved in water and diluted to 10.0 ml with the same solvent. For each impurity, a stock solution was prepared by dissolving 30 mg in water and diluting to 100.0 ml with the same solvent. For methionine solutions, which were spiked with impurities, a stock solution was made by dissolving 300 mg of methionine in water and diluting to 10.0 ml with the same solvent. The solutions for validation with impurities in the range from 0.01 – 0.05 % were prepared by adding 50 µl (0.01 %) or 250 µl (0.05 %) of each impurity to 5.0 ml of methionine stock solution and diluting to 10.0 ml with water, whereas in the range from 0.001 – 0.005 %, the impurity stock solutions were diluted tenfold and then 50 µl (0.001 %), 100 µl (0.002 %) and 250 µl (0.005 %) of this solutions were added to 5.0 ml of methionine stock solution and diluted to 10.0 ml with water.

All solutions were sonicated for 15 minutes and filtrated with 0.45 µm cellulose acetate syringe filters directly into the vial prior use.

*2.5. Synthesis of AcMetMet 1 und AcMetMet 2*

For the preparation of the diastereomers AcMetMet 1 and AcMetMet 2 (see Fig. 3), 477 mg (2.5 mmol) *N*-acetyl-DL-methionine, 623 mg (3.25 mmol) 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) and 10 mg (0.08 mmol) hydroxybenzotriazole (HOBT) were dissolved in 20 ml of dry dimethylformamide and stirred at 0 °C for 15 minutes. Afterwards, 746 mg (5.0 mmol) of DL-methionine were added and the solution was stirred for 18 hours at room temperature. The solvent was evaporated and the oily product purified by means of column chromatography using silica gel and a mixture of ethyl acetate and methanol in a ratio of 9:1 [V%/V%] as eluent. Only 100 ml of the eluent was applied in order to only elute the diastereomers, which elute first. Other compounds like HOBT, methionine and anorganic ions remain on the silica gel and are discarded. The solvent of the eluate was evaporated and an oily product was obtained. To separate the diastereomers, 2 mL of the oily product were diluted with 2 mL of Milli-Q purified water, filtrated with PVDF syringe filters and purified by means of preparative HPLC (chromatographic conditions see 2.3). The peaks eluting at 17.6 and 26.1 minutes were collected. The solvent of the two fractions was evaporated and a white powder was obtained for each diastereomer.

AcMetMet 1: Peak Purity by HPLC 98.6 % [25]; ESI/MS (m/z): 323.3 ($[M+H]^+$, found), 323.1 ($[M+H]^+$, calculated); $^1$H-NMR (DMSO, $\delta_H$ [ppm], J [Hz]): 1.84 (s, 3H, -NH-C(=O)-C**H$_3$**), 1.85 (br, 4H, -S-CH$_2$-C**H$_2$**-CH-NHC(=O)-, -S-CH$_2$-C**H$_2$**-CH-NHC(=O)-), 2.03 (s, 6H, -S-C**H$_3$**, -S-C**H$_3$**), 2.45 (m, 4H, -S-C**H$_2$**-CH$_2$-CH-NHC(=O)-, -S-C**H$_2$**-CH$_2$-CH-NHC(=O)-), 4.23 (td, $^3J_{HH}$ = 4.62, 8.53, 8.40, 1H, -S-CH$_2$-CH$_2$-C**H**-NHC(=O)-), 4.33 (td, $^3J_{HH}$ = 5.55, 8.09, 8.07, 1H, -S-CH$_2$-CH$_2$-C**H**-NHC(=O)-), 8.04 (d, $^3J_{HH}$ = 8.05, -N**H**-C(=O)-CH$_3$), 8.12 (d, $^3J_{HH}$ = 7.65, -N**H**-C(=O)-CH$_3$); $^{13}$C-NMR (MeOH-d$_4$, $\delta_C$ [ppm]): 15.36 (2C, -CH$_2$-CH$_2$-S-**C**H$_3$, -CH$_2$-CH$_2$-S-**C**H$_3$), 22.53 (1C, -NH-C(=O)-**C**H$_3$), 31.11 (1C, -CH$_2$-**C**H$_2$-S-CH$_3$), 31.33 (1C, -CH$_2$-**C**H$_2$-S-CH$_3$), 32.45 (1C, -**C**H$_2$-CH$_2$-S-CH$_3$), 32.91 (1C, -**C**H$_2$-CH$_2$-S-CH$_3$), 53.02 (1C, -**C**(NH-)-C(=O)-NH-), 54.17 (1C, -**C**(NH-)-C(=O)-NH-), 173.49 (1C, **C**OOH), 174.12 (2C, (NH-**C**(=O)-CH$_3$, -C(NH-)-**C**(=O)-NH-)); IR (FT): ν (cm$^{-1}$) = 3308 (w), 3240 (w), 3085 (w), 2908 (w), 1725 (m), 1666 (m), 1600 (m), 1541 (s), 1224 (s), 939 (w), 792 (w), 670 (w).

AcMetMet 2: Peak Purity by HPLC 96.7 % [25]; ESI/MS (m/z): 323.3 ($[M+H]^+$, found), 323.1 ($[M+H]^+$, calculated); $^1$H-NMR (DMSO, $\delta_H$ [ppm], J [Hz]): 1.85 (s, 3H, -NH-C(=O)-C**H$_3$**), 1.86

(br, 4H, -S-$CH_2$-C**$H_2$**-CH-NHC(=O)-, -S-$CH_2$-C**$H_2$**-CH-NHC(=O)-), 2.02 (s, 6H, -S-C**$H_3$**, -S-C**$H_3$**), 2.42 (m, 4H, -S-C**$H_2$**-$CH_2$-CH-NHC(=O)-, -S-C**$H_2$**-$CH_2$-CH-NHC(=O)-), 4.30 (m, 1H, -S-$CH_2$-$CH_2$-C**H**-NHC(=O)-), 4.37 (td, $^3J_{HH}$ = 5.39, 8.26, 8.32, 1H, -S-$CH_2$-$CH_2$-C**H**-NHC(=O)-), 8.00 (d, $^3J_{HH}$ = 8.16, -N**H**-C(=O)-$CH_3$), 8.23 (d, $^3J_{HH}$ = 8.00, -N**H**-C(=O)-$CH_3$); $^{13}$C-NMR (MeOH-$d_4$, $\delta_C$ [ppm]): 15.36 (2C, -$CH_2$-$CH_2$-S-**C**$H_3$, -$CH_2$-$CH_2$-S-**C**$H_3$), 22.64 (1C, -NH-C(=O)-**C**$H_3$), 31.17 (1C, -$CH_2$-**C**$H_2$-S-$CH_3$), 31.37 (1C, -$CH_2$-**C**$H_2$-S-$CH_3$), 31.94 (1C, -**C**$H_2$-$CH_2$-S-$CH_3$), 32.95 (1C, -**C**$H_2$-$CH_2$-S-$CH_3$), 52.79 (1C, -**C**(NH-)-C(=O)-NH-), 54.11 (1C, -**C**(NH-)-C(=O)-NH-), 173.55 (1C, **C**OOH), 174.02 (1C, (NH-**C**(=O)-$CH_3$), 174.87 (1C, -C(NH-)-**C**(=O)-NH-); IR (FT): ν ($cm^{-1}$) = 3284 (w), 2919 (w), 1704 (m), 1667 (m), 1630 (s), 1558 (s), 1301 (s), 914 (m), 811 (m), 744 (m), 681 (m).

## 3. Results and Discussion

Whereas the impurities *N*-acetyl-DL-methionine and DL-methionine-sulfoxide are commercially available, the diastereomers AcMetmet 1 and AcMetMet 2 were synthesized according to Fig. 3.

### *3.1. Method Development*

For the separation of L-methionine from all putative impurities a mixed SIELC Primesep® 100 column was used. This column has a reversed phase stationary phase with embedded anionic ion-paring reagent and is therefore capable of retaining lipophilic and cationic substances. In contrast to normal reversed phase columns where the retention behavior is more or less predictable, the method development on such mixed mode columns is mostly empirical, especially when it comes to changes in chromatographic parameters, the retention times of substances might change unexpectedly.

The selection of additives and solvents is limited to the operating range of the mixed-mode column. The pH of the mobile phase has to be within 1.5 – 6.5 and methanol is not suitable for this column. Furthermore, the zwitterionic nature of amino acids around neutral pH-values is not likely to produce optimal results with a stationary phase that is based on cationic and lipophilic interactions due to the negative charge of the deprotonated carboxylic acid. Thus, an acidic additive that give a pH that is near the lower end of the columns

specification is required. Suitable substances are trifluoric acetic acid, phosphoric acid and formic acid.

In Fig. 4, a chromatogram of a sample spiked with impurities under optimal chromatographic conditions, being 12.5 mM phosphoric acid and acetonitrile (80 : 20), is shown. AcMetMet 1, AcMetMet 2 and AcMet are lipophilic substances which are retained on the mixed mode column primarily by the reversed phase mechanism. With regard to the acetylated amines, cationic interactions are not possible. These impurities are affected mainly by changes in the eluting power of the mobile phase. In Fig. 5, the effect of acetonitrile is shown. In comparison to 20 % acetonitrile (Fig. 4), an increase to 25 % leads to shorter retention times for all substances resulting in a poorer separation of the diastereomers and a bad peak shape of *N*-acetyl-DL-methionine. A reduction of the amount of acetonitrile to 15 % leads to the opposite. In addition, under these conditions, L-methionine-sulfoxide is not separated from L-methionine. The best resolution and peak shape was obtained with 20 % acetonitrile.

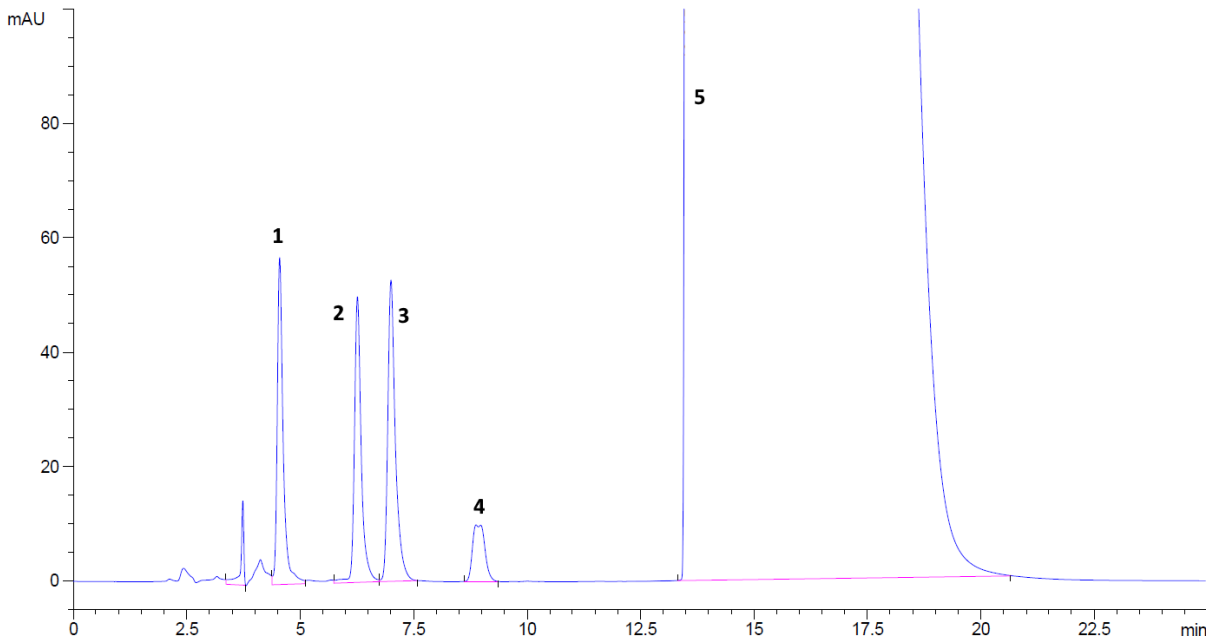


**Fig. 4:** A chromatogram of a sample (15 mg/mL) spiked with all known impurities (0.01 %); order of elution: *AcMet* (1), AcMetMet 1 (2), AcMetMet 2 (3), MetOx (4), L-methionine (5); chromatographic conditions: Flow: 1 mL/min; Temp.: 30 °C; Mobile Phase: 12.5 mM phosphoric acid and acetonitrile in ratio of 80 : 20 [V%/V%]; 210 nm; injection volume: 50 µL;

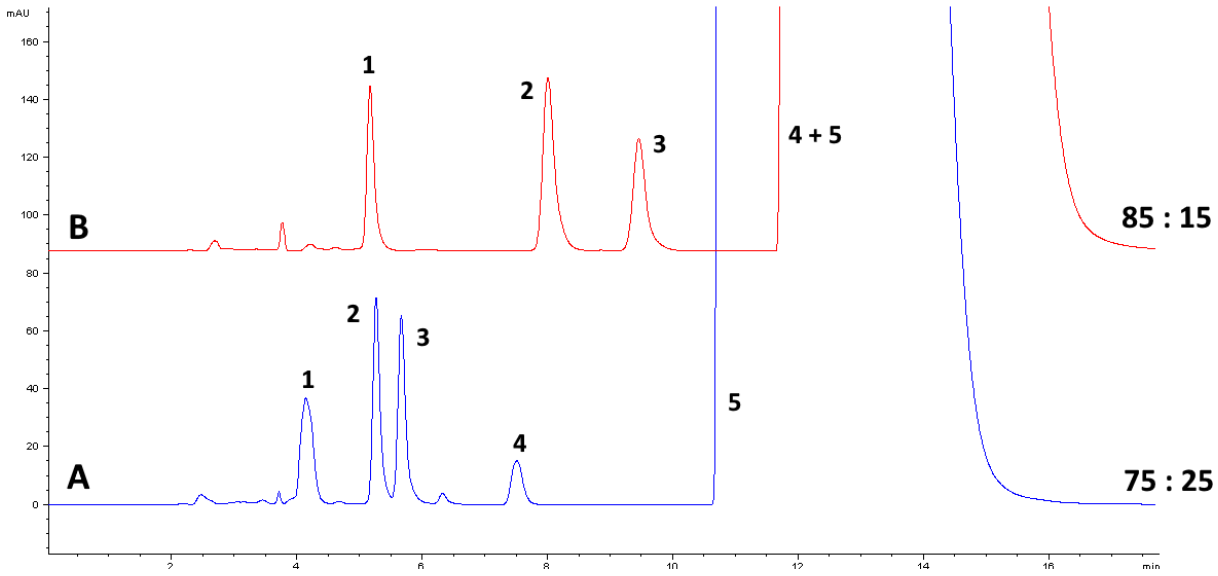


**Fig. 5:** Effect of acetonitrile proportion; mobile phase A: 25 mM phosphoric acid, mobile phase B: acetonitrile; A: (75 : 25); B: (85 : 15) [V%/V%]; chromatographic conditions see Fig. 4

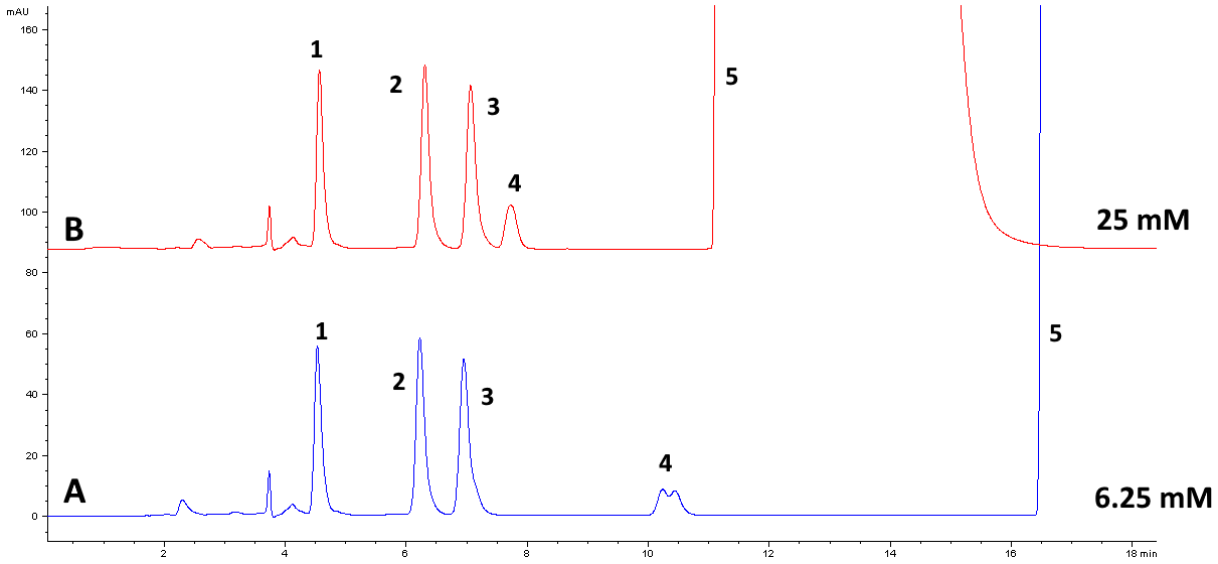


**Fig. 6:** Effect of molarity of the phosphoric acid. A: 6.25 mM; B: 25 mM; chromatographic conditions see Fig. 4

L-methionine-sulfoxide and L-methionine consisting of a free amine moiety are retained primarily by the cationic ion exchange and are sensitive to changes in pH and molarity of the additive, respectively. For the mixed mode column an acidic pH is required, which can be achieved by a buffer or a pure acid. With trifluoric and formic acid an adequate separation of methionine and its impurities was not found possible. The best results were achieved with 12.5 mM phosphoric acid (pH = 2.1). In Fig. 6 the effect of the molarity of the acid is shown. At a concentration of 6.25 mM phosphoric acid (pH = 2.4), the retention times for

L-methionine-sulfoxide and L-methionine are clearly increased and the peak for L-methionine gets very broad. Increasing the concentration of the acid to 25 mM (pH = 1.9) leads to a poor separation of L-methionine-sulfoxide and the diastereomers. Acetylated impurities are not affected by these changes. The effect on the phosphoric acid concentration can be explained by the ionic strength associated with the degree of protonation, which affects the retention of substances with a free amine moiety who are competing with hydronium ions for places on the cationic exchanger of the stationary phase. A concentration of 12.5 mM phosphoric acid was considered best for the separation.

In the monographs of the Ph.Eur. [2], the reporting threshold defines the limit above a impurity has to be reported [26]. For APIs having a maximum daily dose higher than 2 g, the reporting threshold is defined as 0.03 % [27, 28]. This threshold applies to methionine, since it is used in parenteral nutrition with doses higher than 2g/day. Thus, the method must be able to quantify all impurities down to at least 0.03 %.

### *3.2. Validation of the Method*

The method was validated following the guidelines of the International Conference on Harmonization (ICH) [29]. Accuracy, precision, specificity, detection/quantification limit, linearity and range were evaluated.

#### *3.2.1. Specificity*

A sample of L-methionine spiked with all known impurities with a concentration of 15 µg/mL (0.01 %) yielded a resolution of at least 3.0 for all peaks (see Fig. 4).

#### *3.2.2. Limit of Detection/Quantification:*

Limit of detection and quantification were first estimated by the signal-to-noise ratio obtained from a 0.01 % solution of all impurities and then shown by an artificial mixture of these solutions at the estimated concentration. The signal-to-noise ratios at LOD were higher than 3 and the relative standard deviation of the area about 20 %. The signal-to-noise ratios for the peaks at the LOQ were roughly 20 and the relative standard deviation for the area not more than 3 %. L-methionine-sulfoxide showed a higher LOD and LOQ due to the weaker

absorption of the substance at 210 nm and the broad peak caused by a little resolution of the two diastereomers of L-methionine-sulfoxide (see Table 1).

**Table 1:** Limit of detection and limit of quantification of the putative impurities

| Impurity | LOD | S/N | RSD of Area | LOQ | S/N | RSD of Area |
|---|---|---|---|---|---|---|
| **AcMet** | 0.06 µg/mL (0.0004 %) | 4 | 17.1 % | 0.30 µg/mL (0.002 %) | 20 | 1.0 % |
| **AcMetMet 1** | 0.06 µg/mL (0.0004 %) | 5 | 19.7 % | 0.30 µg/mL (0.002 %) | 26 | 1.4 % |
| **AcMetMet 2** | 0.06 µg/mL (0.0004 %) | 5 | 19.4 % | 0.30 µg/mL (0.002 %) | 22 | 2.9 % |
| **MetOx** | 0.30 µg/mL (0.002 %) | 4 | 15.5 % | 0.75 µg/mL (0.005 %) | 17 | 1.1 % |

### *3.2.3. Linearity*

Linearity was determined in the range from 0.3 to 30 µg/mL (0.002 - 0.200 %) for L-methionine and the impurities *N*-acetyl-DL-methionine, AcMetMet 1 and AcMetMet 2 in the range from 0.75 to 30 µg/mL (0.005 - 0.200 %) for L-methionine-sulfoxide. Each calibration curve was constructed of six levels. The coefficient of determination was higher than 0.999 for all impurities (see Table 2). The correction factors were calculated by dividing the slope of the methionine calibration curve by the slope of the impurity.

### *3.2.4. Accuracy*

For accuracy, a sample of L-methionine was spiked with impurities at four levels (0.001, 0.005, 0.01 and 0.05 %) and a three-fold determination was made. Accuracy was calculated in two different ways:

1) with the equations obtained from linear regression

2) with a 10.000-fold dilution of L-methionine as external standard and the correction factors. This conforms to the style of the Ph.Eur.

**Table 2:** Calibration curves, correction factors and coefficient of determination

| Impurity | Calibration Curve | Correction Factor | $R^2$ |
|---|---|---|---|
| **L-Methionine** | y = 29.407x – 7.6082 | 1.00 | 0.9998 |
| **AcMet** | y = 41.888x + 0.8089 | 0.70 | 0.9999 |
| **AcMetMet 1** | y = 52.780x + 4.8535 | 0.56 | 0.9999 |
| **AcMetMet 2** | y = 53.898x + 1.2999 | 0.55 | 0.9999 |
| **MetOx** | y = 17.533x – 1.651 | 1.68 | 0.9998 |

Since pure L-methionine already contains impurities, which would falsify the results, the peak areas of the impurities in the spiked solutions had to be reduced by the peaks areas of the corresponding impurities already existing in the non-spiked L-methionine. Quantification of the sulfoxide is not possible at concentration below 0.005 %. Recovery with linear regression is more accurate in most cases, except for the diastereomers at 0.001 %, where external standard provides better results (see Table 3). Therefore, the limit of quantification is increased to 0.002 % and external standard is used for quantifying the content of impurities.

### *3.3. Precision*

Precision was evaluated with a sample of L-methionine spiked with impurities at 0.01 % each. Repeatability was verified by analyzing the spiked solution six times in series. The solution was then kept at 4 °C for three days and analyzed again six times in series to evaluate the intermediate precision. The relative standard deviation of the repeatability was in the range from 1.28 to 3.40 % (n=6) and of the intermediate precision in the range from 1.96 to 3.72 % (n=2). The results prove that the method is precise.

**Table 3:** Overview of recovery determined by external standard and the equation from linear regression. Each sample was measured three times. The average and the relative standard deviation is given.

| Impurity | Concentration [µg/mL] | Linear Regression | | Ext. Std. | RSD |
|---|---|---|---|---|---|
| | | Measured [µg/mL] | Recovery [%] | Recovery [%] | [%] |
| AcMet | 0.15 (0.001 %) | 0.159 | 106.0 | 115.9 | 10.8 |
| | 0.75 (0.005 %) | 0.747 | 99.6 | 99.7 | 4.9 |
| | 1.50 (0.01 %) | 1.497 | 99.8 | 98.7 | 1.2 |
| | 7.50 (0.05 %) | 7.357 | 98.1 | 96.0 | 0.1 |
| AcMetMet 1 | 0.15 (0.001 %) | 0.062 | 41.0 | 98.9 | 4.0 |
| | 0.75 (0.005 %) | 0.755 | 100.7 | 109.2 | 2.3 |
| | 1.50 (0.01 %) | 1.472 | 98.1 | 100.8 | 3.9 |
| | 7.50 (0.05 %) | 7.636 | 101.8 | 99.6 | 2.5 |
| AcMetMet 2 | 0.15 (0.001 %) | 0.130 | 86.7 | 106.2 | 4.0 |
| | 0.75 (0.005 %) | 0.774 | 103.3 | 110.0 | 2.4 |
| | 1.50 (0.01 %) | 1.527 | 98.5 | 107.9 | 1.2 |
| | 7.50 (0.05 %) | 7.195 | 95.9 | 100.5 | 0.5 |
| MetOx | 0.15 (0.001 %) | N.D. | N.D. | N.D. | N.D. |
| | 0.75 (0.005 %) | 0.805 | 107.3 | 121.4 | 3.8 |
| | 1.50 (0.01 %) | 1.604 | 107.0 | 115.0 | 3.1 |
| | 7.50 (0.05 %) | 7.455 | 99.4 | 102.5 | 2.6 |

### *3.4. Robustness*

To assure the robustness of the method, following method parameters have been varied: temperature ±10 °C; molarity of the phosphoric acid ±2.5 %; flow rate ±0.1 mL/min; injection volume ±5 µl; detection wavelength ±2 nm; acetonitrile proportion ±2 %. A sample of L-methionine spiked with impurities at 0.1 % was injected three times for each change of conditions. Changes in resolution, retention time and signal-to-noise ratio were studied. None of the changes did interfere with the separation of the impurities. Signal-to-noise ratio was reduced not more than 20 % and L-methionine was eluted within 20 minutes. Therefore, the method can be regarded to be robust.

### *3.5. Batch Samples*

Different batches of L-methionine and also DL- and D-methionine were analyzed (see Table 4). The most often impurities found were L-methionine-sulfoxide, detected in all samples tested, and *N*-acetyl-DL-methionine, observed in most of the samples. The two diastereomers were present only in a few batches and the content was low. Besides the known impurities, depending on the manufacture, unknown impurities were also present. However, the amount of these impurities was lower than the reporting threshold and none of these observed impurities did interfere with the separation of the other impurities. None of the samples contained the impurity L-methionine-sulfone.

In general, the amount of impurities in methionine is low. Since the reporting threshold is set to 0.03 %, only L-methionine-sulfoxide would have to be listed as an impurity in certificates of analysis in some batches.

**Table 4:** Overview of Batch Data results. The content of impurities is expressed in [%]. For unknown impurities, a correction factor of 1.0 was used.

| | L-Met | | | | D-Met | DL-Met |
|---|---|---|---|---|---|---|
| **Supplier** | **EDQM** | **EDQM** | **Sigma** | **Fluka** | **Sigma** | **Sigma** |
| **MetOx** | 0.030 | 0.017 | 0.017 | 0.021 | 0.019 | 0.040 |
| **AcMet** | 0.026 | 0.014 | 0.009 | 0.011 | N.Q. | N.D. |
| **AcMetMet 1** | N.Q. | N.Q. | N.D. | N.D. | N.D. | N.D. |
| **AcMetMet 2** | N.Q. | N.Q. | N.D. | N.D. | N.D. | N.D. |
| **Unknown Imp @ 8.4 min** | 0.017 | 0.016 | N.D. | 0.019 | N.D. | 0.028 |
| **Unknown Imp @ 10.8 min** | 0.009 | 0.007 | N.D. | 0.005 | N.D. | 0.009 |

## 4. Conclusion

A sensitive, accurate and precise method for the determination of impurities in L-methionine has been developed and validated. The method is also suitable for impurity profiling of DL- and D-methionine. The amount of impurities in methionine batches were found to be very small.

**Conflict of Interest**

The authors declare, that they have no conflict of interest.

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# 2. Indirect Enantioseparation of Amino Acids by CE Using Automated In-Capillary Derivatization with *Ortho*-Phthalaldehyde and *N*-Acetyl-L-Cysteine

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**ABSTRACT**

A simple CE method for the assessment of enantiomeric purity of individual amino acids (AAs) is presented. The AAs were derivatized with *ortho*-phthalaldehyde and a thiol in the capillary by means of successive hydrodynamic injections (5 sec, 0.5 psi) of derivatization solution, sample and derivatization solution. A voltage of 1 kV was applied to blend the solutions and to start the reaction. Subsequently, the derivatives were separated using a background electrolyte (BGE) composed of 50 mM borate buffer (pH = 9.25) and 7.5 mM of a cyclodextrin (CD). In order to find the optimal derivatization reagent and CD, the impact of the thiols 3-mercaptopropionic acid (MPA), *N*-acetyl-L-cysteine (NAC) and *N*-isobutyryl-L-cysteine (NIBLC), and various neutral and negative charged CDs on the resolution were assessed. The use of NAC and γ-CD enabled the best indirect enantioseparation of 16 AAs. Serine could be separated with β-CD, and proline and cysteine are not suitable for automated OPA-derivatization. The resolutions were ranging from 2.3 to 16.6. A method was validated representative for the AAs methionine, aspartic acid, tryptophan and phenylalanine. The recovery (n=3) of the minor enantiomers was found to be within 95.1 and 107.5 %. The relative standard deviation (RSD) was ranging from 1.2 to 6.4 %. The method is accurate, precise, linear, and robust. For methionine it could be demonstrated, that the ratio of L- and D-AA ranging from 1 – 99 % can be evaluated by direct comparison of the corrected peak areas with satisfying accuracy ranging between 92.1 and 115.9 %.

*Abbreviations*: AA, amino acid; OPA, *ortho*-phthalaldehyde; MPA, 3-mercaptopropionic acid; NAC, *N*-acetyl-L-cysteine, NIBLC, *N*-isobutyryl-L-cysteine; CD, cyclodextrin; HPBCD, (2-hydroxypropyl)-β-cyclodextrin; HPGCD, (2-hydroxypropyl)-γ-cyclodextrin; SBCD, sulfated β-cyclodextrin sodium salt; CMBCD, carboxymethyl-β-cyclodextrin; DMCD, 2,6-di-O-methyl-β-cyclodextrin; TMCD, 2,3,6-tri-O-methyl-β-cyclodextrin; BGE, background electrolyte; IS, internal standard; CDA, chiral derivatization agent;

## 1. Introduction

The assessment of enantiomeric purity of amino acids (AAs) is an important field of analysis due to the importance of D-amino acids in living organism [1-8]. Several methods for the analysis of AAs were reported. Modern approaches make use of liquid chromatography, partially with sensitive detection methods like fluorescence detection or mass spectrometry. Newer methods employ evaporative light scattering detector (ELSD) or charged aerosol detector (CAD) [9,10]. However, capillary electrophoresis has been proved to be worth especially when it comes to the separation of enantiomers [11]. It benefits from a low consumption of chemicals and maintenance costs.

Enantioseparation of AAs can be performed either directly or indirectly. While the latter requires treatment of the AAs with a derivatization agent prior to separation, a direct enantioseparation is achieved by interaction between the pure analyte and a chiral selector. A major disadvantage of the direct enantioseparation of most of the AA is the lack of a chromophore and the short optical light path of the CE instrument, making a sensitive detection often impossible. In most cases, the AAs are derivatized before or after analysis to improve the separation behavior and introduce a strong chromophore or fluorophore in order to enable a sensitive detection. For an indirect enantioseparation, a selector is usually also necessary to resolve the derivatives. Selectors that are suitable for direct and indirect enantioseparation include cyclodextrins (CD) [12-14], crown ethers [15-17], macrocyclic antibiotics [18-20], chiral surfactants [21,22] and ligand-exchange complexes [23-26].

CDs are the most common type of selectors for CE, which are used for the separation of all types of isomers and structurally related compounds in CE, not only enantiomers. They are cyclic oligosaccharides which consist of 6 to 8 α(1,4)-glycosidic linked glucopyranose molecules. By derivatization, new moieties and a negative of positive charge can be introduced, giving CDs that show different selectivities to analytes, which is crucial for method development.

Various derivatization reagents have been applied for AAs when CE is used e.g. fluorescein isothiocyanate (FITC) [27], fluorenylmethyloxycarbonyl chloride (FMOC-Cl) [28], 3-(4-carboxybenzoyl)quinoline-2-carboxaldehyde (CBQCA) [29,30], and *ortho*-phthalaldehyde

(OPA) [31]. OPA is a proven reagent which allows for a fast derivatization of AAs. A fluorophore is introduced upon reaction to allow sensitive detection of the derivatives by fluorescence but UV detection is also possible. Disadvantages of OPA are preeminently the poor stability of the derivatives. For this reason, the procedure is often automatized and in-capillary derivatization is applied [32,3,33]. Derivatization of AAs inside the capillary has been mostly performed with a BGE that contains OPA and a thiol [3,34]. The AAs are then derivatized during the separation. For this, the composition of the BGE is limited to provide the necessary chemical environment for the OPA derivatization to take place. Here, a procedure is used with a BGE without OPA. Sample and derivatization solution were injected in the capillary and the derivatization takes place prior to separation. This procedure gives more freedom in the choice of the composition of the BGE and the absence of OPA in the BGE reduces the UV absorbance of the buffer itself.

The aim of this study was to find simple methods for the assessment of enantiomeric purity for individual AAs, which does not require special instrumentation such as an amino acid analyzer in the European Pharmacopoeia [35], besides the capillary electrophoresis system.

## 2. Material and Methods

### *2.1. Apparatus*

The CE system consisted of a Beckman P/ACE MDQ capillary electrophoresis system from Beckman Coulter (Krefeld, Germany) equipped with a PAD detector unit. Data acquisition and analysis was performed with the 32 Karat software version 8.0.

For pH adjustments a PHM 220 Lab pH-Meter with a combined pH electrode from Radiometer Analytical (Villeurbanne, France) or a Metrohm 744 pH-Meter with a combined pH electrode from Deutsche METROHM GmbH & Co. KG (Filderstadt, Germany) were used.

### *2.2. Chemicals and Materials*

HPLC grade methanol was obtained from VWR International GmbH (Darmstadt, Germany). Water was purified using the Milli-Q purification system by Merck Millipore

(Schwalbach, Germany). Sodium tetraborate decahydrate, γ-cyclodextrin (γ-CD), (2-hydroxypropyl)-β-cyclodextrin (HPBCD), (2-hydroxypropyl)-γ-cyclodextrin (HPGCD), sulfated β-cyclodextrin sodium salt (SBCD), carboxymethyl-β-cyclodextrin (CMBCD), 2,6-di-O-methyl-β-cyclodextrin (DMCD), and 2,3,6-tri-O-methyl-β-cyclodextrin (TMCD), *ortho*-phthalaldehyde (OPA), *N*-acetyl-L-cysteine (NAC) and *N*-isobutyryl-L-cysteine (NIBLC) were purchased from Sigma-Aldrich Chemie GmbH (Schnelldorf, Germany), β-cyclodextrin (β-CD) from Wacker Chemie GmbH (Burghausen, Germany), and D-, L-, and DL- amino acids were obtained by Sigma-Aldrich Chemie GmbH/Fluka (Schnelldorf, Germany) and Alfa Aesar (Lancashire, United Kingdom).

Fused silica capillaries (50 and 75 µm inner diameter, 375 µm outer diameter) with polyimide coating were purchased from BGB Analytik Vertrieb GmbH (Rheinfelden, Germany).

### *2.3. Preparation of Solutions*

The borate buffer for the BGE (50 mM, pH = 9.25) was prepared by dissolving 1.907 g sodium tetraborate decahydrate in 100.0 mL water. No pH adjustment was necessary. For the borate buffer for the derivatization solution (pH = 10.0, 20 mM), 0.763 g sodium tetraborate decahydrate was dissolved in 90 mL water. The pH was adjusted to 10.0 with 2 M sodium hydroxide solution by means of a calibrated pH-electrode and the solution was diluted to 100.0 mL.

The derivatization solution was prepared by dissolving 40.2 mg OPA (0.30 mmol) in 2.0 ml pure methanol, adding either 31.8 mg MPA, 57.5 mg NIBLC, or 49.0 mg NAC (0.30 mmol each) and diluting with 20 mM borate buffer (pH = 10.0) to 10.0 mL. The solutions were kept in the refrigerator for not more than three days. The BGE was prepared by dissolving 75 µmol of the corresponding CD in 10.0 mL borate buffer (pH = 9.25).

The AA solutions, used for screening, were prepared by dissolving 0.1 mmol AA individually in water and diluting to 20.0 mL with the same solvent to give a 5 mM solution of each AA. The solutions for linearity ranging from 1 to 10 % were obtained by dissolving 100.0 mg of L-AA in water, adding 10.0 mL of 0.5 mg $mL^{-1}$ L-alanine and diluting with water to 100.0 mL. A solution of the corresponding D-AA was prepared accordingly. For preparation of a mixture of both enantiomers, the respective solutions were mixed in the right ratio, e.g.

0.1 mL of D-phenylalanine solution was mixed with 9.9 mL of L-phenylalanine solution to obtain a 1 % solution of D-phenylalanine in L-phenylalanine. The solutions for linearity ranging from 0.1 to 1.0 % were prepared correspondingly, but 500.0 mg of the L- or D-AA were used and 10.0 mL of 0.05 mg $mL^{-1}$ L-alanine was added. The same procedure was used for the solutions used for recovery. Samples for quantification by area normalization consisted of 1 mg $mL^{-1}$ AA in Milli-Q water.

All solutions were sonicated for 15 min prior to use and filtrated with syringe filters directly into the vial.

### *2.4. Electrophoretic Conditions*

The capillary used had a total length of 62.5 cm (50 cm to the detector) and an inner diameter of 50 µm. It was rinsed with 0.1 M NaOH for 20 min at 40 psi (1 psi = 6894.76 Pa) before and after every sequence of runs. Before each run, the capillary was rinsed with 0.1 M NaOH for 2 min, followed by water for 2 min and with BGE for 2 min each at 40 psi. In-capillary derivatization was performed by means of the OPA/NAC derivatization scheme. The BGE consisted of a 50 mM borate buffer (pH = 9.25) with 7.5 mM of γ-CD. The voltage was set to 20 kV, the temperature to 25 °C and the total run time was 30 min. The derivatives were detected with a PDA detector at 238 nm with a 10 nm bandwidth.

## 3. Results and Discussion

The aim of this study was to develop a simple method for the assessment of enantiomeric purity for individual AAs by means of CE which does not require expensive chemicals or special instrumentation as the amino acid analyzer (AAA). The AAs are derivatized inside the capillary by injecting the derivatization solution as a sandwich i.e. before and after the AA. Next, a low voltage is applied for one minute, to blend the solutions in order to start the reaction and to give two diastereomers in case a chiral thiol was used. The two isomers are then separated with a suitable cyclodextrin in an aqueous borate buffer.

### *3.1. In-Capillary Derivatization*

Here, a method was developed using a derivatization of the AAs with OPA in combination with a thiol e.g. 3-mercaptopropionic acid (MPA), *N*-acetyl-L-cysteine (NAC) or *N*-isobutyryl-L-cysteine (NIBLC) (see *Figure 1*), because these lead to more stable derivatives than with 2-mercaptoethanol [36-38]. NAC and NIBLC are also non-toxic solids and therefore safer to handle. The derivatization takes place inside the capillary, by injecting the derivatization solution in sequence before and after the sample (sandwich injection). A similar method has been described previously for aminoglycosides [39-41]. The method takes care of the instability of the OPA derivatives. The solutions were injected with a pressure of 0.5 psi for 5 sec. A voltage of 1 kV is applied afterwards for one minute in order to blend the solutions to start the reaction. For a fast reaction time, the pH value of the buffer was set to 10.0. The separation was started afterwards by increasing the voltage to the value used by the method. Proline has a secondary amine moiety and is therefore not suitable for the derivatization with OPA. A derivatization of cysteine was found to be impossible, probably due to side reactions of OPA with the thiol moiety of cysteine. A possible solution for this issue is the alkylation of cysteine with iodoacetic acid [42,43]. However, this procedure is not suitable for an automated derivatization since it would also alkylate the thiol needed for the OPA derivatization.

To verify the efficiency of the in-capillary derivatization procedure, three AAs carrying a chromophore (phenylalanine, tryptophan and methionine) were used. They can be detected without the OPA label. 5 mM of each AA were injected individually and derivatized with the in-capillary derivatization procedure (OPA/NAC, sandwich). Residual non-derivatized AA was separated at 15 kV in a BGE consisting of 50 mM borate buffer (pH = 9.25) and 7.5 mM γ-CD. The peak area for the underivatized AA was below the limit of detection in all cases. Hence, the AAs are quantitatively converted into its derivatives. If the derivatization solutions were injected only prior or after the sample, peaks of underivatized AAs are clearly visible and the degree of derivatization is reduced by 10 – 40 %.

Besides the peaks visible in the blank solution and the two derivatives of the AA, no additional peaks were found in the electropherograms, indicating that the derivatives are stable for the duration of the separation.

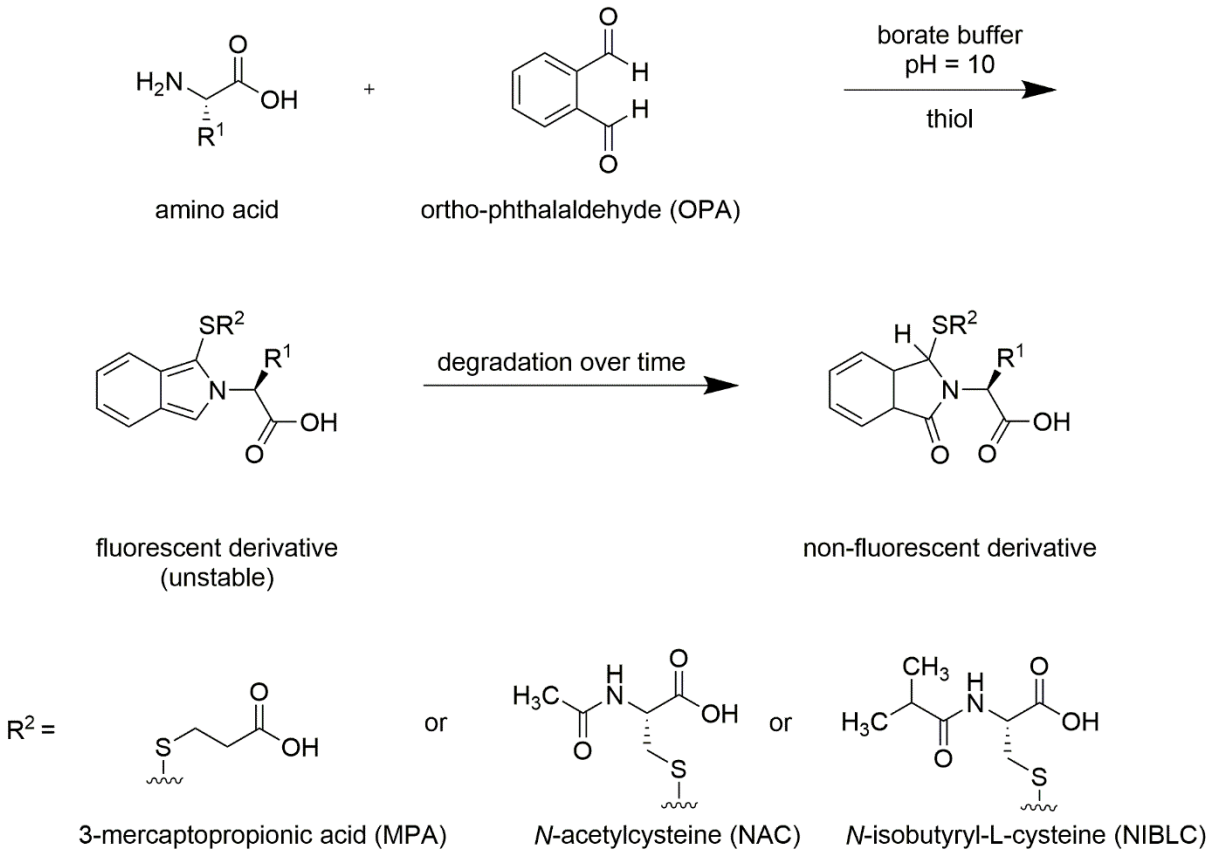



**Figure. 1:** Reaction of AAs with OPA and the thiol

### *3.2. Screening of Thiol and Cyclodextrins*

If MPA is used as the thiol for derivatization, the products are still enantiomers; diastereomers are formed due to the chiral carbon atom present in NAC and NIBLC.

Even if the products of the derivatization of the AAs are no longer enantiomers, a selector is still necessary as in principle capillary zone electrophoresis cannot separate compounds with the same size and *z/m* ratio. Therefore, the thiol used for the derivatization with OPA and the CD in the BGE is crucial.

To find the optimal combination of thiol and CD, a screening was carried out in which each AA was derivatized with either MPA, NAC or NIBLC as thiol and then separated with one of the CDs. For this, various neutral and negative charged CDs were evaluated. The background electrolyte consisted of a 5 mM tetraborate buffer (pH = 9.25) with 7.5 mM of either CD. The separation voltage was 15 kV for all the derivatives of all AAs except aspartic and glutamic

acid. Here, the voltage was set to 25 kV due to lower migration times of these compounds caused by the negative charge of the additional carboxylic acid.

In *Table 1* the results of the screening experiments are shown. The resolutions obtained between the peaks due to the OPA-derivatives of the L- and D-AA are given. Data of the separation of OPA-labeled AAs with a combination of a thiol and CD that achieved only very low resolutions at all is not shown. The use of γ-CD and NAC as thiol enables the indirect enantioseparation of all AAs tested with resolutions between 2.3 and 16.6, except for serine. However, serine can be separated with β-CD/NAC with a resolution of 1.5. Besides this, β-CD is inferior to γ-CD in most cases due to the lower resolutions that were observed in comparison to γ-CD. Out of all other CDs tested, only with HPBCD satisfying results are obtained for most of the AAs. All other CDs allow a separation only for a few individual AAs. Regarding the thiol, it is advisable to use NAC, since the use of NIBLC or MPA mostly leads to decreased resolution. The enantiomer migration order (EMO) is dependent on AA, CD and thiol being used and differs therefore with the various conditions that were tested during this study. It was not evaluated in detail, but it was determined for the AAs methionine, aspartic acid, glutamic acid, leucine, isoleucine, valine, phenylalanine and tryptophan, that the migration order is L-AA before D-AA, when γ-CD and NAC is used for the separation.

The separation voltage had minor impact on the resolution of the diastereomeric derivatives. Depending on the AA, the resolutions either increased or decreased slightly. A higher voltage led to shorter retention times and a better peak shape. A good compromise between migration time, peak shape, resolution and current was established with 20 kV.

Changes of the pH (9.25 ± 1.00) and molarity (50 ± 10 mM) of the BGE, and the temperature (25 ± 5 °C) were found to be negligible.

The UV-spectrum of OPA-labeled AAs has two maxima at 238 and 345 nm. Detection at 238 nm is about five times more sensitive in comparison to 345 nm but the excess of the OPA-reagent is also detected at 238 nm (see *Figure 2*). Since the OPA-peak is separated and does hence not interfere with the quantification, detection was performed at 238 nm.

Overview of the resolutions ($R_s$) due to the peaks of the derivatives of the L- and D-AA obtained with different thiols and CDs. Resolut
n 0.5 are marked with "-". The voltage applied was 15 kV, except for the derivatives of aspartic and glutamic acid, which was set to 25
d of 50 mM borate buffer (pH = 9.25) and 7.5 mM of the CD, T = 25 °C; λ = 238 nm.

| | γ-CD | | | β-CD | | | DMCD | | TMCD | | HPBCD | | | HPGCD | | CMBCD | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | **NAC** | **MPA** | **NIBLC** | **NAC** | **MPA** | **NIBLC** | **NAC** | **MPA** | **NAC** | **MPA** | **NAC** | **NIBLC** | **MPA** | **NAC** | **MPA** | **NAC** | **NIBLC** |
| | 3.2 | 0.9 | 1.2 | 3.8 | - | 3.0 | 2.9 | - | - | - | 2.0 | 2.3 | - | 0.5 | - | 1.3 | 1.2 |
| e | 4.3 | - | - | 1.0 | - | - | - | - | 0.9 | - | 1.4 | - | - | - | - | - | - |
| gine | 5.7 | - | - | - | - | - | - | 1.0 | 0.9 | - | 0.9 | - | 0.9 | - | - | 0.8 | 1.1 |
| c acid | 5.1 | 0.6 | 2.1 | 6.0 | - | 7.0 | 0.5 | - | ns[1] | ns[1] | 1.0 | ns[1] | ns[1] | 1.0 | - | - | ns[1] |
| ine | 9.7 | - | - | 2.1 | - | - | 1.2 | - | 2.4 | - | 2.7 | - | - | 0.5 | - | 2.5 | 2.1 |
| ic acid | 16.6 | 0.7 | 3.2 | 0.8 | 1.8 | 1.3 | 1.9 | - | ns[1] | ns[1] | 2.4 | 2.2 | 1.3 | 1.6 | ns[1] | - | 2.2 |
| e | 11.5 | - | - | 1.7 | 2.0 | 1.8 | 1.2 | - | 1.4 | - | 4.8 | 2.2 | 2.0 | 2.0 | 0.7 | 1.7 | 1.3 |
| ne | 5.5 | 8.3 | 2.8 | 4.1 | 2.4 | 5.1 | 0.8 | - | 3.5 | 1.7 | 5.0 | 4.1 | 1.9 | 0.6 | 4.9 | 1.9 | 1.5 |
| | 11.8 | 1.1 | - | 0.7 | 0.6 | 2.3 | 1.2 | 1.5 | 4.8 | - | 1.0 | - | - | 3.6 | 1.4 | 1.7 | - |
| | 2.3 | - | - | 1.3 | - | - | - | - | ns[1] | 1.0 | 1.8 | 1.4 | - | - | - | 1.8 | 0.8 |
| nine | 13.8 | - | 0.9 | 1.0 | 1.1 | 1.5 | 1.4 | 1.8 | 1.2 | - | 1.1 | 1.5 | 0.6 | 3.9 | 1.4 | 1.7 | 1.5 |
| lanine | 3.4 | 0.5 | - | 1.9 | 2.5 | - | 2.6 | 3.6 | - | 1.1 | 3.5 | - | 3.7 | 3.6 | - | - | - |
| | - | - | 1.3 | 1.5 | - | 1.4 | 1.5 | - | 1.2 | - | - | 0.9 | - | 0.6 | - | 0.9 | - |
| ne | 2.8 | - | 2.5 | 4.9 | 1.6 | 3.7 | 0.5 | - | 1.9 | - | 3.0 | 1.3 | 1 | - | - | 1.4 | 1.1 |
| han | 4.4 | 1.5 | - | 0.7 | 2.1 | - | - | - | 0.7 | - | 1.5 | 2.2 | 1.4 | 3.1 | 3.7 | - | 1.0 |
| e | 8.3 | 1.1 | - | 1.6 | 4.0 | - | 4.5 | 5.8 | 2.1 | 2.0 | 5.2 | - | 4.2 | 5.2 | 1.7 | - | - |
| | 8.9 | 1.5 | 0.7 | 4.1 | - | 4.4 | 1.1 | - | 3.5 | 1.6 | 4.2 | 2.0 | - | 2.3 | - | 1.8 | 1.7 |

etection within 30 min due to slow migration of the derivatives

To improve sensitivity, a capillary with a diameter of 75 µm was tested. This resulted in a decrease of resolution between the peaks of the L- and D-AA derivatives for all AAs. Derivatives which already showed a low resolution with a 50 µm capillary, could not be separated with the 75 µm capillary any more (e.g. lysine, threonine). Shorter and longer capillaries led to lower or higher resolutions, respectively. A length of 50 cm to the detector of the capillary was necessary for achieving satisfying resolutions. Longer capillaries increase the run time of the method with only slight improvement of the resolution.

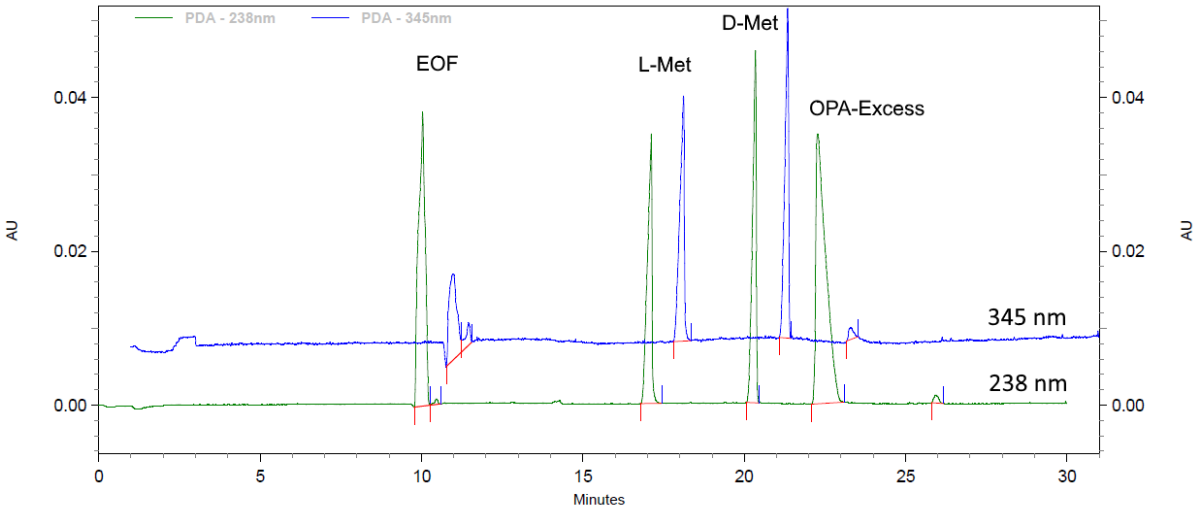


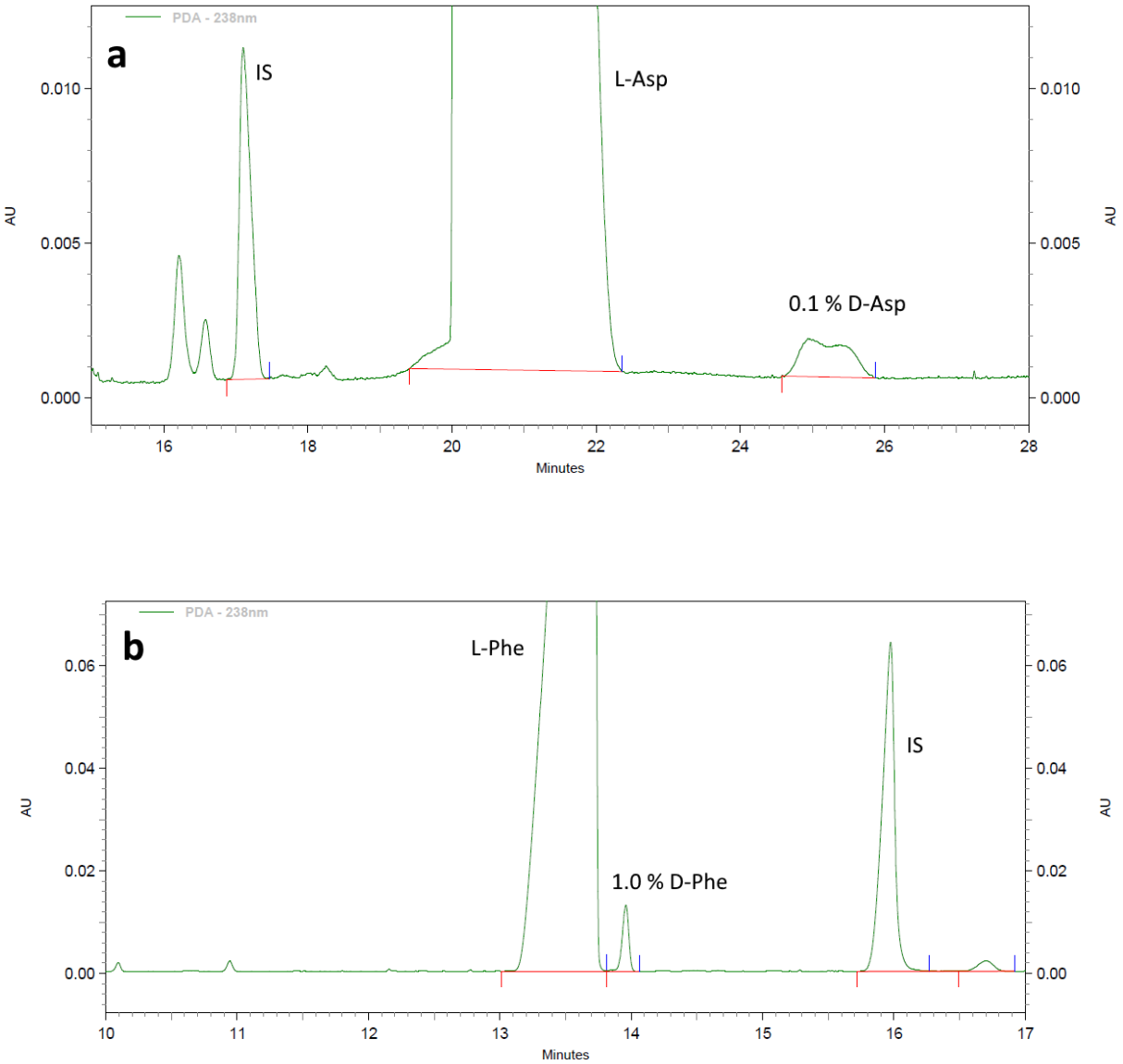

**Figure 2:** Electropherogram of DL-methionine with detection of the derivatives at 238 and 345 nm. At 238 nm the excess of OPA and the thiol can be seen. Electrophoretic conditions: 15 kV, 25 °C, BGE: 50 mM borate buffer (pH = 9.25) with 7.5 mM γ-CD

Taken all together, the best results were obtained using a 50 mM borate buffer (pH = 9.25) with 7.5 mM γ-CD at a voltage of 20 kV, NAC as thiol, a temperature of 25 °C, a 50 µm capillary with a total length of 62.5 cm (50 cm to the detector) and a detection at 238 nm.

*Figure 3* shows the electropherograms of four L-AAs with the corresponding D-AA at the level of quantification. L-alanine is used as an internal standard (IS) to cope with the inaccuracies of the hydrodynamic injection. The percentage LOQ of the minor diastereomer is 0.1 % for the AAs methionine and aspartic acid, which were injected with a concentration of 5 mg $mL^{-1}$. The poor peak shape of D-aspartic acid could not be avoided. It seems to be related to the electrophoretic conditions of the method and the poor solubility of aspartic acid at pH 9.25. However, quantification is still feasible. The AAs phenylalanine and tryptophan have to

be injected in lower concentrations in order to enable fully separation, which results in a LOQ of 1.0 %.

The ratio of L- and D-AA can also be calculated by direct comparison of the corrected areas of both peaks without using an IS. However, the concentration of the sample should be lower than 1 mg $mL^{-1}$, otherwise the signals of the peaks are outside the linear range of the detector and an accurate quantification is not possible. Differences in the signal response of both diastereomers are neglected. *Figure 4* displays the electropherograms of 1 % L- and D-methionine, respectively, in presence of the 99 % major diastereomer without IS.



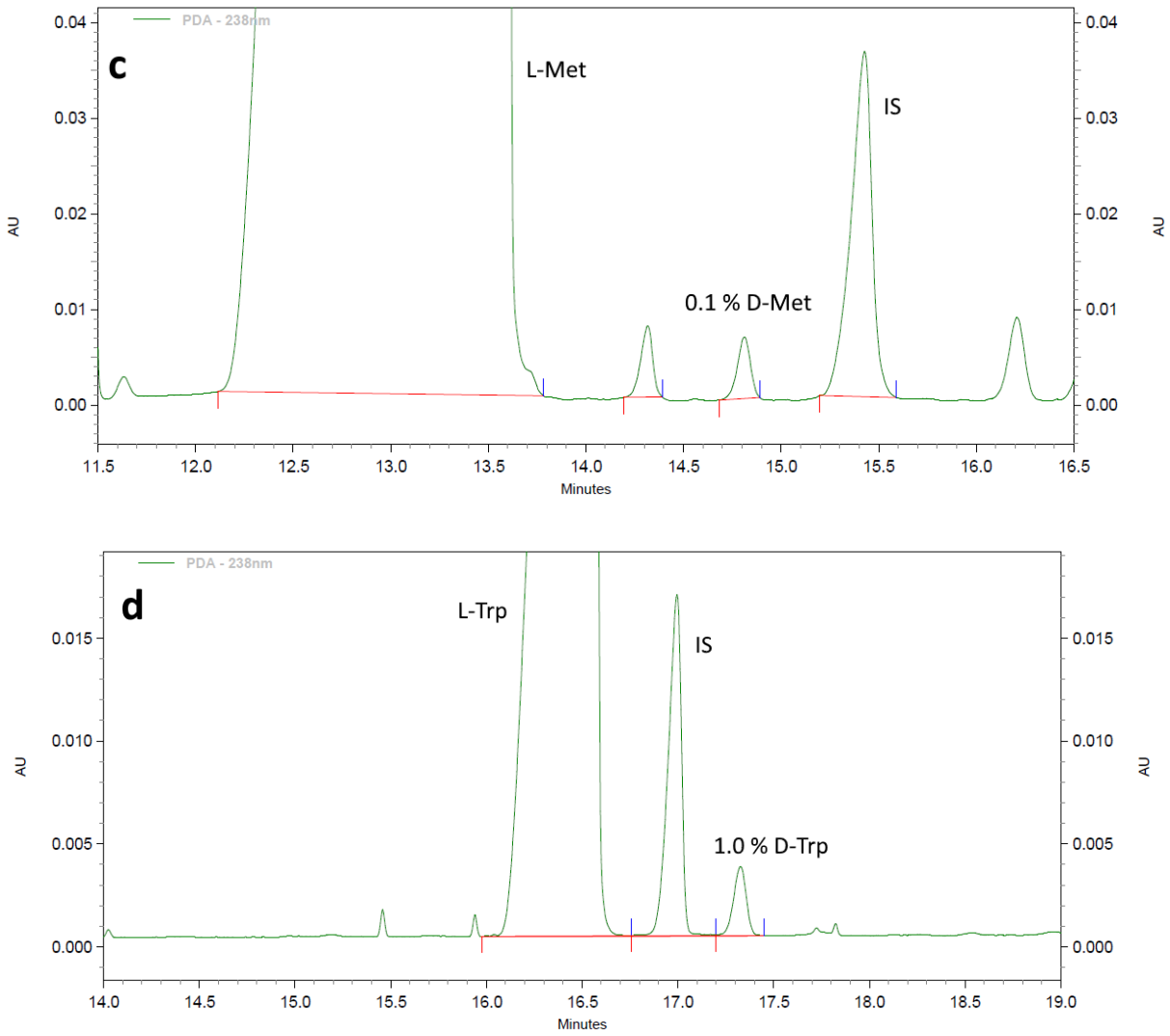



**Figure 3:** Assessment of enantiomeric purity; a: aspartic acid (5 mg $mL^{-1}$, 0.1 % D-Asp); b: phenylalanine (1 mg $mL^{-1}$, 1.0 % D-Phen); c: methionine (5 mg $mL^{-1}$, 0.1 % D-Met); d: tryptophan (1 mg $mL^{-1}$, 1.0 % D-Trp); L-alanine is used as IS, Electrophoretic conditions: 20 kV, 25 °C, BGE: 50 mM borate buffer (pH = 9.25) with 7.5 mM γ-CD, UV at 238 nm

The optical purity of the chiral derivatization agents (CDA) NAC and NIBLC has to be considered, since it can limit the LOQ of the method. The major enantiomer of an AA may form a derivative with the chiral impurity of the CDA, that is enantiomeric to the derivative formed by the minor enantiomer of the AA with the CDA. These compounds have to be separated in order to avoid the interference of the quantification of the minor enantiomer. The optical purity of NIBLC is 100.0 % according to the certificate of analysis. For NAC no certificate is available, however the electropherograms of pure L or D-methionine did not show any additional peaks besides the main peak neither with NAC nor NIBLC as thiol.

Therefore, if any, a derivative with the chiral impurity of the CDA is lower than the limit of detection and does hence not interfere the quantification.

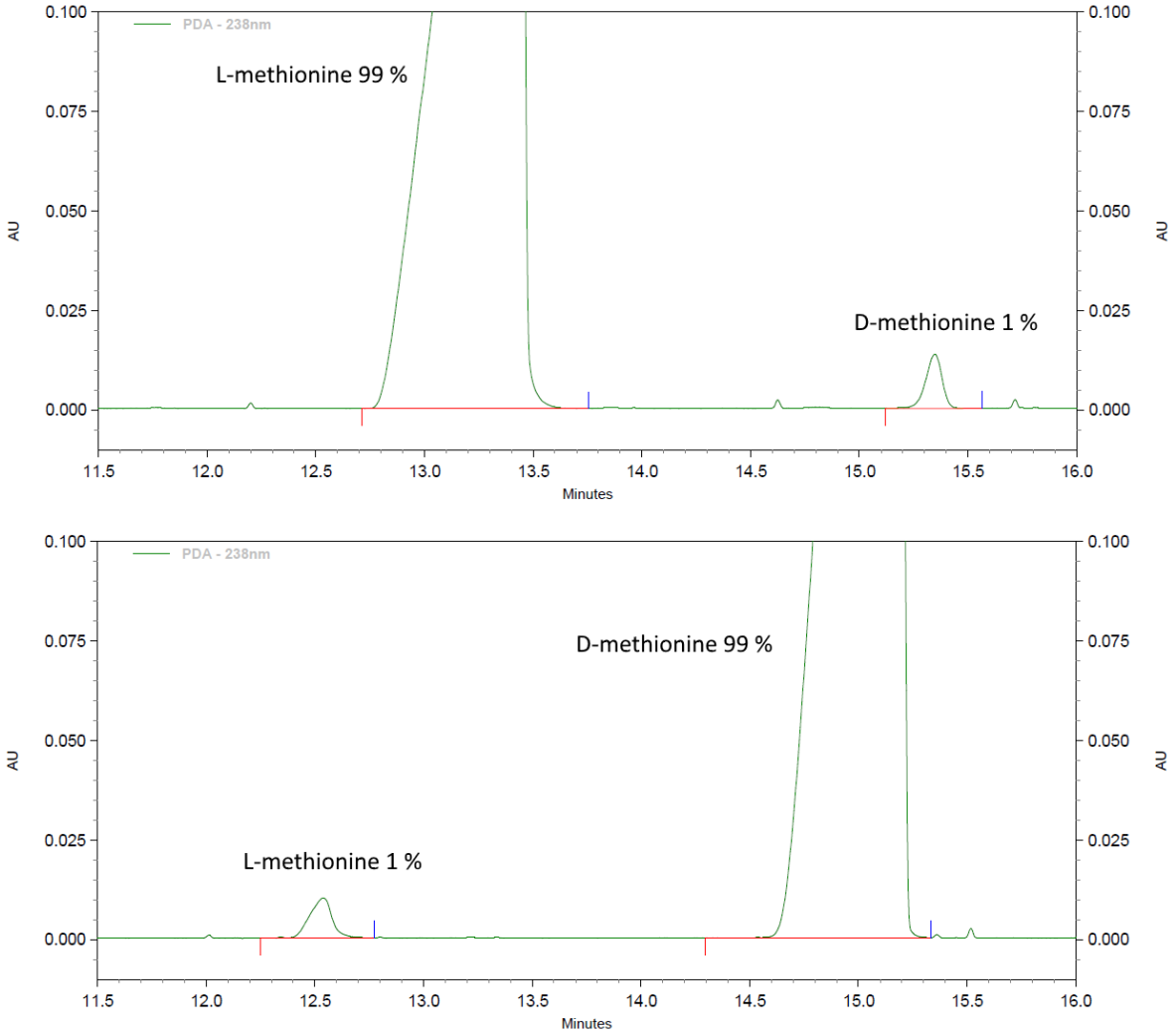



**Figure 4:** Direct quantification of a methionine without IS; electrophoretic conditions see Figure 3

### *3.3. Validation*

The validation was performed with the in-capillary derivatization of section 3.1 and the method elaborated in section 3.3.

#### *3.3.1. Limit of Detection and Quantification*

For the determination of the LOQ the signal-to-noise ratio of at least 10 was applied. It was determined by continuous dilutions of a stock solution with a concentration of

0.1 mg $mL^{-1}$. The LOQ and LOD for the derivatives of the AAs varies slightly. However, at a concentration of 5 µg $mL^{-1}$ a signal-to-noise ratio of more than 10 could be achieved for all AAs. The LOD was not determined experimentally but specified as one third of the concentration of the LOQ being 1.67 µg $mL^{-1}$.

### *3.3.2. Linearity*

The highest total concentration of the sample that makes a sufficient separation of the minor diastereomer possible was applied. If the resolution between the diastereomers was high, higher concentrations of the sample could be used to achieve a lower percentage LOQ. The linearity for the minor diastereomer was evaluated ranging from 5 µg $mL^{-1}$ to 50 µg $mL^{-1}$ (0.1 – 1.0 %) for the AAs methionine and aspartic acid (total concentration 5 mg $mL^{-1}$) and from 10 to 100 µg $mL^{-1}$ (1 – 10 %) for the AAs phenylalanine and tryptophan (total concentration 1 mg $mL^{-1}$). A calibration curve was constructed with 5 data points (n = 3) and linear regression was performed. Due to the inaccuracy with hydrodynamic injection L-alanine was used as internal standard. The coefficient of determination is higher than 0.999 in all cases, showing that the method is linear.

### *3.3.3. Precision*

The precision of the in-capillary derivatization procedure was evaluated by injecting 1 mg $mL^{-1}$ of the DL-AAs methionine, phenylalanine and isoleucine individually, each with 0.2 mg $mL^{-1}$ of L-alanine as internal standard (n=10). The relative standard deviation of the corrected area ratio of AA and internal standard was ranging between 1.2 to 1.7 %. The relative standard deviation of the accuracy (see *Table 2*) was found to be within 1.2 and 6.4 %. The method can be considered to be precise.

### *3.3.4. Accuracy*

The accuracy is summarized in *Table 2*. Three replicate injections of each AA with its corresponding enantiomer at the LOQ level were performed. The enantiomeric impurity was

quantified using the equation obtained by linear regression. The recovery was found to be within 95.1 to 107.5 %, showing that the method is accurate.

**Table 2:** Accuracy of the determination of enantiomeric purity (n=3). The concentration of methionine and glutamic acid was 5 mg $mL^{-1}$ and of phenylalanine and tryptophan 1 mg $mL^{-1}$. The AAs contained the minor enantiomer at its LOQ.

| AA | Measured [µg $mL^{-1}$] | Recovery [%] | RSD [%] |
|---|---|---|---|
| **D-Methionine 0.1 %** | 4.76 ± 0.16 | 95.1 | 3.3 |
| **L-Methionine 0.1 %** | 4.87 ± 0.25 | 97.4 | 5.1 |
| **D-Aspartic acid 0.1 %** | 5.27 ± 0.34 | 105.3 | 6.4 |
| **L-Aspartic acid 0.1 %** | 4.92 ± 0.11 | 98.3 | 2.2 |
| **D-Phenylalanine 1.0 %** | 9.81 ± 0.12 | 98.1 | 1.2 |
| **L-Phenylalanine 1.0 %** | 10.37 ± 0.33 | 103.7 | 3.2 |
| **D-Tryptophan 1.0 %** | 10.75 ± 0.59 | 107.5 | 5.5 |
| **L-Tryptophan 1.0 %** | 10.68 ± 0.50 | 106.8 | 4.7 |

Accuracy was additionally evaluated by samples of methionine without an IS by means of direct comparison of the corrected area of the peaks ranging from 1 to 50 % for the derivatives of L- and D-methionine (see *Table 3*). A potential difference in detector response of both diastereomers was neglected. The accuracy is within 92.1 and 115.9 % which is considered as accurate, but less accurate in comparison to the usage of an IS.

**Table 3:** Recovery of L- and D-AAs by means of direct comparison of the corrected peak areas of the derivatives (n=3).

| Content [%] | D-Methionine | | L-Methionine | |
|---|---|---|---|---|
| | Measured [%] | Recovery [%] | Measured [%] | Recovery [%] |
| **1** | 1.09 ± 0.02 | 111.4 | 1.16 ± 0.03 | 115.9 |
| **2** | 1.87 ± 0.02 | 93.4 | 1.91 ± 0.15 | 95.5 |
| **5** | 5.36 ± 0.09 | 107.2 | 5.44 ± 0.05 | 108.8 |
| **10** | 10.74 ± 0.11 | 107.4 | 9.21 ± 0.16 | 92.1 |
| **20** | 20.36 ± 0.05 | 101.8 | 21.57 ± 0.23 | 107.8 |
| **50** | 49.07 ± 0.11 | 98.1 | 50.93 ± 0.11 | 101.9 |

### *3.3.5. Robustness*

Robustness was evaluated by changing one of the following parameters at a time: concentration of the BGE: 50 mM ± 5 mM; temperature: 25 °C ± 5 °C; concentration of the CD: 7.5 mM ± 1 mM; separation voltage: 15 kV ± 2 kV and detection wavelength: 238 nm ± 2 nm. DL-phenylalanine was injected and the change in resolution and signal-to-noise ratio were monitored. None of these changes did impair with the separation of the derivatives and the changes in signal-to-noise ratio was found to be negligible.

## 4. Conclusion

A robust, linear, precise and accurate CE method with an in-capillary derivatization for the assessment of enantiomeric purity of AAs was developed and validated. Depending on the AA the enantiomeric impurity can be determined at a level between 0.1 to 1.0 %. The method enables the indirect enantioseparation of AAs with minimal amount of chemicals and without the need of an expensive chiral column. The method can be used as an alternative for the insensitive test with optical rotation used in the European Pharmacopoeia. The sensitivity can be improved with the use of a fluorescence detector without any changes of the method.

**Compliance with Ethical Standards**

The authors declare no conflict of interest.

**Acknowledgment**

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reversed-phase high-performance liquid chromatography with fluorimetric detection. J Chromatogr A 913 (1–2):309-313. doi:http://dx.doi.org/10.1016/S0021-9673(00)01201-2

# 3. High Performance Liquid Chromatography Evaluation of the Enantiomeric Purity of Amino Acids by Means of Automated Precolumn Derivatization with *Ortho*-Phthalaldehyde and Chiral Thiols

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**ABSTRACT**

The use of *ortho*-phthalaldehyde (OPA) for the derivatization of amino acids (AA) is well known. It enables the separation of the derivatives on common reversed phase columns and improves the sensitivity with fluorescence detection. With the use of a chiral thiol an indirect enantioseparation of chiral amines and AAs is feasible. The major drawback of the OPA-derivatization is the poor stability of the products. Here, a method with an in-needle-derivatization procedure is optimized to facilitate a quantitative conversion of the AA with OPA and the chiral thiols *N*-acetyl-L-cysteine or *N*-isobutyryl-L-cysteine, followed by a subsequent analysis, eluding the stability issue. Both enantiomers of a single AA were separated as OPA-derivatives with a pentafluorophenyl column and a gradient program consisting of 50 mM sodium acetate buffer pH = 5.0 and acetonitrile. Fluorescence detection is commonly used to achieve sufficient sensitivity. In this study, the enantiomeric impurity of an AA can be detected indirectly with common UV spectrophotometric detection with a LOQ of 0.04 %. 17 different L-AAs were tested and the amount of D-AA for each individual AA was calculated by means of area normalization, which was ranging from not detectable up to 4.29 %. The recovery of the minor enantiomer of L- and D-AA was demonstrated for three AAs at a 0.04 % level and ranged between 92.3 and 113.3 % with the relative standard deviation being in between 1.7 and 8.2 %.

*Abbreviations*: AA, amino acid; OPA, *ortho*-phthalaldehyde; ME, 2-mercaptoethanol; MPA, 3-mercaptopropionic acid; NAC, *N*-acetyl-L-cysteine; NIBLC, *N*-isobutyryl-L-cysteine; NIBDC, *N*-isobutyryl-D-cysteine; FLD, fluorescence detection; PFP, pentafluorophenyl; AAA, amino acid analysis; CDA, chiral derivatization agent;

## 1. Introduction

Amino acids (AA) are the fundamental building stocks of proteins and living organism in general. In addition, AAs are also used as drugs, dietary supplement and are raw material. The enantiomeric purity of the AAs has to be controlled, because both enantiomers can have different physiological significance, which has already been reported.[1-5] Major pharmacopoeias like the European Pharmacopoeia[6] and the United States Pharmacopoeia[7] have monographed the AAs. Normally, the test for impurities is performed by means of reversed phase HPLC with UV spectrophotometric detection. However, AAs are not suitable for a direct analysis, due to the weak retention of the polar AAs on reversed phase columns, the low absorption of UV radiation of most of the AAs due to the lack of a chromophore and the absence of a chiral selector. Therefore, the Ph.Eur. conducts the evaluation of enantiomeric purity by means of optical rotation. However, this test lacks specificity and sensitivity and a more powerful method is needed.

The use of an evaporative light scattering detector (ELSD) or a charged aerosol detector (CAD) enables the detection of all AAs and if a chiral column is applied, a direct enantioseparation is feasible. Methods for the use of these detectors in the analysis of AAs have been published.[8-10] However, these detectors are still rare and do not always deliver the sensitivity needed for the low amounts of chiral impurities that can be found in most of the AAs. Furthermore, there are more downsides e.g. nonlinear response, limitation of buffer selection and the response is depended on the organic share of the mobile phase which makes a gradient elution problematic. For a better sensitivity in AA impurity profiling, amino acid analysis (AAA) using cation ion exchange columns and post-column ninhydrin derivatization is performed in most monographs of the European Pharmacopoeia, replacing the thin-layer-chromatography with ninhydrin treatment.[11] Since this achiral method cannot be utilized for the evaluation of enantiomeric purity, a derivatization reagent that converts the chiral AAs into diastereomers to separate them on common reversed phase columns is necessary. *Ortho*-phthalaldehyde (OPA)[12] in combination with a chiral thiol is very suitable for the indirect enantioseparation of AAs, because it is relatively cheap, exhibits short reaction times, the AAs are fully derivatized, no toxic compounds are necessary for this process and the products can be detected either with an UV or a fluorescence detector. In the latter case, the excess of the

reagent does not interfere with the AA detection, due to the formation of the fluorophore upon derivatization. The major drawbacks of OPA are the limitation to primary amines and especially the poor stability of the products, which may degrade to non-fluorescent compounds.[13-15] However, the addition of sodium hypochlorite enables the derivatization of secondary amines like proline.[16] The automation of the derivatization process by the autosampler of the HPLC system eludes the stability issue, by putting the derivatization procedure directly before the analysis, reducing the time in which the derivatives have to be stable to a minimum. This has also been reported for the analysis of OPA derivatized AAs by capillary electrophoresis.[17,18] OPA reacts within a few minutes in an aqueous borate buffer (pH = 10.0) in the presence of these thiols with primary AAs to give a fluorescent isoindole product (see Figure 1).

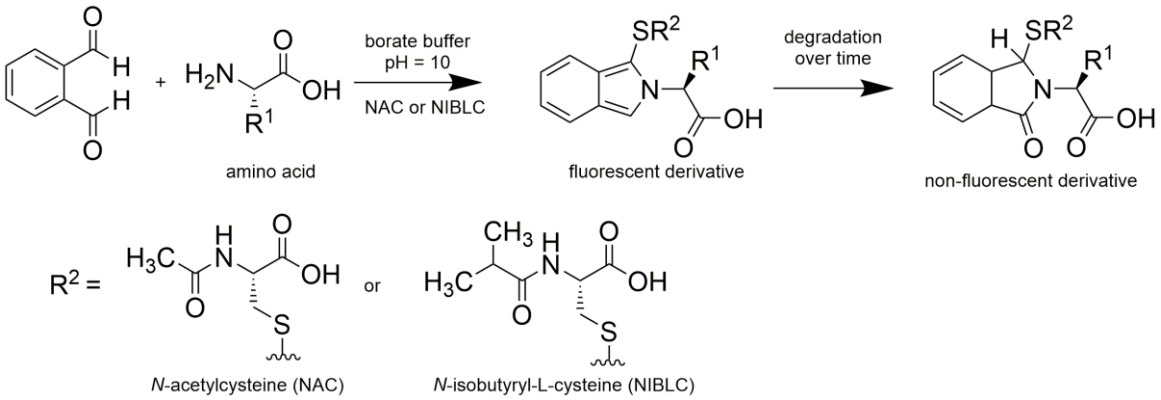



**Figure 1:** reaction of ortho-phthalaldehyde with AAs

Here, we study and optimize the automated precolumn derivatization procedure that takes place inside the needle of an autosampler, which is crucial for the quantitative conversion of the AAs into its derivatives and specially for a low carry-over effect. A method with a similar approach by means of capillary electrophoresis has been published previously by the authors.[19] It is aimed to derivatize the AAs using OPA and either NAC or NIBLC as thiol. The principle of the derivatization process is to draw consecutively various reagent solutions in a specific order into the needle of the autosampler. This can be achieved with a custom injection program of the autosampler. Since the derivatization takes place immediately before the separation on the column, the often reported instability of the OPA derivatives does not

play a role. However, the stability of the derivatives with this procedure was studied by means of liquid chromatography / mass spectrometry (LC/MS).

Methods for the indirect enantioseparation of AAs with a chiral derivatization agent (CDA) have been published previously.[20-24] The derivatives are detected by means of fluorescence detection (FLD), which is not always available. For this study, it was aimed to develop a method that is capable of assessing the enantiomeric purity of individual AAs with good sensitivity even with UV spectrophotometric detection and see how it performs in comparison to traditional fluorescence detection. To achieve this good sensitivity, relatively high concentrated samples had to be used to enable a limit of quantification of the minor enantiomer below 0.1 % with UV detection and new methods for the separation of these samples had to be developed accordingly.

## 2. Experimental

### *2.1. Apparatus*

The experiments were performed either on an Agilent (Waldbronn, Germany) 1200 series HPLC instrument with a vacuum degasser (G1322A), a quaternary pump (G1311A), an autosampler (G1329A), a thermostated column department (G1316A), a variable wavelength detector (G1314B) and a 1100 series fluorescence detector (G1321A) or an Agilent 1100 series HPLC instrument equipped with a vacuum degasser (G1379A), binary pump (G1312A), autosampler (G1313A), variable wavelength detector (G1314A) and a fluorescence detector (G1321A). For pH adjustments a PHM 220 Lab pH-Meter with a combined pH electrode from Radiometer Analytical (Villeurbanne, France) and a Metrohm 744 pH-Meter with a combined pH electrode from Deutsche METROHM GmbH & Co. KG (Filderstadt, Germany) were used. LC-MS measurements were performed using a LC/MSD Trap G2445D ion trap from Agilent with electro spray ionization (ESI) attached to a HPLC equipped with a vacuum degasser (G1379B), a binary pump (G1312A), an autosampler (G1329A) with a thermostat (G1330B), a thermostated column compartment (G1316A) and a diode array detector (G1315B).

### *2.2. Chemicals and materials*

HPLC grade acetonitrile and methanol were obtained from VWR International GmbH (Darmstadt, Germany). Water for HPLC was purified using the Milli-Q purification system by Merck Millipore (Schwalbach, Germany). *Ortho*-phthalaldehyde, *N*-acetyl-L-cysteine (total purity > 99 %), *N*-isobuturyl-L-cysteine (optical purity > 99.5 %), acetic acid solution 97 %, formic acid solution 50 %, sodium tetraborate decahydrate, and sodium hydroxide solution 50 % for HPLC was purchased from Sigma-Aldrich Chemie GmbH (Schnelldorf, Germany). D-, L-, and DL- amino acids were obtained by Sigma-Aldrich Chemie GmbH/Fluka (Schnelldorf, Germany), and Alfa Aesar (Lancashire, United Kingdom). Columns were a Kinetex C18 (100x2.1 mm, 2.6 µm particles), a Kinetex PFP (150x4.6 mm; 2.6 µm particles) both from Phenomenex (Aschaffenburg, Germany) and a Nucleodur Phenylhexyl (150x4.6 mm; 3 µm particles) from Machery-Nagel (Düren, Germany).

### *2.3. Preparation of Solutions*

For the derivatization solution, 8.0 mg OPA (60 mmol) were dissolved in 200 µl pure methanol, 11.5 mg NIBLC or 9.8 mg NAC (60 mmol each) were added and diluted to 2.0 ml with 20 mM borate buffer (pH = 10.0). The solution was kept in the refrigerator for not more than three days.

For the aqueous mobile phase in the pH range of 3.0 to 4.4, 4.603 g of 50 % m/m formic acid solution was diluted with Milli-Q-water to approximately 950 ml and the pH was adjusted with 50 % m/m sodium hydroxide solution for HPLC using a calibrated pH-electrode, to give the desired pH value. The solution was diluted to 1.0 L afterwards. For an aqueous mobile phase in the pH range of 4.5 to 6.0, the same procedure was applied, but 3.003 g of glacial acetic acid was used instead of formic acid.

For the determination of recovery, stock solutions of each L- and D-AA of a concentration of 10 mM were prepared. Afterwards, 5.0 ml of one enantiomer solution was spiked with 5.0 ml of a 2,500-fold dilution of the corresponding other enantiomer to obtain a mixture of an AA with 0.04 % of its enantiomer.

The reference solutions used for quantification were prepared accordingly but with Milli-Q-water instead of the 10 mM AA solution.

For linearity, stock solutions of a concentration of 1 mM were prepared and 100-fold diluted. To obtain the solution for linearity in the range 0.002 – 0.04 %, 10, 50, 100, 150 and 200 µl of the diluted stock solution were further diluted to 1000 µl. For the solutions for linearity in the range 0.04 – 1.0 %, a 1 mM solution of each AA was diluted 10-fold and 20, 100, 200, 300, 400 and 500 µL were diluted to 1000 µl to obtain the desired concentrations.

All final solutions were filtrated through cellulose acetate syringe filters directly into the HPLC vial prior use.

## 3. Results and Discussion

The aim of this study was to elaborate a method for the indirect enantioseparation of AAs by means of an effective online derivatization with OPA and a chiral thiol that overcomes the problem of the instability of the OPA derivatives by performing the derivatization immediately before separation. The method should provide a good sensitivity with regards to the enantiomeric impurity of an AA. Since fluorescence detectors are not always available, satisfying results should already be achieved by UV spectrophotometric detection

The derivatization reaction takes place inside the needle prior to the injection. In principle, 2 µl of the derivatization solution (pH = 10.0), 2 µl of the sample and again 2 µl of the derivatization solution (pH = 10.0) were drawn into the needle of the autosampler from different vials. The solutions within the needle were mixed three times and the program was halted for one minute for the reaction to take place. Finally, the needle content was injected into the HPLC system.

### *3.1. Stability of the Derivatives*

For OPA derivatives it is reported, that the stability is related to the composition of the derivatization solution. The type of thiol and the ratio of the thiol and OPA is vital.[25-29] In addition, the chemical environment of the derivatization solution facilitates degradation. Thus, extracting the derivatives improves the stability.[13,15] In this study, *N*-acetyl-L-cysteine (NAC) and *N*-isobutytyl-L-cysteine (NIBLC) is used which leads to more stable derivatives and

due to the separation of the derivatives straight off the derivatization procedure, no degradation of the derivatives was observed.

To confirm the products being formed by the derivatization procedure with OPA/NIBLC and being stable throughout the run, all derivatives were analyzed by means of an ESI-ion-trap mass spectrometer. Instead of sodium acetate, ammonium acetate was used to meet the requirements of a volatile mobile phase for LC/MS. The m/z found for each individual derivative, corresponds to the expected $[M+H]^+ \pm 0.5$. Due to the two amino groups, lysine and ornithine are derivatized twice, which explains why they are the most retained compounds on reversed phase columns.

To further ensure the identity of the derivatives, fragmentation was applied to all AAs. The derivatives were cleaved into NIBLC without the sulfur (m/z 158.2) and a second fragment that is composed of OPA, the AA and the sulfur (Figure 2). Besides the expected products no other compounds were detected for any AA, indicating that the OPA labeled AAs are stable throughout the time of HPLC separation.

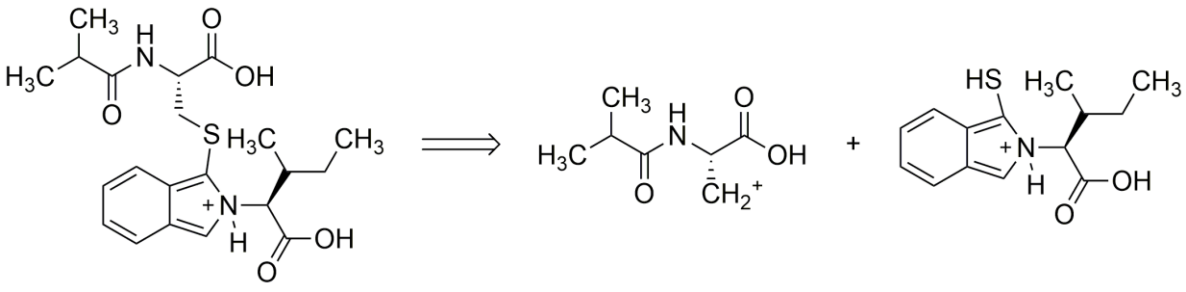



**Figure 2:** Fragmentation of derivatives on the example of L-isoleucine

The ratio of OPA and the thiol was varied in the range of 1:10 to 10:1. As long as the thiol and OPA are present in the excess needed, the ratio has no effect either on the degree of derivatization or the stability. A possible racemization of the AAs during derivatization or the HPLC run is considered as unlikely due to the mild conditions of the process. Additionally, no reports in literature have been found that describe such thing.

### *3.2. Optimization of the In-Needle Derivatization Procedure*

The injection program consists of four parts. Washing the needle, drawing reagent and sample solution in the needle, waiting for the reaction to complete and injecting the mixture into the HPLC system. The order of these steps has great impact on the degree of derivatization and the carry-over effects which were observed. To optimize the in-needle derivatization procedure, parameters like amount and drawing order of derivatization solution, pH value of the derivatization solution, and hold time for the reaction were varied. Isoleucine was chosen as a representative AA. 2 µl of a solution of 5 mM DL-isoleucine were derivatized (OPA/NIBLC), injected and the peak area due to L- and D-isoleucine was monitored.

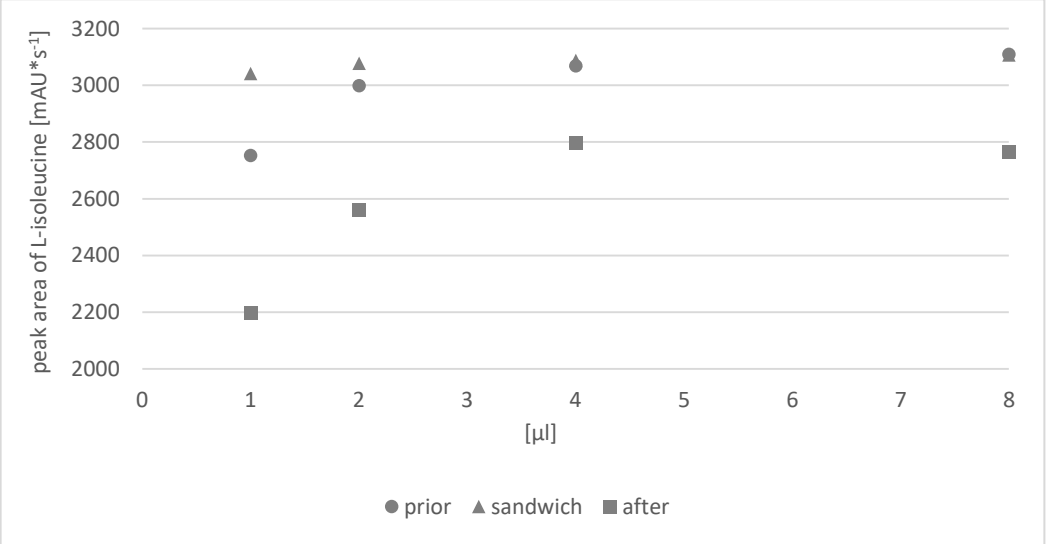
Results of the variations of the procedure are described below:

1. Drawing order: The sequence of drawing the samples and the derivatization reagents was evaluated. It is possible to draw different volumes of the OPA-derivatization solution prior to the sample, after the sample and both, prior and after the sample (sandwich). Figure 3a shows, that drawing 2 µl OPA-derivatization solutions either as a sandwich or 4 µl before the sample is most effective and gives the maximum degree of derivatization. Drawing the derivatization solution after the sample only results in a 10 – 40 % reduction of the peak area.
2. Mixing steps: One mixing step is performed by the movement of the plunger of the autosampler back and forth. If the derivatization solutions are drawn as a sandwich, the highest degree of derivatization is achieved without any mixing step. If the derivatization solution is drawn before the sample, one mixing step is necessary. Of note, more mixing steps result in less response (Figure 3b).
3. Position of the needle: The mixing can take place while the needle is withdrawn from the sample vial or in the needle seat; the degree of derivatization is higher with the needle withdrawn from the sample vial.
4. pH value: Higher pH values increase the reaction rate. A pH value of 10.0 was found to be adequate for a short reaction time (Figure 3c).
5. Halt time: If the derivatization solutions are drawn as sandwich, the degree of derivatization is not dependent on the halt time. When the derivatization solution is drawn prior to the sample only, a halt time of one minute is required (Figure 3d).

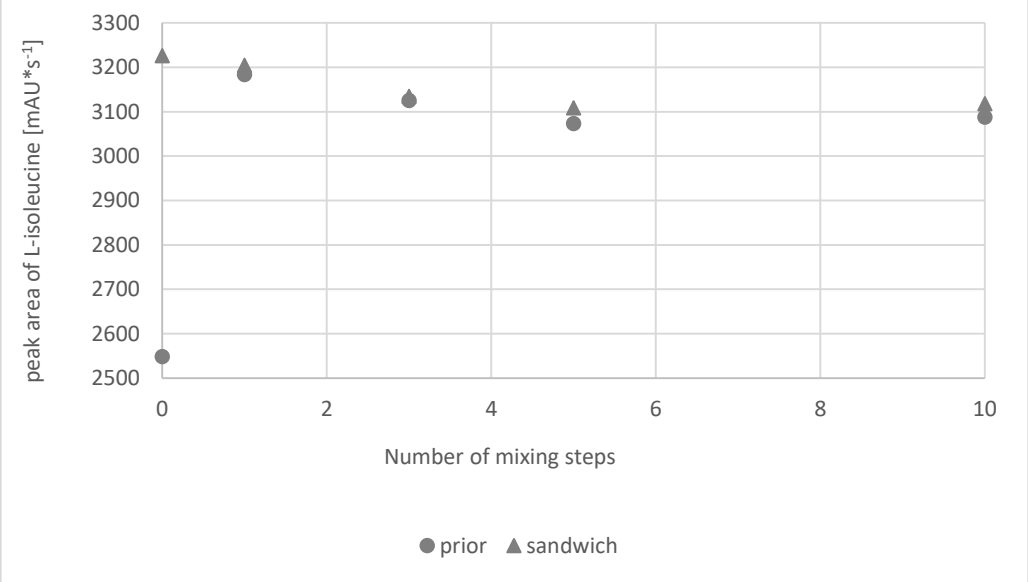
For a better clarity, figure 3a-d display the data of L-isoleucine only. However, the results for D-isoleucine were identical.

Based on these results, the most effective derivatization program is a sandwich of drawing 2 µl of the derivatization solution, 2 µl of the sample solution and 2 µl of the derivatization solution and no mixing step. Although not necessary in case of a sandwich injection, the halt time was set to 30 sec to ensure an effective derivatization for both enantiomers of the different AAs. Because of this and the fast reaction rate of the OPA derivatization in general, no kinetic resolution effects are expected.

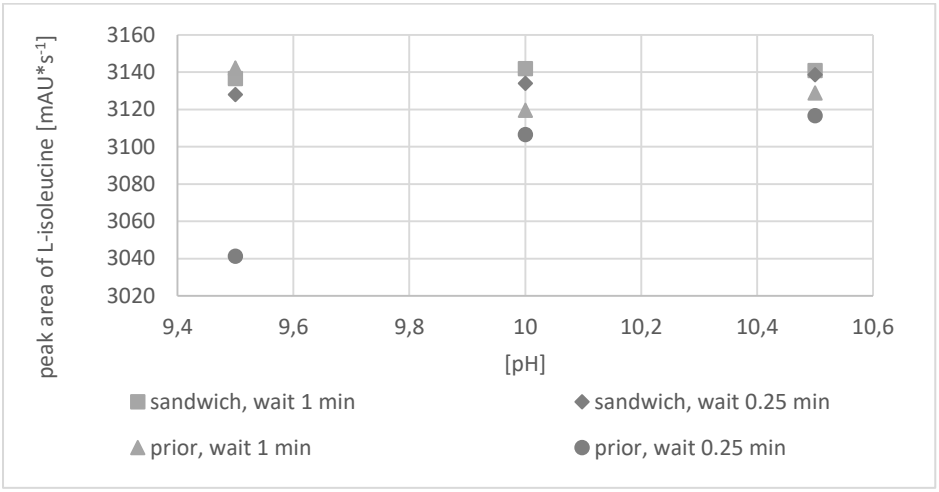
a)




b)



c)




d)

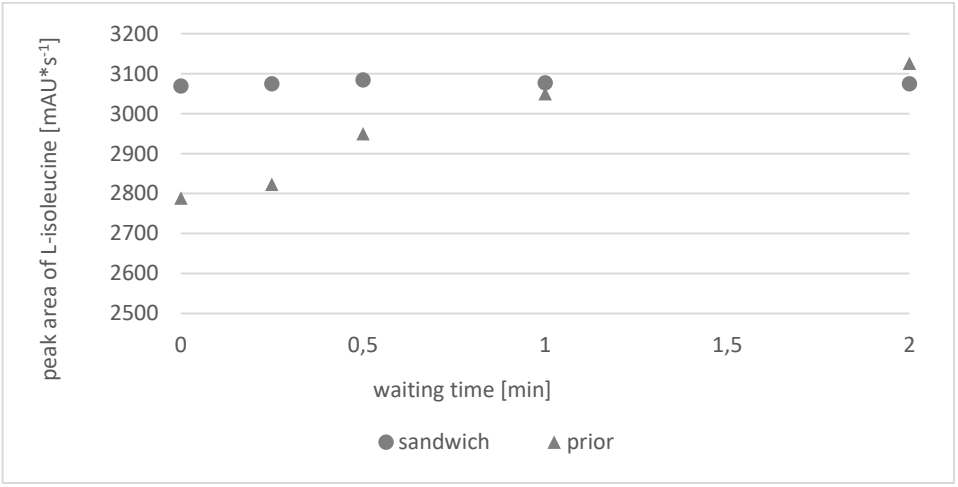



**Figure 3:** Effect of various parameters on derivatization of DL-isoleucine with OPA and NIBLC. a) amount and drawing order of derivatization solution, b) number of mixing steps, c) pH Value of derivatization solution, d) halt time; prior: 4 µl of derivatization solutions is drawn before the sample; sandwich: 2 µl of derivatization solution is drawn before and after the sample.

In order to check whether this procedure converts the AAs quantitatively to the derivatives, tryptophan was chosen as a test AA because of its fluorophore in an underivatized state. Hence, any residues from the derivatization can be detected with good sensitivity (excitation 280 nm, emission 360 nm). A solution of 5 mM tryptophan was derivatized, injected and the peak area of residual underivatized tryptophan determined. The peak was quantified by means of an external standard of tryptophan with a concentration of 0.1 mM without any derivatization to calculate the effectiveness of the procedure (n=3): the aforementioned derivatization program for UV revealed > 99.9 % derivatization and the

derivatization program for fluorescence detection (see section 3.3) > 99.5 % derivatization, indicating a sufficient conversion with the derivatization reagent in both cases.

An analogous procedure was used for the AAs methionine and phenylalanine, which can be detected by UV at 210 nm. In both cases, the residual non-derivatized AA was below the limit of quantification.

### *3.3. Carry-Over Effects*

To study the observed carry-over effects, the derivatization program was repeated 20 times with a mixture of 20 AAs as sample and the column removed from the HPLC system. Afterwards, the column was reinstalled, a blank solution was injected and the extend of carry-over effects was evaluated. Even though the derivatization scheme was simple and effective, carry-over effects were observed upon fluorescence detection.

The carry-over effects might be explained by small drops of the corresponding solutions adhering outside the needle when the solutions are drawn and released and the containments were carried from one vial to the next. To overcome this problem, several needle washing procedures with buffer before and between the drawing steps were applied, but resulted either in reduced derivatization or in carry-over-effects, albeit smaller. Alternatively, the needle was washed at the beginning and the derivatization solution was only drawn prior to the sample to avoid contamination of the derivatization with the sample solution. This program reduced the carry-over effects, while the degree of derivatization was reduced by less than 1 %. However same carry-over effects not related to the vials were still observed.

These were caused by residues of the sample inside the switching valve of the autosampler; it could be resolved by valve switching following the subsequent procedure: The needle is washed three times in a mixture of 20 mM borate buffer (pH=10.0) and methanol 70:30 % v/v. Then, 4 µl of derivatization solution, 2 µl of sample solution and 2 µl of 20 mM borate buffer (pH = 10.0) is drawn subsequently and mixed once with the needle withdrawn from the sample vial. The program is halted for one minute and the contents of the needle is injected into the HPLC system. Then, the valve of the autosampler is switched to bypass, the

program is halted for 15 seconds, the valve is switched to mainpass, to bypass and back to mainpass again.

Even though, this procedure reduced the carry-over effects to an acceptable level, it is recommended to replace wash solution, derivatization solution and borate buffer at least once a day.

Interestingly, the carry-over effects were observed upon fluorescence detection only and hence this derivatization program is applied, when fluorescence detection is used. Due to the lower sensitivity of the UV detector, the derivatization program described in section 3.2 is sufficient for UV spectrophotometric detection when the derivatization solution is replaced regularly.

### *3.4. Indirect Enantioseparation of DL-AAs*

To find optimal chromatographic conditions for the enantioseparation of each derivatized DL-AA, different columns (C18, pentafluorophenyl (PFP), phenylhexyl), organic modifiers (acetonitrile, methanol), thiols, gradient profiles, and pH values of mobile phase (3.0 – 6.0) were evaluated. It is aimed to develop one method which is capable of assessing the enantiomeric purity of all individual AAs except proline, which lacks the primary amino group necessary for the OPA derivatization.

NAC and NIBLC as chiral thiol reagent are ideal for the separation of the obtained diastereomers in terms of resolutions with the latter being higher in almost all cases.

In general, reversed phase columns are well-suited for the separation of the derivatives of one distinct AAs due to the good resolutions that were observed throughout (see Table 1). The best results were obtained with a PFP column, because resolutions were found to be higher with this column than with the C18 and phenylhexyl columns.

For the PFP column, the impact of organic solvent of the mobile phase, the temperature, and the flow rate on the resolutions were studied. Acetonitrile was found to be more suitable than methanol due to a higher resolution and a lower back pressure observed. The column temperature was varied between 10 to 30 °C in 5 °C steps, but the effect on the resolution between the derivatives was found to be negligible. This also applied to the flow

rate which was varied between 0.60 ml·min$^{-1}$ to 0.90 ml·min$^{-1}$ in 0.15 ml·min$^{-1}$ steps. Depending on the pH value of the mobile phase, the gradient profile had to be adjusted due to the significant change of hydrophobicity of the derivatives.

In Table 1 the resolutions between the peaks of the two diastereomers for the individual derivatized AA achieved with different conditions are shown. The optimal gradient profile and pH value of the mobile phase for each AA varies slightly. Using the PFP column, a 50 mM aqueous sodium acetate buffer at pH = 5.0, an acetonitrile gradient, and NIBLC as thiol reagent, the best overall results in terms of resolution between the derivatives of single AAs were found. This method was used for the evaluation of enantiomeric purity of individual AAs and validation parameters. The elution order for all AAs was found to be L before the D derivative with this method.

If UV spectrophotometric detection is used, the excess of the OPA reagent may interfere with the quantification of the minor diastereomer due to coelution. In this case, *N*-isobutyryl-D-cysteine (NIBDC) can be used to reverse the elution order of the derivatives.

The chromatogram of cysteine showed three peaks with UV spectrophotometric detection with a relative low response. The third one was probably due to a side reaction of OPA with the amine moiety of one cysteine molecule and the thiol moiety of another cysteine molecule. Hence, cysteine cannot be directly analyzed with this method. The problem with the derivatization of cysteine has been reported previously and a possible solution is the alkylation of the free thiol moiety with iodoacetic acid.[30,31] Since iodoacetic acid would also alkylate the free thiol group of NIBLC, it cannot be used with automated derivatization.

Therefore, this method excludes the evaluation of cysteine, the achiral AA glycine and proline with its secondary amine from the 20 proteinogenic AAs.

To demonstrate the applicability of this method for the evaluation of enantiomeric purity, 17 different L-AAs were injected and the amount of D-AA by means of area normalization was determined (*Table 2*). The method described previously with UV spectrophotometric detections was used. The content of the minor enantiomer is ranging between not detectable and 0.06 % for most AAs. Serine, leucine, and lysine show higher amounts up to 4.29 %. However, it has to be considered, that the amount of the minor enantiomer is dependent on factors like storage and origin or synthesis.

solutions between the peaks due to the two derivatized enantiomers of one specific AA at a time obtained with different columns,
e mobile phase. Resolutions lower than 0.5 are marked with "-"

| | Phenylhexyl | | | | PFP | | | | C18 | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | NAC | | NIBLC | | NAC | | NIBLC | | NAC | | NIBL | |
| | pH 3.5 | pH 5.0 | pH 3.5 | pH 5.0 | pH 3.5 | pH 5.0 | pH 3.5 | pH 5.0 | pH 3.5 | pH 5.0 | pH 3.5 | |
| | 1.10 | - | 2.98 | 3.29 | 0.89 | 1.02 | 2.87 | 3.77 | 0.65 | - | 2.46 | |
| | 2.67 | 3.23 | 2.68 | 3.59 | 3.00 | 3.49 | 2.48 | 5.54 | 1.67 | 3.13 | 2.43 | |
| **e** | 0.88 | - | 2.96 | 3.02 | 0.64 | - | 2.87 | 5.27 | 0.70 | 0.86 | 2.71 | |
| **cid** | 0.80 | 2.47 | 2.64 | 1.19 | 0.55 | 4.56 | 3.25 | 3.13 | 0.66 | 1.85 | 3.11 | |
| | 2.06 | 2.08 | 2.79 | 2.78 | 1.86 | 3.01 | 1.92 | 4.98 | 1.23 | 2.31 | 2.52 | |
| **cid** | 2.50 | 1.33 | 3.31 | 2.55 | 2.51 | 1.76 | 4.43 | 4.69 | 1.69 | 1.25 | 3.65 | |
| | - | - | 1.71 | 0.81 | 1.69 | - | 2.74 | 1.78 | 1.06 | - | 1.78 | |
| | 7.44 | 5.15 | 8.99 | 6.24 | 3.93 | 5.02 | 7.11 | 7.69 | 4.65 | 3.53 | 7.52 | |
| | 4.03 | 0.89 | 6.17 | 3.55 | 2.83 | 1.40 | 5.87 | 5.68 | 2.88 | 0.94 | 5.98 | |
| | 1.45 | 0.98 | 2.43 | 1.34 | 1.20 | 0.56 | 1.30 | 1.77 | 0.86 | 0.57 | 2.49 | |
| **e** | 3.71 | 0.61 | 6.81 | 3.72 | 3.08 | 0.97 | 6.69 | 5.55 | 2.86 | 0.74 | 5.30 | |
| **hine** | 3.43 | 1.15 | 4.70 | 2.00 | 2.30 | 0.49 | 3.03 | 1.96 | 2.65 | - | 4.32 | |
| | 0.77 | - | 2.06 | 2.41 | 0.58 | 1.37 | 2.62 | 4.02 | 0.63 | - | 2.24 | |
| | - | 2.52 | 2.53 | 1.95 | 1.11 | 2.95 | 2.77 | 2.85 | 1.25 | 1.48 | 2.70 | |
| **n** | 4.88 | 1.34 | 6.78 | 3.82 | 2.81 | 1.74 | 5.74 | 4.15 | 3.50 | 1.37 | 6.22 | |
| | 2.99 | 2.35 | 3.65 | 2.36 | 2.33 | 2.39 | 3.06 | 2.86 | 2.26 | 3.02 | 3.05 | |
| | 5.10 | 4.26 | 6.58 | 5.15 | 3.20 | 4.88 | 5.94 | 6.58 | 3.72 | 3.74 | 5.30 | |

**aphic conditions:**
: 20 °C; detection UV at 345 nm; derivatization program for UV; 5 mM AA; all % are % v/v
Flow 1 ml·min$^{-1}$; Mobile Phase: NAC/pH=3.5: A: 50 mM sodium formiate, B: ACN; 0 to 5 min: 25 % B; 5 to 30 min: 25 – 35 % B; NAC/pH=5.0: A: 50 m
CN; 0 to 5 min: 10 % B; 5 to 30 min: 10 – 35 % B; NIBLC/pH=3.5: A: 50 mM sodium formiate, B: ACN; 0 to 5 min: 25 % B; 5 to 40 min: 25 – 45 % B; NIB
dium acetate, B: ACN; 0 to 5 min: 10 % B; 5 to 30 min: 10 – 35 % B;
Flow 0.4 ml·min$^{-1}$; Mobile Phase: NAC/pH=3.5: A: 50 mM sodium formiate, B: ACN; 0 to 5 min: 20 % B; 5 to 30 min: 20 – 35 % B; NAC/pH=5.0: A: 50 m
CN; 0 to 5 min: 5 % B; 5 to 30 min: 5 – 25 % B; NIBLC/pH=3.5: A: 50 mM sodium formiate, B: ACN; 0 to 5 min: 10 % B; 5 to 30 min: 10 – 35 % B; NIB
dium acetate, B: ACN; 0 to 5 min: 5 % B; 5 to 30 min: 5 – 25 % B;
Flow 0.75 ml·min$^{-1}$; Mobile Phase: NAC/pH=3.5: A: 50 mM sodium formiate, B: ACN; 0 to 5 min: 10 % B; 5 to 35 min: 10 – 40 % B; NAC/pH=5.0
ate, B: ACN; 0 to 5 min: 5 % B; 5 to 30 min: 5 – 35 % B; NIBLC/pH=3.5: A: 50 mM sodium formiate, B: ACN; 0 to 5 min: 20 % B; 5 to 40 min: 20
0: A: 50 mM sodium acetate, B: ACN; 0 to 5 min: 5 % B; 5 to 35 min: 5 – 35 % B;

**Table 2:** Evaluation of enantiomeric purity for 17 L-AAs. Percentage content is given by means of area normalization. n.q.= not quantifiable; n.d. = not detectable

| L-AA | D-AA [%] |
|---|---|
| **alanine** | 0.06 |
| **arginine** | 0.05 |
| **asparagine** | 0.05 |
| **aspartic acid** | 0.04 |
| **glutamine** | 0.17 |
| **glutamic acid** | 0.04 |
| **histidine** | N.D. |
| **isoleucine** | N.Q. |
| **leucine** | 1.05 |
| **lysine** | 4.29 |
| **methionine** | 0.05 |
| **phenylalanine** | N.D. |
| **serine** | 0.39 |
| **threonine** | N.D. |
| **tryptophan** | N.D. |
| **tyrosine** | N.D. |
| **valine** | 0.06 |

### *3.5. Comparison of fluorescence and UV spectrophotometric detection*

The excitation and emission wavelength for OPA-AAs differ in literature.[22,25,32-35] The values for the excitation wavelength varies in between 229 to 240 nm and 330 to 360 nm. For the emission wavelength, the values are within 418 to 455 nm. This might be explained by the influence of different mobile phases, AAs and thiols on excitation and emission maxima of the derivatives. To achieve the highest sensitivity for this method with both detection modes, the optimal detector settings were determined experimentally by recording spectra with the fluorescence detector of the HPLC system. Excitation was found to be optimal at 233 and 345 nm and emission at 445 nm. Excitation at 233 nm gives a higher signal, but using 345 nm as the excitation wavelength is more suitable due to the lower noise and higher specificity for the OPA-derivatives. For UV spectrophotometric detection, 345 nm as detection wavelength was used for the same reasons.

For the determination of the limit of detection (LOD) and quantification (LOQ), the signal-to-noise levels of L-AAs at low concentration (0.1 µM for fluorescence detection and 10 µM for UV/Vis detection) were first evaluated and then the theoretical LOD (signal-to-noise level of 3) and LOQ (signal-to-noise level of 10) calculated. The limit of quantification for fluorescence detection varies in the range of 0.02 – 0.03 µM (equals to 40 – 60 fmol actually injected amount of AA) for both excitation wavelengths ($\lambda_{ex}$ = 345 and $\lambda_{ex}$ = 233). At a concentration of the sample of 5 mM, the LOQ expressed in percentage units is 0.002 % and the LOD is 0.0006 % (0.009 µM). For UV spectrophotometric detection on the other hand, the limit of quantification is 1.5 – 2.0 µM (3 – 4 pmol AA). Due to the significantly higher baseline noise that is present in comparison to 345 nm in the UV-spectrum, the LOQ does not benefit from the higher signal intensity at 233 nm. At a concentration of the sample of 5 mM, the LOQ expressed in percentage units is 0.04 % and the LOD is 0.012 % (0.6 µM). As expected UV spectrophotometric detection is about 50-100 times less sensitive than fluorescence detection, but does still provide the desired sensitivity for the method and is therefore used for validation.

As described previously,[25-27] the limit of quantification and detection is also strongly dependent on the purity of the reagents and solvents used for the derivatization solution, which may be contaminated with compounds that have a free amine moiety. Therefore, peaks can be present in the chromatogram of a blank, possibly interfering with the quantification of coeluting impurities at a low level. Therefore, the blank value of the reagent has to be taken into consideration and the content of AAs in samples has to be corrected accordingly if necessary.

Moreover, the LOQ for the evaluation of enantiomeric purity is also determined by the optical purity of the CDA, which is 100.0 % according to the certificate of analysis of the manufacture. However, the derivatization of the purest L-methionine found revealed the content of D-methionine to be 0.005 %. It is not possible to decide whether this is related to an actual amount of 0.005 % D-methionine present in L-methionine or due to the limited purity of the chiral agent, which would lead to a derivative consisting of L-methionine and NIBDC. Since both derivatives are enantiomeric to each other, they are not separated with reversed phase chromatography and therefore cannot be distinguished. Thus, it must be assumed that the optical purity is not more than 99.995 %. This means, that the theoretical LOQ with fluorescence detection of 0.002 % cannot be reached and the method does not

benefit from the higher sensitivity of a fluorescence detector due to practical limitations. However, 0.04 %, which is the LOQ of UV spectrophotometric detection is possible. For this reason, UV detection is used further on. It has to be noted, that the optical purity of the CDA has to be determined again, when a new lot is being used.

### *3.6. Validation Parameters*

#### *3.6.1. LOD, LOQ and Linearity*

LOQ and LOQ were determined in chapter 3.5. The LOD was found to be 0.012 % and the LOQ 0.04 %.

The linearity of the method was tested by injecting the 17 different L-AAs at different concentrations in triplicate in the range from 2.0 µM to 50 µM (0.04 – 1.00 %, 6 data points) with UV spectrophotometric detection. The coefficient of determination obtained by linear regression was higher than 0.999 in all cases.

#### *3.6.2. Precision*

The precision of the enantiomeric purity assessment tests that were performed with the AAs phenylalanine, methionine and tryptophan (see Table 3) varies between 1.7 and 8.2 %. For the determination of impurities close to the limit of quantification, this is considered acceptable and the method is precise.

To evaluate the precision of the automated derivatization procedure itself, the chemically different DL-AAs isoleucine, phenylalanine, lysine, methionine and glutamic acid were chosen. They were injected six times with the derivatization program for UV and FLD at a concentration of 5 mM. The relative standard deviation for the area was not higher than 0.89 %, indicating that the online-derivatization is precise.

### *3.6.3. Recovery*

To determine the recovery of the enantiomeric impurity, 5 mM of the L-enantiomers of the AAs methionine, phenylalanine and tryptophan respectively were spiked with its corresponding D-enantiomer at a concentration of 2 µM (0.04 %) and *vice versa*. A PFP column, OPA/NIBLC as derivatization reagent and a 50 mM sodium acetate buffer pH=5.0 were used (see *Table 1*). Quantification was performed by means of an external standard at the same concentration. In order to correct for impurities naturally present in the AA or the impurities present in the derivatization solution, the peak area was corrected by the formula $P_c = P_s - P_n$, where $P_c$ is the corrected peak area of the derivative to be determined, $P_s$ is the peak area of the derivative to be determined of a spiked solution and $P_n$ is the peak area of the derivative to be determined of a non-spiked solution. The recovery is shown in *Table 3*. It varies between 92.3 and 113.3 %, which is considered to be acceptable for impurities close to the limit of quantification. In Figure 4, the chromatogram of the separation of 0.04 % D-methionine from L-methionine is shown as an example.

**Table 3:** Recovery of the minor enantiomer of AAs with UV spectrophotometric detection, each AA was spiked with 2.0 µM (0.04 %) of its corresponding enantiomer.

| AA | Measured concentration [µM] | Recovery [%] | RSD [%] |
|---|---|---|---|
| **D-phenylalanine** | 1.86 ± 0.09 | 92.8 | 4.6 |
| **L-phenylalanine** | 2.17 ± 0.08 | 108.3 | 4.2 |
| **D-methionine** | 2.19 ± 0.16 | 109.6 | 8.2 |
| **L-methionine** | 1.93 ± 0.03 | 96.4 | 1.7 |
| **D-tryptophan** | 2.15 ± 0.12 | 107.5 | 5.7 |
| **L-tryptophan** | 2.27 ± 0.12 | 113.3 | 5.8 |

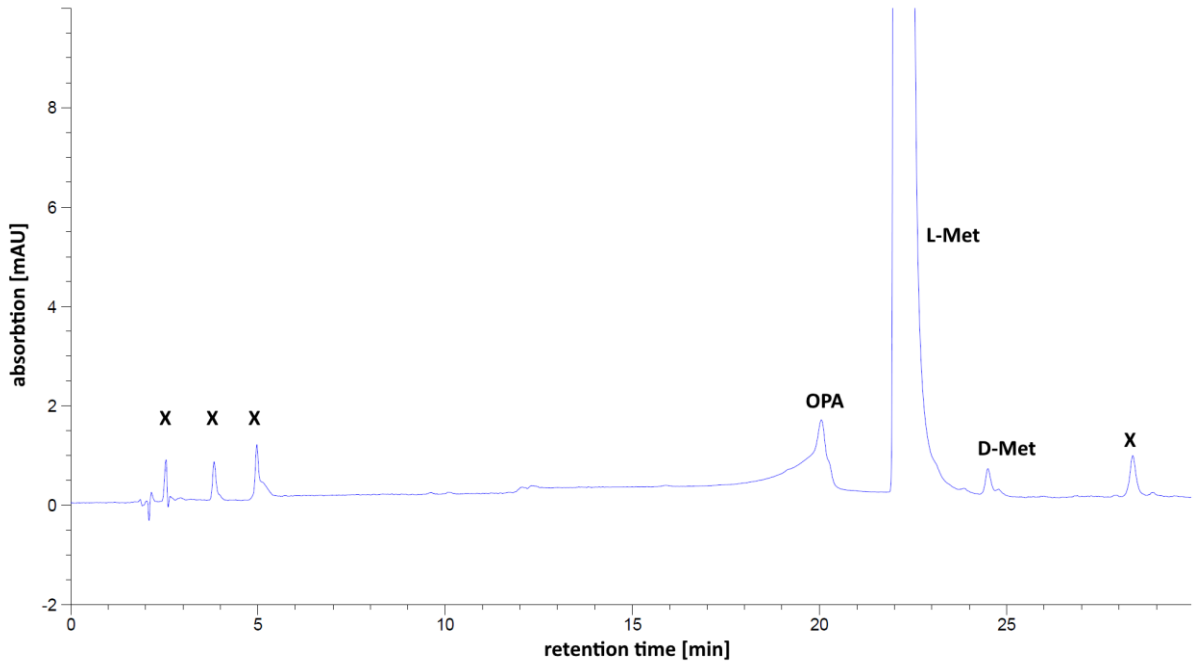



**Figure 4:** Separation of 0.04 % D-methionine from L-methionine. The excess of the OPA-reagent is also detected. x: peaks from the blank solution; Chromatographic conditions: Temp.: 20 °C; Flow: 0.75 ml·min$^{-1}$; mobile phase A: 50 mM sodium acetate (pH: 5.00), mobile phase B: acetonitrile; 0 to 5 min: 5 % B, 5 to 35 min: 5 to 35 % B, UV spectrophotometric detection at 345 nm.

## 4. Conclusions

The aim of this study was to establish methods for the assessment of enantiomeric purity of a single AA. The procedure for the OPA-derivatization of AAs, taking place inside the needle of the autosampler, transforms the AA quantitatively into its derivatives and eludes the issue of poor stability of the derivatives. The derivatization is fast, can be automated and needs only very small amounts of sample and derivatization reagents compared to an offline derivatization or an online derivatization that takes place in a reaction vial. In addition, the proposed method is more sensitive and specific that the test for optical rotation described in the European Pharmacopoeia. The type of autosampler used in this study is quite common nowadays. Therefore, this procedure should be possible with a large number of HPLC instruments.

As shown in previous publications,[20-24] the amounts of L- and D-AAs in plants, peptidic antibiotics, toxins and drugs can be detected with fluorescence detection down to 1-2 pmol

(equivalent to an amount of 0.1 % due to the lower sample concentration that was used by the authors). Other publications focus on enantioseparation of a subset of all proteinogenic AAs or non-proteinogenic AA in various matrixes. Here, the assessment of enantiomeric purity down to a level of 0.04 % of the minor enantiomer can be easily achieved with common UV spectrophotometric detection and it is shown that indirect enantioseparation is feasible for 17 proteinogenic AA with the same method within 35 min. All in all, this method provides a better sensitivity for the minor enantiomer with a simpler instrument setup than other publications and is directed to evaluate the enantiomeric purity of proteinogenic AAs, that are used for pharmaceutical purposes.

**Conflict of Interest**

The authors declare no conflict of interest.

**Acknowledgment**

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# 4. Development of a Method for Related Substances of Amino Acids

## 1. Introduction

In current monographs of the European Pharmacopoeia [1] amino acid analysis (AAA) is utilized for the control of related substances of amino acids (AAs) [2]. This is done by using cation ion exchange columns and postcolumn ninhydrin derivatization for alanine, arginine, cysteine, glycine, isoleucine, leucine, lysine, phenylalanine, proline, serine, threonine, tryptophan, tyrosine, and valine. In the monographs of asparagine, aspartic acid, glutamic acid and histidine thin-layer-chromatography is still applied. Since ninhydrin derivatization only works for compounds with a free amine and carboxylate moiety, non-AAs cannot be detected. In the case, the AA are accompanied by non-AA impurities, reversed phase chromatography is used instead (methionine) or additionally (glycine and tryptophan).

The aim of this study was to test whether the precolumn derivatization with OPA, that was applied for the evaluation of enantiomeric purity with HPLC described in [3], can also be used for the evaluation of related substances of AAs instead of the AAA. As it is the case of AAA, only impurities with an amine can be detected with this method.

## 2. Experimental

### *2.1. Apparatus*

The apparatus was the same as described in [3].

### *2.2. Chemicals and Materials*

Chemical and materials were the same as described in [3].

### *2.3. Preparation of Solutions*

Preparation of the derivatization solution and the mobile phase are described in [3].

## 3. Results and Discussion

### *3.1. Separation of a Mixture of Derivatized L-AAs*

The aim of this study was to find generic chromatographic conditions that are well suited for the separation of OPA-labeled L-AAs on reversed phase columns. However, several methods for the separation of OPA-labeled L-AAs have been described already [4-8]. Due to the challenging task to separate about 20 substances in one run, the sensitiveness of these substances to changes in chromatographic conditions, and the lot-to-lot consistency of columns, the robustness of these methods is poor and the results were found to be difficult to reproduce in our lab.

For method development, the impact on type of column, pH value of the mobile phase, gradient steepness, and different thiol reagents for the derivatization on a mixture of 22 L-AAs were evaluated. These values were optimized for best selectivity, which means to achieve a baseline separation of as much AA-derivatives as possible.

1. The pH of the mobile phase was varied in the range of 3.0 – 6.0 in 0.5 steps. Using a low pH leads to a poor selectivity and bad peak shape on all columns tested. The best results in terms of resolution and peak shape for the separation of L-AA derivatives were achieved with a pH of 5.5 and 6.0. For the phenylhexyl column, a pH of 5.75 was additionally tested and gave the best results.
2. Due to the big differences in lipophilicity of the OPA derivatives of the early eluting aspartic acid and the late eluting lysine, a gradient elution is indispensable. In general, the gradient starts with an isocratic step of about 7 % v/v acetonitrile for 5 minutes, followed by a flat gradient to about 30 % v/v acetonitrile within 30 to 40 minutes, and with another isocratic step for 5 minutes. The exact profile has to be adjusted for each column tested to improve separation. Methanol was found to be inferior to acetonitrile because less derivatives were separated with a resolution more than 1.5.

3. The phenylhexyl and pentafluorophenyl column were found to be suitable for the separation, whereas the C18 column could separate less derivatives. Since the results with the phenylhexyl column looked most promising, it was used for further study.
4. Since the variation of the column temperature in case of the phenylhexyl column between 10 to 40 °C was negligible, 20 °C was applied throughout.
5. The flow rate had to be adjusted depending on the dimensions and particle size of the column being used for the backpressure to be within the specifications of the HPLC system and the total run time of the method to be less than 45 min. Variations of the flow rate in case of the phenylhexyl column (0.60 to 0.90 mL/min, 0.15 mL/min steps) showed only little changes with regard to resolution, besides the overall runtime.
6. Unlike for the enantioseparation, the derivatives do not have to be diastereomers, thus the application of 3-mercaptopropionic acid (MPA) is also feasible. However, the selectivity is increased from MPA to *N*-acetyl-L-cysteine (NAC) and to *N*-isobutyryl-L-cysteine (NIBLC), independently from column and other parameters.

In Figure 1 the separation of 20 L-AAs is shown with the best parameters found: The AAs labelled with OPA/NIBLC were separated on a Phenylhexyl column using a gradient with mobile phase A: 50 mM sodium acetate (pH= 5.75) and mobile phase B: acetonitrile; gradient program: 0 to 5 minutes: 7 % v/v mobile phase B; 5 to 30 minutes: 7 to 25 % v/v mobile phase B; 30 to 40 minutes: 25 to 30 % v/v mobile phase B, 40 to 45 minutes: 30 % v/v mobile phase B. The flow was set to 0.75 mL/min and the column temperature to 20 °C. The separation is strongly dependent on the pH value of mobile phase A and the gradient steepness. This method can be used for the determination of the AA impurities that are given in the European Pharmacopoeia in the corresponding monographs of an AA, which are covered by means of amino acid analysis (AAA). However, since AAs can also contain various types of non-AA impurities alongside with oxidation product, a full coverage of all impurities is not possible with this method. Some might not have an amino group and are therefore not detected. If a different reversed phase column is being used, a reoptimization of the gradient profile may be necessary and the flow rate has to be adjusted to a value that is suitable for the dimensions of the column. However, the remaining chromatographic conditions should remain unchanged.

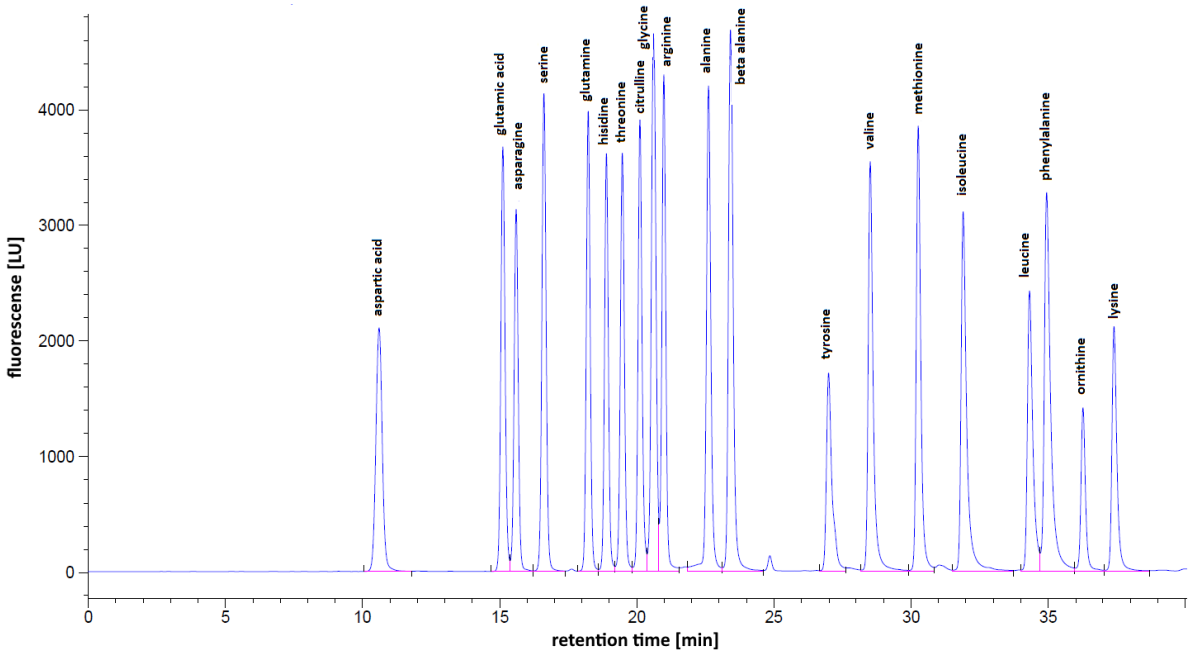


**Figure 1:** Separation of 20 derivatized L-AAs with OPA/NIBLC; derivatization program for FLD; column: phenylhexyl; chromatographic conditions: Temp.: 20 °C; Flow: 0.75 mL/min; mobile phase A: 50 mM sodium acetate (pH: 5.75), mobile phase B: acetonitrile; 0 to 5 min: 7 % B, 5 to 30 min: 7 to 25 % B, 30 to 40 min: 25 to 30 % B, 40 to 45 min: 30 % B; excitation: 345 nm, emission: 445 nm;

Tryptophan was excluded in the chromatogram, because it more or less coelutes with leucine and phenylalanine. For impurity profiling it is sufficient to separate tryptophan from its putative impurities. Since leucine and phenylalanine are no impurities of tryptophan the coleution is not important.

As an example of impurity profiling, the separation of phenylalanine from its impurities leucine, valine, methionine and tyrosine is given in Figure 2 with the aforementioned conditions. The shoulder of the peak due to L-phenylalanine seems to be related to the high concentration of the main compound, which can partly be seen with various AAs depending on the chromatographic conditions. Tailing can also be observed. However, this does not interfere with the quantification of impurities. If quantification of L-phenylalanine is desired, a diluted solution is necessary.

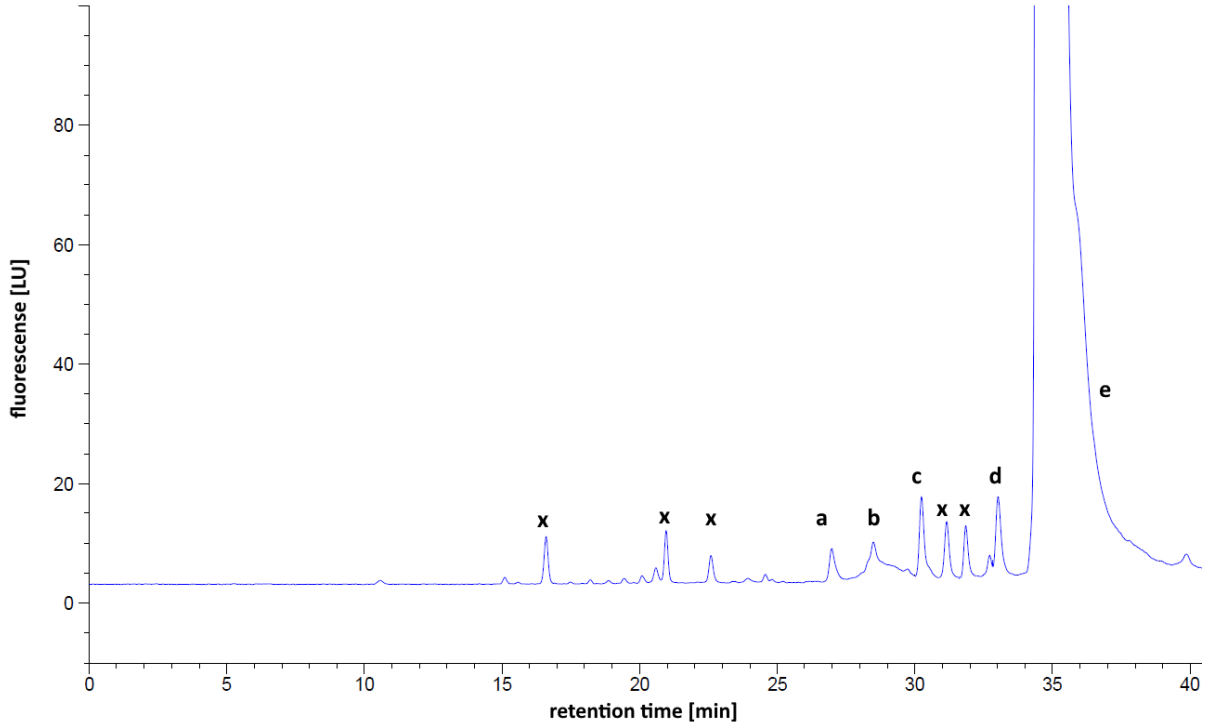


**Figure 2:** Chromatogram showing impurity profiling of 5 mM L-phenylalanine (e) spiked with 0.02 % of tyrosine (a), valine (b), methionine (c) and leucine (d). The area of the peaks of the AAs were corrected by the area of the corresponding peaks of pure L-phenylalanine. Non-identified impurities are marked with "x". Chromatographic conditions see Figure 1

### *3.2. Validation Parameters*

Validation parameters were evaluated with the method for impurity profiling described in paragraph 3.1 using NIBLC as thiol. The work focuses on recovery, LOD/LOQ and linearity of L-AAs for impurity profiling. To demonstrate the general feasibility of this procedure as an alternative for the AAA used in the European Pharmacopoeia, the precision and accuracy of the determination of impurities at a 0.02 % level in L-phenylalanine was evaluated.

#### *3.2.1. Recovery*

To evaluate recovery for impurity profiling of L-phenylalanine, a sample of 5 mM L-phenylalanine was spiked with its potential impurities L-tyrosine, L-methionine, L-leucine and L-valine at a 0.02 % level (see Figure 2). Quantification was performed by means of external standards. The recovery was in the range of 93.0 to 95.3 % (see Table 1), which is considered acceptable (chromatographic conditions see section 3.5.1; $\lambda_{ex}$ = 345, $\lambda_{em}$ = 445).

### *3.2.2. Limit of Detection/Quantification for Impurity Profiling*

In addition to the results given in [3], the detailed values for limit of quantification and detection is shown in Table 2, for both fluorescence and UV spectrophotometric detection.

**Table 2:** LOD and LOQ for OPA-labeled AAs with fluorescence (FLD) and UV spectrophotometric detection. The LOD is defined as the concentration of a OPA-labeled AA that gives a peak with a signal-to-noise ratio of 3 and the LOQ as the concentration that gives a peak with a signal-to-noise ratio of 10 accordingly. $\lambda_{ex}$=excitation wavelength; $\lambda_{em}$= emission wavelength

| | **LOQ [µM]** | | | **LOD [µM]** | | |
|---|---|---|---|---|---|---|
| | FLD ($\lambda_{em}$ = 445) | | UV | FLD ($\lambda_{em}$ = 445) | | UV |
| | $\lambda_{ex}$ = 345 | $\lambda_{ex}$ = 235 | $\lambda$ = 345 | $\lambda_{ex}$ = 345 | $\lambda_{ex}$ = 235 | $\lambda$ = 345 |
| **alanine** | 0.019 | 0.020 | 1.530 | 0.006 | 0.006 | 0.459 |
| **arginine** | 0.021 | 0.021 | 1.408 | 0.006 | 0.006 | 0.423 |
| **asparagine** | 0.029 | 0.030 | 1.471 | 0.009 | 0.009 | 0.441 |
| **aspartic acid** | 0.027 | 0.027 | 2.345 | 0.008 | 0.008 | 0.704 |
| **beta-alanine** | 0.019 | 0.019 | 1.390 | 0.006 | 0.006 | 0.417 |
| **citrulline** | 0.028 | 0.028 | 1.499 | 0.008 | 0.008 | 0.450 |
| **glutamine** | 0.026 | 0.026 | 1.639 | 0.008 | 0.008 | 0.492 |
| **glutamic acid** | 0.025 | 0.025 | 1.251 | 0.007 | 0.007 | 0.375 |
| **glycine** | 0.023 | 0.023 | 2.028 | 0.007 | 0.007 | 0.609 |
| **histidine** | 0.031 | 0.030 | 1.570 | 0.009 | 0.009 | 0.471 |
| **isoleucine** | 0.020 | 0.020 | 2.407 | 0.006 | 0.006 | 0.722 |
| **leucine** | 0.020 | 0.020 | 2.108 | 0.006 | 0.006 | 0.632 |
| **lysine** | 0.028 | 0.028 | 1.352 | 0.008 | 0.009 | 0.406 |
| **methionine** | 0.020 | 0.021 | 1.686 | 0.006 | 0.006 | 0.506 |
| **ornithine** | 0.029 | 0.030 | 1.396 | 0.009 | 0.009 | 0.419 |
| **phenylalanine** | 0.024 | 0.023 | 2.143 | 0.007 | 0.007 | 0.643 |
| **serine** | 0.018 | 0.019 | 1.217 | 0.005 | 0.006 | 0.365 |
| **threonine** | 0.019 | 0.020 | 1.320 | 0.006 | 0.006 | 0.396 |
| **tryptophan** | 0.024 | 0.023 | 2.194 | 0.007 | 0.007 | 0.658 |
| **tyrosine** | 0.026 | 0.022 | 1.645 | 0.008 | 0.007 | 0.494 |
| **valine** | 0.021 | 0.021 | 2.064 | 0.006 | 0.006 | 0.619 |

### *3.2.3. Linearity*

Table 3 lists the results for evaluation of linearity of OPA derivatives of L-AAs for both, fluorescence (FLD) and UV spectrophotometric detection. Each calibration curve was constructed with 5 data points (n=3). The coefficient of determination is higher than 0.999 in all cases, showing that the method is linear.

**Table 3:** Linear regression for OPA derivatives of AAs. For fluorescence detection, ranging from 0.1 to 2.0 µM and for UV spectrophotometric detection ranging from 2 to 50 µM

| L-AA | FLD | | UV | |
|---|---|---|---|---|
| | regression equation | $R^2$ | regression equation | $R^2$ |
| **alanine** | y = 311.8x - 1.9 | 0.9994 | y = 0.810x - 0.113 | 0.9995 |
| **arginine** | y = 238.3x + 2.3 | 0.9994 | y = 0.752x - 0.536 | 0.9996 |
| **asparagine** | y = 238.7x + 0.6 | 0.9990 | y = 0.856x - 0.701 | 0.9997 |
| **aspartic acid** | y = 263.5x + 1.9 | 0.9993 | y = 0.749x + 0.169 | 0.9992 |
| **beta alanine** | y = 388.9x + 2.9 | 0.9999 | y = 0.941x - 0.233 | 0.9992 |
| **citrulline** | y = 259.8x + 1.9 | 0.9995 | y = 0.837x + 0.020 | 0.9998 |
| **glutamine** | y = 222.1x - 0.3 | 0.9996 | y = 0.684x - 0.252 | 0.9995 |
| **glutamic acid** | y = 294.3x + 6.7 | 0.9991 | y = 0.986x + 0.706 | 0.9998 |
| **glycine** | y = 294.8x - 0.8 | 0.9994 | y = 0.663x - 0.309 | 0.9995 |
| **histidine** | y = 194.9x + 1.7 | 0.9991 | y = 0.682x + 0.320 | 0.9990 |
| **isoleucine** | y = 287.4x - 4.3 | 0.9990 | y = 0.650x - 0.153 | 0.9993 |
| **leucine** | y = 287.9x - 2.3 | 0.9992 | y = 0.736x + 0.253 | 0.9998 |
| **lysine** | y = 136.1x + 3.0 | 0.9992 | y = 1.286x - 0.142 | 0.9994 |
| **methionine** | y = 271.9x + 3.1 | 0.9993 | y = 0.959x - 0.519 | 0.9996 |
| **ornithine** | y = 133.5x + 2.6 | 0.9990 | y = 1.430x - 0.114 | 0.9993 |
| **phenylalanine** | y = 302.7x - 6.8 | 0.9996 | y = 0.907x + 0.038 | 0.9999 |
| **serine** | y = 322.7x – 1.0 | 0.9990 | y = 1.001x - 0.302 | 0.9996 |
| **threonine** | y = 307.5x - 4.8 | 0.9991 | y = 0.838x - 0.039 | 0.9999 |
| **tryptophan** | y = 193.1x - 4.4 | 0.9994 | y = 0.794x - 0.060 | 0.9998 |
| **tyrosine** | y = 215.8x - 5.2 | 0.9996 | y = 0.743x + 0.188 | 0.9993 |
| **valine** | y = 300.4x - 2.1 | 0.9993 | y = 0.753x + 0.503 | 0.9994 |

**Table 1:** Recovery of the AA impurities (1 µM; 0.02 %) in L-phenylalanine (5 mM)

| Impurity | Measured concentration [µM] | Recovery [%] | RSD [%] |
|---|---|---|---|
| **tyrosine** | 0.95 ± 0.04 | 94.5 | 3.9 |
| **valine** | 0.93 ± 0.04 | 93.0 | 4.1 |
| **methionine** | 0.95 ± 0.02 | 95.3 | 2.3 |
| **leucine** | 0.94 ± 0.02 | 93.4 | 1.8 |

## 4. Conclusion

The aim of this study was to establish an alternative method for the amino acid analysis for related substances used in the European Pharmacopoeia. The proposed sensitive method does not need a dedicated expensive instrumentation as is the case for the AAA described in the European Pharmacopoeia. It enables the separation of 20 AAs as the OPA derivatives and is therefore applicable for the evaluation of related substances of most AAs that are monographed in the European Pharmacopoeia. The validation has to be done for each AA separately. It was shown, that the accuracy for impurity profiling of L-phenylalanine is sufficient. The type of autosampler used in this study is quite common nowadays. Therefore, this procedure should be possible with a large number of HPLC instruments. Of note, as it is the case with the AAA, the newly developed method is not suitable for non-AA impurities.

## References

[1] EDQM, European Pharmacopoeia 8.6, in, Strasbourg, 2016.

[2] EDQM, European Pharmacopoeia 8th Edition, 2.2.56. Amino Acid Analysis, Strasbourg, 2016.

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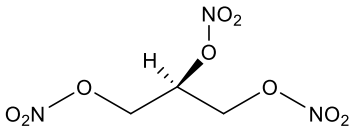
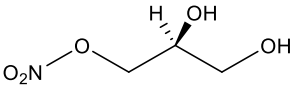
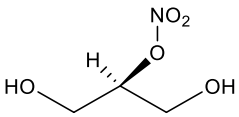
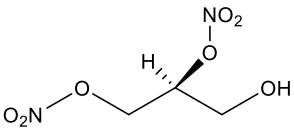
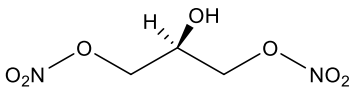
# 5. Impurity Profiling and Assay of Glyceryl Trinitrate Solution

## 1. Introduction

Glyceryl trinitrate (GTN) is a drug that is used to treat angina pectoris and chronic heart failure by liberating nitric oxide causing vasodilatation. The nitrate ester is synthesized by the threefold esterification of glycerin with nitric acid and sulphuric acid [1]. The impurities are nitrate and compounds that are obtained by an incomplete esterification (see Table 1-1).

**Table 1-1:** Glyceryl trinitrate and its known impurities

| Impurity | Impurity-name | Structure | MW |
|---|---|---|---|
| | Glyceryl trinitrate (GTN) |  and enantiomer | 227.09 |
| **A*** | Nitrate |  | 62.00 |
| **B** | 1-Glycerinmononitrate (1-Mononitroglycerin**, 1-GMN) |  and enantiomer | 137.09 |
| **C** | 2-Glycerinmononitrate (2-Mononitroglycerin**, 2-GMN) |  and enantiomer | 137.09 |
| **D** | 1,2-Glycerindinitrate (1,2 Dinitroglycerin**, 1,2-GDN) |  and enantiomer | 182.09 |
| **E** | 1,3-Glycerindinitrate (1,3 Dinitroglycerin**, 1,3-GDN) |  and enantiomer | 182.09 |

*Impurity A as an inorganic nitrate is separated by thin-layer-chromatography and detected with potassium iodide and starch solution and not subject of this HPLC method.

** Labeling by Cerilliant

In Edition 8.6 of the European Pharmacopoeia [2] related substances are evaluated with an isocratic HPLC method [3]. This method lacks sensitivity and selectivity, especially for impurities 1-GMN and 2-GMN. Hence, an improved method is necessary. A method suggested by a manufacture using a reversed phase column (C18, 250x4.6 mm, 5 µm particles) and a methanol-water gradient was tested (see Figure 1-1). While this method is an improvement, there are still several disadvantages. Separation of the impurities 1-GMN and 2-GMN is not sufficient, sensitivity is low (LOQ of 0.1 %), and because of the rising baseline an accurate integration of the peaks due to 1,2-GDN and 1,3-GDN is not possible. A new method was developed in order to overcome these shortcomings.

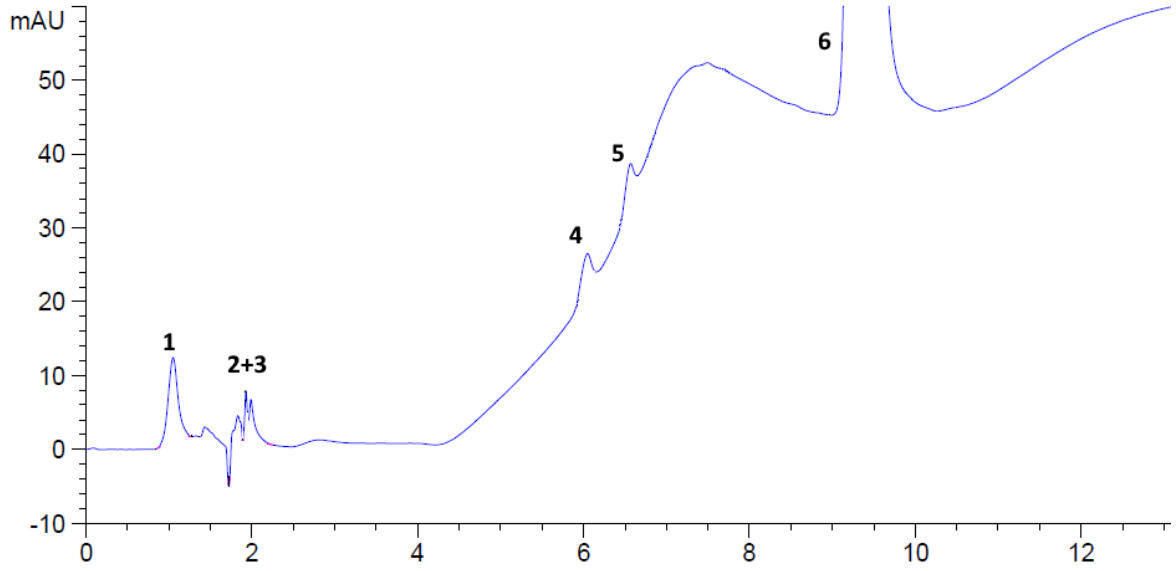



**Figure 1-1:** Chromatogram of a sample of glyceryl trinitrate solution spiked with all impurities (0.3 %) with the method suggested by a manufacture. 1: injection peak; 2: 2-GMN; 3: 1-GMN; 4: 1,3-GDN; 5: 1,2-GDN; 6: GTN

## 2. Experimental

### *2.1. Apparatus*

The validation HPLC mainly used (System 1) was an Agilent 1200 system with a quaternary pump (low pressure mixing) and a variable wavelength detector (VWD). For comparison, another two Agilent HPLC-systems were used as well. System 2 consists of a binary pump (high pressure mixing) and a variable wavelength detector and System 3 of a binary pump and a diode array detector (DAD). System 3 was also used during method

development. For details see Table 2-1. Chromatographic data was evaluated with Agilent ChemStation for LC 3D Systems Rev. B.03.02 [341].

**Table 2-1:** HPLC systems used during validation

| Component | System 1<br>Agilent 1200 HPLC | System 2<br>Agilent 1100 HPLC | System 3<br>Agilent 1100 HPLC |
|---|---|---|---|
| **Degasser** | G1322A | G1379A | G1322A |
| **Pump** | G1311A (Quaternary) | G1312A (Binary) | G1312A (Binary) |
| **Autosampler** | G1329A | G1313A | G1313A |
| **Column Oven** | G1316A | G1316A | G1316A |
| **Detector** | G1314B (VWD) | G1314A (VWD) | G1315B (DAD) |
| **Dwell Volume** | 1.27 mL | 0.95 mL | 0.99 mL |

### *2.2. Chemicals and Materials*

Glyceryl trinitrate solution spiked with 0.3 % of the impurities 1-GMN, 2-GMN, 1-2-GDN and 1,3-GDN and the impurities individually were obtained from the EDQM (Strasbourg, France). These were used for method development only. The standards used for the validation of the method are listed in Table 2-2. Acetonitrile and methanol were purchased from Sigma–Aldrich (Steinheim, Germany). Water was purified with the Milli-Q purification system (Schwalbach, Germany).

**Table 2-2:** List of the standards that were used for calibration curves and reference solutions

| Name | Supplier | Batch | CAS | Concentration |
|---|---|---|---|---|
| **1-Mononitroglycerin** | Cerilliant, Sigma Aldrich | FN05031101 | 624-43-1 | 100 µg/ml in acetonitrile |
| **2-Mononitroglycerin** | Cerilliant, Sigma Aldrich | FN07231201 | 620-12-2 | 100 µg/ml in acetonitrile |
| **1,2 Dinitroglycerine** | Cerilliant, Sigma Aldrich | FN05021404 | 621-65-8 | 100 µg/ml in acetonitrile |
| **1,3 Dinitroglycerine** | Cerilliant, Sigma Aldrich | FN01131104 | 623-87-0 | 100 µg/ml in acetonitrile |
| **Glycerin trinitrate solution** | EDQM, Sigma Aldrich | 2.4 | 55-63-0 | 10 mg/ml in ethanol |

*2.3. Method description*

An Eurosphere II 18H column (250x4.6 mm, 5 µm particle size, Knauer Wissenschaftliche Geräte GmbH, Berlin, Germany) was used for method development and validation. A gradient elution mode with a flow rate of 1.5 ml/min was used (see Table 2-3). The reequilibration time after the end of the gradient was 5 min. The temperature was set to 40 °C, UV spectrophotometric detection at 210 nm was applied, and the injection volume was 20 µl.

**Table 2-3:** Gradient program for the method (Flow 1.5 ml/min)

| Time [min] | Water % [V/V] | Acetonitrile % [V/V] |
|---|---|---|
| **0** | 90 | 10 |
| **2** | 90 | 10 |
| **12** | 60 | 40 |
| **20** | 20 | 80 |

Solutions:

Test solution (a): Glyceryl trinitrate solution (containing glyceryl trinitrate in ethanol, 10 mg/mL) is diluted with water to a final concentration of 0.5 mg/mL. (e.g. dilute 500 µl of glyceryl trinitrate solution to 10.0 ml with water)

Test solution (b): A 5 % solution of test solution (a) with a final concentration of 25 µg/mL. (*Sample solution for the assay. It has to be diluted to fit into the linear range of the detector)*

Reference solution (a): A 0.25 % dilution of the test solution (a). (*Used as an external standard for the quantification of impurities*)

Reference solution (b): A solution of glyceryl trinitrate solution standard with a final concentration of 25 µg/mL. (*Serves as an external standard for the quantification of glyceryl trinitrate)*

Reference solution (c): A solution of glyceryl trinitrate solution for system suitability CRS. (*Contains impurities B, C, D and E; used to determine the resolution between peaks)*

Evaluation:

System suitability: The resolution between peaks due to the impurities 1-GMN and 2-GMN has to be at least 1.5 and at least 2.0 for 1,2-GDN and 1,3-GDN.

For related substances, test solution (a) and reference solution (a) are injected. The content of impurities is calculated using the following formula:

$$Imp\ \% = \frac{peak\ area\ of\ the\ impurity\ in\ test\ solution(a)}{peak\ area\ of\ glyceryl\ trinitrate\ in\ reference\ solution(a)} \times\ 0.25\ \%$$

The maximum for each impurity is 0.3 %, the maximum for all impurities together is 1.0 %, and the reporting threshold is 0.05 %.

The assay is performed as described above with the modification, that test solution (b) and reference solution (b) are injected. The content of glyceryl trinitrate is calculated using the following formula:

$$GTN\ \% = \frac{peak\ area\ of\ glyceryl\ trinitrate\ in\ test\ solution(b)}{peak\ area\ of\ glyceryl\ trinitrate\ in\ reference\ solution(b)} \times\ 100\ \%$$

## 3. Results and Discussion

### *3.1. Method Development*

To further improve the method of the manufacture, several key changes were made. First of all, acetonitrile was used instead of methanol as organic solvent in the mobile phase. Acetonitrile has a lower UV cutoff (< 190 nm) than methanol (205 nm) and therefore shows a lower absorption of the mobile phase at 210 nm with UV spectrophotometric detection. This reduces the rise of the baseline in the chromatogram after the gradient starts. Due to the different solvent and in order to increase the resolution, the gradient profile had to be adjusted. The slope at the beginning was decreased and the final hold at 50 % acetonitrile was changed to a further increase of acetonitrile proportion in the mobile phase to elute glyceryl trinitrate within 20 minutes. By this, the baseline showed a steady slope which improves the accurate integration of the later eluting peaks. The resolution between the peaks due to 1,2-GDN and 1,3-GDN was further improved but not satisfying. Hence, the temperature of the column was increased to 30, 40, and 50 °C. Higher temperatures lead to a higher resolution for these peaks. A temperature of 40 °C was found to be a good compromise of column lifetime, simplicity and resolution which was hereby increased to 3.1. To address the poor

resolution and peak shape of impurities 1-GMN and 2-GMN, water was used as sample solvent instead of ethanol. Since ethanol has a considerably higher elution strength than the initial mobile phase, the early eluting peaks are negatively affected by the solvent. The use of water gave a much better peak shape and a resolution of 4.7 could be achieved. Water as the diluent for glyceryl trinitrate solution could lead to precipitation of glyceryl trinitrate. However, during the development and the validation of this method, no precipitation problems were encountered. The solubility of glyceryl trinitrate is higher than 1 mg/mL in water and therefore more than double the amount used in this method (0.5 mg/mL).

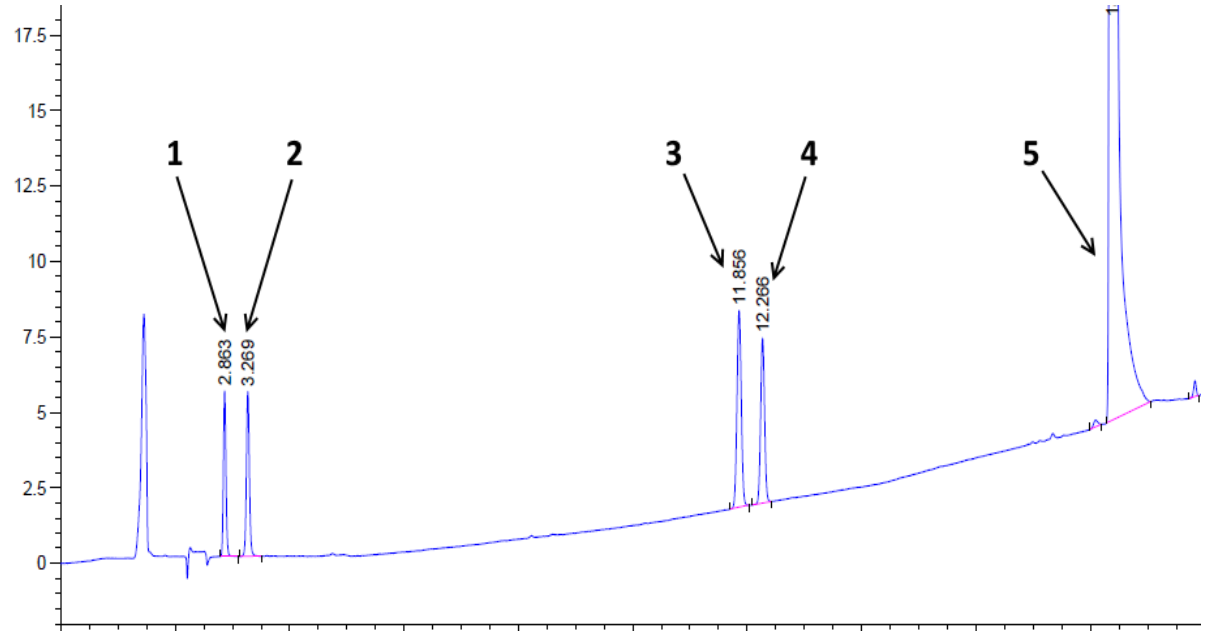
Thus, the method can be summarized as follows: An acetonitrile/water gradient is used (0 – 2 min: 10 % ACN; 2 – 12 min: 10 – 40 % ACN; 12 – 20 min: 40 – 80 % ACN), the flow rate is 1.5 ml/min, the temperature is 40 °C and the compounds are detected by means of UV spectrophotometric detection at 210 nm.

### *3.2. Method Validation*

The newly developed method was subject for validation. Precision (system repeatability, method repeatability, reproducibility), accuracy, selectivity, limit of detection, limit of quantification, linearity, relative response factors, robustness and stability were evaluated. Furthermore, the validation for an assay was performed. The validation was executed in accordance with the standards given by the International Council for Harmonization of Technical Requirements for Pharmaceuticals for Human Use (ICH) in the document Validation of Analytical Procedures: Text and Methodology Q2 (R1) [4].

### *3.3. Selectivity*

A preparation of an artificial impurity mixture, with a concentration of 1.25 µg/ml (0.25 % of the nominal concentration of the sample required by the method) for impurities 1-GMN, 2-GMN, 1,2-GDN, 1,3-GDN and 0.50 mg/ml for glycerin trinitrate (100 % of the nominal concentration of the sample required by the method) was injected once (Figure 3-1). All peaks are separated with a resolution of at least 3.0. Thus, the method is selective and specific for the impurities 1-GMN, 2-GMN, 1,2-GDN, 1,3-GDN and glyceryl trinitrate.




| # | Name | Time [min] | Area [mAU*s] | Height [mAU] | Sym. | Width [min] | S/N | Rs |
|---|---|---|---|---|---|---|---|---|
| **1** | 2-GMN | 2.863 | 17.262 | 5.486 | 0.87 | 0.0467 | 385 | - |
| **2** | 1-GMN | 3.269 | 20.145 | 5.455 | 0.89 | 0.0546 | 382 | 4.7 |
| **3** | 1,3-GDN | 11.856 | 34.124 | 6.512 | 0.96 | 0.0807 | 376 | 74.6 |
| **4** | 1,2-GDN | 12.266 | 26.170 | 5.452 | 0.95 | 0.0744 | 315 | 3.1 |
| **5** | GTN | 18.383 | 7748.221 | 2136.944 | 0.90 | 0.0546 | 12.273 | 56.1 |

**Figure 3-1:** Chromatogram with data of the selectivity solution; 1: 2-GMN; 2: 1-GMN, 3: 1,3-GDN; 4: 1,2-GDN; 5: GTN; All other peaks are due to the solvent of the sample. Chromatographic conditions: flow rate 1.5 ml/min; temp.: 40 °C; UV detection at 210 nm; mobile phase A: water, B: acetonitrile; gradient: 0 to 2 min, 10 % B; 2 to 12 min 10 – 40 % b; 12 to 20 min, 40 – 80 % B;

### *3.4. Precision*

#### *3.4.1. System Repeatability*

A preparation of an artificial impurity mixture, with a concentration of 1.25 µg/ml (0.25 % of the nominal concentration of the sample required by the method) for all impurities and 125 µg/ml (25 % of the nominal concentration of the sample required by the method) for glycerin trinitrate solution was injected ten times and the average, the standard deviation and the relative standard deviations of retention times and areas were calculated. The results are

shown in Table 3-1 and Table 3-2. The relative standard deviation for retention time and area is below 1 %. The method fulfills the requirements for system repeatability.

**Table 3-1:** Repeatability retention time

| | Retention Time [min] | | | | |
|---|---|---|---|---|---|
| | **2-GMN** | **1-GMN** | **1,3-GDN** | **1,2-GDN** | **GTN** |
| **1.** | 2.867 | 3.275 | 11.803 | 12.213 | 18.315 |
| **2.** | 2.865 | 3.272 | 11.798 | 12.207 | 18.313 |
| **3.** | 2.864 | 3.271 | 11.796 | 12.205 | 18.316 |
| **4.** | 2.865 | 3.272 | 11.799 | 12.209 | 18.314 |
| **5.** | 2.863 | 3.270 | 11.799 | 12.209 | 18.320 |
| **6.** | 2.862 | 3.270 | 11.797 | 12.206 | 18.311 |
| **7.** | 2.865 | 3.272 | 11.794 | 12.203 | 18.311 |
| **8.** | 2.861 | 3.267 | 11.794 | 12.203 | 18.308 |
| **9.** | 2.859 | 3.266 | 11.789 | 12.198 | 18.306 |
| **10.** | 2.859 | 3.264 | 11.785 | 12.193 | 18.306 |
| **Average** | 2.863 | 3.270 | 11.795 | 12.205 | 18.312 |
| **SD** | 0.003 | 0.003 | 0.005 | 0.006 | 0.005 |
| **RSD [%]** | 0.1 | 0.1 | 0.0 | 0.0 | 0.0 |

**Table 3-2:** Repeatability peak area

| | Peak Area [mAU*s] | | | | |
|---|---|---|---|---|---|
| | **2-GMN** | **1-GMN** | **1,3-GDN** | **1,2-GDN** | **GTN** |
| **1.** | 15.545 | 18.117 | 27.708 | 27.266 | 2046.197 |
| **2.** | 15.651 | 18.340 | 27.561 | 27.120 | 2047.313 |
| **3.** | 15.485 | 18.066 | 27.801 | 27.190 | 2045.620 |
| **4.** | 15.382 | 18.059 | 27.738 | 27.195 | 2050.865 |
| **5.** | 15.476 | 18.107 | 27.809 | 27.138 | 2051.136 |
| **6.** | 15.452 | 17.967 | 27.787 | 27.215 | 2050.467 |
| **7.** | 15.555 | 17.979 | 27.632 | 27.160 | 2051.025 |
| **8.** | 15.386 | 18.013 | 27.625 | 27.300 | 2049.698 |
| **9.** | 15.438 | 18.026 | 27.828 | 27.185 | 2051.476 |
| **10.** | 15.409 | 18.098 | 27.799 | 27.225 | 2050.696 |
| **Average** | 15.478 | 18.077 | 27.729 | 27.199 | 2049.449 |
| **SD** | 0.085 | 0.106 | 0.093 | 0.055 | 2.208 |
| **RSD [%]** | 0.6 | 0.6 | 0.3 | 0.2 | 0.1 |

### *3.4.2. Method Repeatability*

A sample of glyceryl trinitrate CRS was prepared three times at a concentration of 0.50 mg/ml (100 % of the nominal concentration of the sample required by the method) on different days with different equipment and each one was injected three times. In Table 3-3, the average values for retention time and impurity content of three injections of each sample are given. The relative standard deviation for the content of impurities is below 5 %. Thus, the method fulfills the requirements for method repeatability.

**Table 3-3:** Intermediate precision results

| | **2-GMN** | **1-GMN** | | **1,3-GDN** | | **1,2-GDN** | |
|---|---|---|---|---|---|---|---|
| | Time [min] | Time [min] | Content [%] | Time [min] | Content [%] | Time [min] | Content [%] |
| **1.** | N.D. | 3.312 | N.Q. | 11.859 | 0.080 | 12.269 | 0.039 |
| **2.** | N.D. | 3.276 | N.Q. | 11.851 | 0.078 | 12.262 | 0.037 |
| **3.** | N.D. | 3.212 | N.Q. | 11.844 | 0.085 | 12.255 | 0.041 |
| **Average** | - | 3.276 | - | 11.852 | 0.081 | 12.262 | 0.039 |
| **SD** | - | 0.051 | - | 0.008 | 0.003 | 0.007 | 0.002 |
| **RSD** | - | 1.5 | - | 0.1 | 4.1 | 0.1 | 4.9 |

### *3.4.3. Reproducibility*

A sample of glyceryl trinitrate solution CRS was spiked with impurities 1-GMN, 2-GMN, 1,2-GDN and 1,3-GDN at a concentration of 1.25 µg/ml (0.25 % of the nominal concentration of the sample required by the method) and was injected six times on System 1, System 2 and System 3 (Table 2-1). The content of impurities was determined (see Table 3-4).

Following columns were used:

System 1: Knauer Eurosphere II 18H; 5 µm; 250x4.6 mm (see Figure 3-2)

System 2: Knauer Eurosphere II 18H; 5 µm; 150x4.6 mm (see Figure 3-3)

System 3: Phenomenex Luna 5u C18(2) 100A; 5 µm; 250x4.6mm (see Figure 3-4)

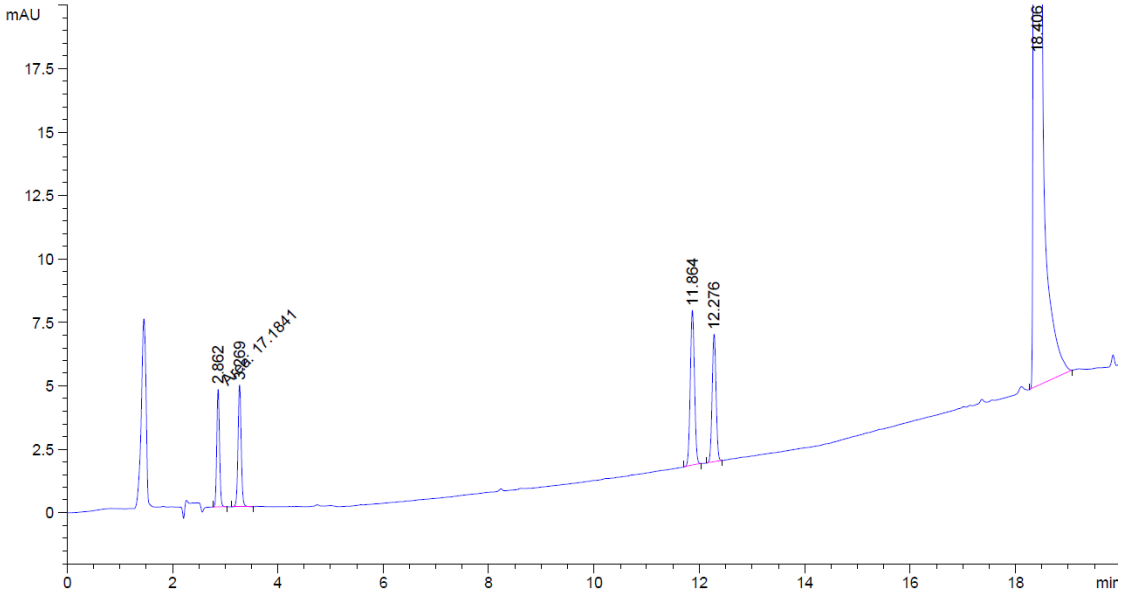



**Figure 3-2:** System1; Knauer Eurosphere II 18H; 5 µm; 250x4.6 mm

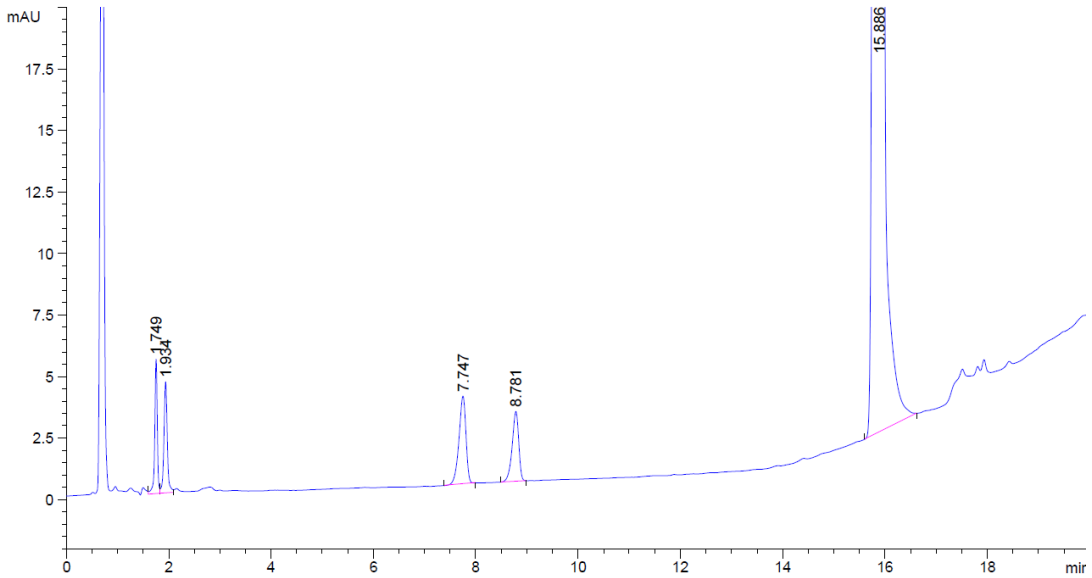



**Figure 3-3:** System 2; Knauer Eurosphere II 18H; 5 µm; 150x4.6 mm

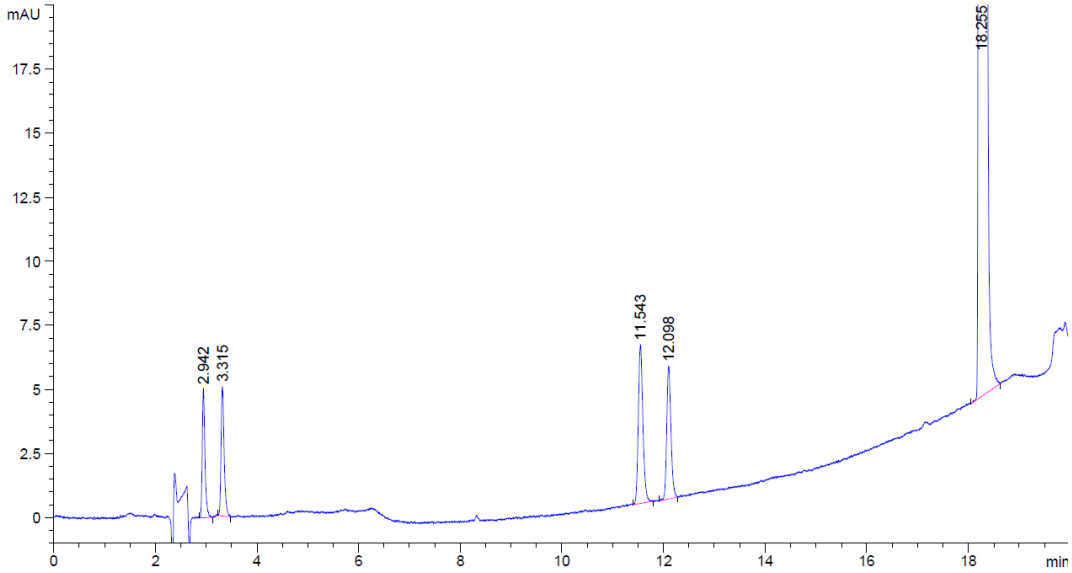



**Figure 3-4:** System 3; Phenomenex Luna 5u C18(2) 100A; 5 µm; 250x4.6mm

**Table 3-4:** Content of impurities determined with different systems.

| | **System 1** | **System 2** | | **System 3** | |
|---|---|---|---|---|---|
| | [%] | [%] | Diff [%] | [%] | Diff [%] |
| **1-GMN** | 0.23 | 0.25 | + 6.0 | 0.22 | - 6.7 |
| **2-GMN** | 0.20 | 0.23 | + 13.3 | 0.19 | - 2.8 |
| **1,2-GDN** | 0.30 | 0.30 | +1.5 | 0.31 | + 2.2 |
| **1,3-GDN** | 0.39 | 0.41 | + 3.9 | 0.39 | + 0.3 |

The content has been determined by means of an external standard and not been corrected with the relative response factors of the impurities. The difference in content of impurities is below 20 %. The highest difference is 13.3 % for impurity 2-GMN on system 2 and is related with the fact that a shorter column was used, so that the separation between the peaks of impurity 1-GMN and 2-GMN was poor. However, at this level, the difference is considered as acceptable. The method fulfills the requirements for reproducibility.

### *3.5. Accuracy*

Accuracy was determined by injecting mixtures of impurities 1-GMN, 2-GMN, 1,2-GDN, and 1,3-GDN with a concentration of 0.25 µg/ml (corresponding to 0.05 % of the nominal concentration required by the method and to the reporting threshold by the Ph.Eur.), 1.25 µg/ml (corresponding to 0.25 % of the nominal concentration required by the method) and 2.50 µg/ml (corresponding to 0.50 % of the nominal concentration required by the method). Each solution was injected three times. The average, standard deviation, relative standard deviation and the content of each impurity was calculated (see Table 3-5 and Table 3-6). The recovery for impurities is below 10 % when calculated with linear regression. Due to the fact that the Ph.Eur. does not use correction factors in the range from 0.8 to 1.2, the recovery is worse when the amount of impurities is calculated in style of the Ph.Eur by using a reference solution only. The recovery is then in the range from 81.8 % to 124.0 %. The method is accurate.

The recovery is defined as:

$$Recovery\ [\%] = \frac{calculated\ content}{true\ content} \cdot 100\ \%$$

The calculated content and the recovery was determined in three ways:

1. By using the equation from linearity.
2. By using the area of the peak in the reference solution and the correction factors (CF) from chapter 3.9.
3. By using the area of the peak in the reference solution without the correction factors. This is how the content of impurities is calculated according to the Ph.Eur.

**Table 3-5:** Accuracy for 1-GMN and 2-GMN, peak area is given in mAU*s, calculated content (CC), and recovery are given in %. CF, correction factor

| | **1-GMN** | | | **2-GMN** | | |
|---|---|---|---|---|---|---|
| | **0.05 %** | **0.25 %** | **0.50 %** | **0.05 %** | **0.25 %** | **0.50 %** |
| | | | | | | |
| **1.** | 3.737 | 19.055 | 38.125 | 3.567 | 18.024 | 35.875 |
| **2.** | 3.758 | 19.071 | 38.126 | 3.690 | 17.810 | 35.730 |
| **3.** | 3.827 | 19.084 | 38.172 | 3.514 | 17.842 | 35.733 |
| | | | | | | |
| **Average** | 3.774 | 19.070 | 38.141 | 3.590 | 17.892 | 35.779 |
| **SD** | 0.047 | 0.015 | 0.027 | 0.090 | 0.115 | 0.083 |
| **RSD** | 1.244 | 0.077 | 0.070 | 2.512 | 0.643 | 0.232 |
| | | | | | | |
| **CC with Linearity** | 0.050 | 0.256 | 0.513 | 0.049 | 0.250 | 0.501 |
| **Recovery** | 100.3 | 102.5 | 102.6 | 97.5 | 99.8 | 100.1 |
| | | | | | | |
| **CC with CF** | 0.049 | 0.246 | 0.492 | 0.048 | 0.241 | 0.483 |
| **Recovery** | 97.4 | 98.4 | 98.4 | 96.9 | 96.5 | 96.5 |
| | | | | | | |
| **CC without CF** | 0.043 | 0.218 | 0.436 | 0.041 | 0.205 | 0.409 |
| **Recovery** | 86.3 | 87.2 | 87.2 | 82.1 | 81.8 | 81.8 |

**Table 3-6:** Accuracy for 1,2-GDN and 1,3-GDN, peak area given in mAU*s, calculated content (CC), and recovery are given in %. CF, correction factor

| | 1,2-GDN | | | 1,3-GDN | | |
|---|---|---|---|---|---|---|
| | **0.05 %** | **0.25 %** | **0.50 %** | **0.05 %** | **0.25 %** | **0.50 %** |
| | | | | | | |
| **1.** | 4.588 | 23.382 | 46.193 | 5.167 | 26.765 | 54.257 |
| **2.** | 4.631 | 23.204 | 46.318 | 5.268 | 26.812 | 54.208 |
| **3.** | 4.685 | 23.328 | 46.206 | 5.287 | 27.014 | 54.228 |
| | | | | | | |
| **Average** | 4.635 | 23.304 | 46.239 | 5.241 | 26.863 | 54.231 |
| **SD** | 0.049 | 0.091 | 0.069 | 0.064 | 0.132 | 0.025 |
| **RSD** | 1.056 | 0.392 | 0.148 | 1.228 | 0.492 | 0.046 |
| | | | | | | |
| **CC with Linearity** | 0.048 | 0.247 | 0.492 | 0.048 | 0.251 | 0.509 |
| **Recovery** | 96.1 | 98.9 | 98.4 | 96.7 | 100.6 | 101.7 |
| | | | | | | |
| **CC with CF** | 0.048 | 0.239 | 0.475 | 0.047 | 0.243 | 0.490 |
| **Recovery** | 95.1 | 95.7 | 94.9 | 94.7 | 97.0 | 97.9 |
| | | | | | | |
| **CC without CF** | 0.053 | 0.266 | 0.529 | 0.060 | 0.307 | 0.620 |
| **Recovery** | 106.0 | 106.6 | 105.7 | 119.8 | 122.8 | 124.0 |

### *3.6. Limit of Detection*

A solution containing the impurities 1-GMN, 2-GMN, 1,2-GDN and 1,3-GDN in a concentration of 0.0125 µg/ml (corresponding to 0.0025 % of the nominal concentration required by the method) was injected six times. The average of the areas, the standard deviation, the relative standard deviation and the signal-to-noise ratio (Ph.Eur. 2.2.46.) were calculated (Table 3-7). The signal-to-noise ratio for the peak at the limit of detection has to be at least 3. The signal-to-noise ratio was found higher than 3 for all impurities with the aforementioned concentration. To ensure that the limit of detection can be achieved on different systems at any time 0.0125 µg/ml (0.0025 %) is defined as the LOD for this method. In Figure 3-5 and 3-6, the peaks of the impurities at a concentration of 0.0125 µg/ml are shown.

**Table 3-7:** Limit of detection

| | Peak Area [mAU*s] | | | |
|---|---|---|---|---|
| **Area** | **1-GMN** | **2-GMN** | **1,2-GDN** | **1,3-GDN** |
| **1.** | 0.201 | 0.378 | 0.325 | 0.416 |
| **2.** | 0.322 | 0.352 | 0.354 | 0.394 |
| **3.** | 0.297 | 0.340 | 0.302 | 0.492 |
| **4.** | 0.281 | 0.331 | 0.351 | 0.412 |
| **5.** | 0.183 | 0.277 | 0.244 | 0.482 |
| **6.** | 0.263 | 0.310 | 0.347 | 0.432 |
| | | | | |
| **Average** | 0.258 | 0.331 | 0.321 | 0.438 |
| **SD** | 0.055 | 0.035 | 0.042 | 0.040 |
| **RSD [%]** | 21.3 | 10.5 | 13.2 | 9.2 |
| **S/N** | 5 | 7 | 5 | 7 |

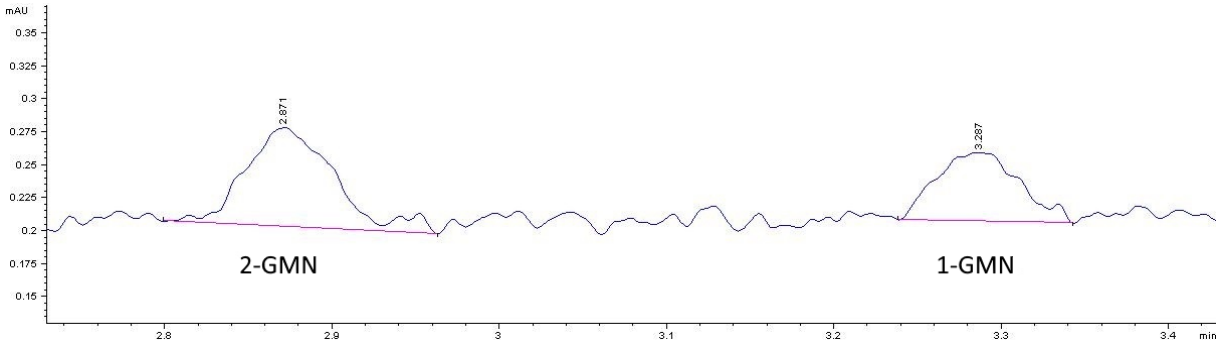



**Figure 3-5:** 0.0125 µg/ml solution (LOD) of impurities 2-GMN and 1-GMN

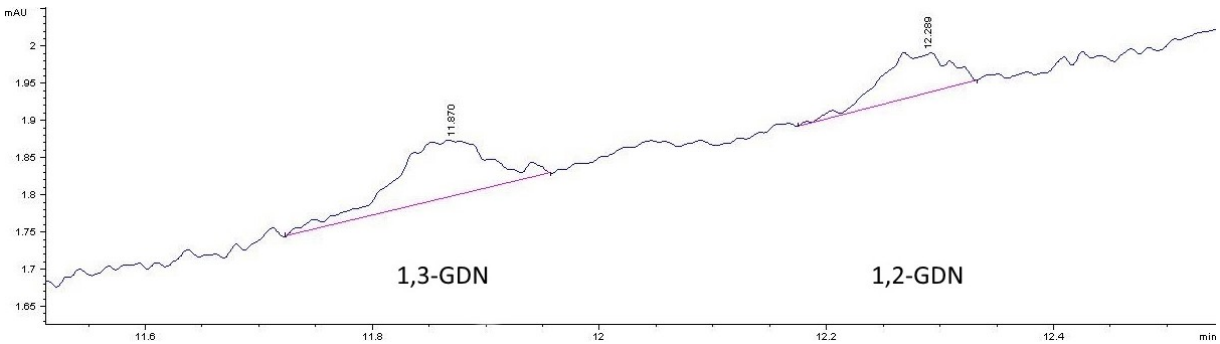



**Figure 3-6**: 0.0125 µg/ml solution (LOD) of impurities 1,2-GDN and 1,3-GDN

### *3.7. Limit of Quantification*

For the determination of the limit of quantification, solutions at a concentration of 0.05 µg/ml (corresponding to 0.01 % of the nominal concentration of the sample required by the method) were prepared for 1-GMN, 2-GMN, 1,2-GDN, 1,3-GDN and GTN. Each solution was injected six times. The average of the area, the standard deviation, the relative standard deviation and signal-to-noise ratios were calculated (see Table 3-8). The signal-to-noise ratio at the LOQ has to be over 10 and the relative standard deviation should be under 10 %. In Figure 3-7, the peaks of the impurities at a concentration of 0.05 µg/ml are shown.

**Table 3-8:** Limit of quantification, peak area is given as mAU*s

| | **Area [mAU*s]** | | | | |
|---|---|---|---|---|---|
| | 1-GMN | 2-GMN | 1,2-GDN | 1,3-GDN | GTN |
| **1.** | 0.869 | 0.787 | 0.915 | 1.031 | 1.099 |
| **2.** | 0.780 | 0.793 | 0.896 | 1.094 | 1.087 |
| **3.** | 0.844 | 0.773 | 0.989 | 1.103 | 1.111 |
| **4.** | 0.808 | 0.781 | 1.012 | 1.146 | 1.101 |
| **5.** | 0.811 | 0.750 | 0.936 | 1.092 | 1.047 |
| **6.** | 0.834 | 0.734 | 0.978 | 1.090 | 1.073 |
| | | | | | |
| **Average** | 0.824 | 0.770 | 0.954 | 1.093 | 1.086 |
| **SD** | 0.031 | 0.023 | 0.046 | 0.037 | 0.023 |
| **RSD [%]** | 3.8 | 3.0 | 4.8 | 3.4 | 2.1 |
| **S/N** | 22 | 24 | 19 | 21 | 28 |

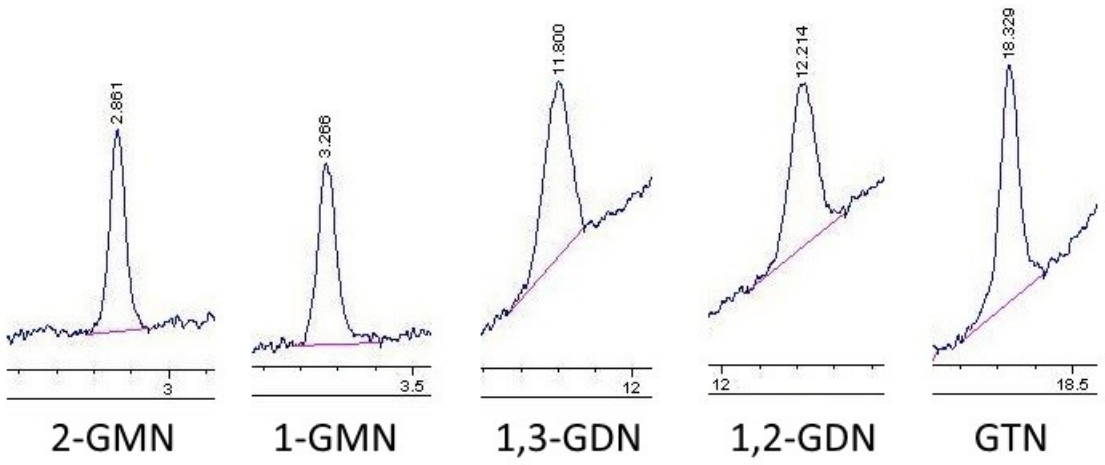



**Figure 3-7:** Limit of quantification, 0.05 µg/ml for each impurity

The RSD for the area is below 10 % for all peaks. Signal-to-noise ratio determined by Ph.Eur. (2.2.46) is higher than 10 for all peaks. A further reduction of the LOQ on this system would be possible. However, to ensure that the limit of quantification can be achieved on different systems at any time 0.05 µg/ml (0.01 %) is defined as the LOQ for this method.

### *3.8. Linearity*

Six solutions of impurities 1-GMN, 2-GMN, 1,2-GDN, 1,3-GDN and glyceryl trinitrate at levels in the range of 0.05 to 2.5 µg/ml (corresponding to 0.01 - 0.50 % of the nominal concentration of the sample required by the method) were prepared and each one was injected three times. The average was plotted against the concentration and a linear regression was made. In Table 3-9 an overview of the results in linearity is given.

**Table 3-9:** Overview linearity results

| | **Slope** | **Y-Intercept** | **$R^2$** | **Y-Intercept at 0.05 %** | **Residual sum of squares** |
|---|---|---|---|---|---|
| **1-GMN** | 74.218 | 0.0523 | 0.99999 | 1.4 | 0.01599 |
| **2-GMN** | 71.224 | 0.119 | 0.99999 | 3.2 | 0.00523 |
| **1,2-GDN** | 93.722 | 0.1302 | 0.99997 | 2.7 | 0.09474 |
| **1,3-GDN** | 106.440 | 0.0939 | 0.99999 | 1.7 | 0.03172 |
| **GTN** | 83.655 | 0.138 | 0.99998 | 3.2 | 0.05128 |

The coefficient of determination $R^2$ is higher than 0.999 and the ratio of the Y-Intercept of the area at the reporting threshold (0.05 %) is below 10 % for the impurities and GTN. The method is linear for the impurities 1-GMN, 2-GMN, 1,2-GDN and 1,3-GDN in the range 0.05 to 2.5 µg/ml (0.01 – 0.50 %).

### *3.9. Relative Response and Correction Factors*

The relative response factors and the correction factors for the impurities 1-GMN, 2-GMN, 1,2-GDN and 1,3-GDN were determined by using the slope of the impurity obtained by

linear regression in chapter 3.8 and the slope of glyceryl trinitrate (see Table 3-10). Relative response factors (RRF) and correction factors (CF) are calculated by following formulae:

$$RRF = \frac{slope\ of\ impurity\ from\ linear\ regression}{slope\ of\ GTN\ from\ linear\ regression} \qquad CF = \frac{1}{RRF}$$

**Table 3-10:** Relative response factors (RRF) and correction factors (CF)

| Impurity | RRF | CF |
|---|---|---|
| **1-GMN** | 0.887 | 1.127 |
| **2-GMN** | 0.851 | 1.175 |
| **1,2-GDN** | 1.121 | 0.891 |
| **1,3-GDN** | 1.272 | 0.786 |

Since the correction factor are given with one decimal place in the Ph.Eur. and only values outside the range from 0.8 to 1.2 are considered, these correction factors will not be included in the monograph.

Taken together, the method is precise, linear and accurate in the range of 0.25 to 2.50 µg/ml (0.05 to 0.50 %) for the impurities 1-GMN, 2-GMN, 1,2-GDN and 1,3-GDN as shown in the corresponding chapters.

### *3.10. Robustness*

Following parameters were evaluated:

- Flow rate
- Composition of mobile phase
- Temperature
- Initial hold
- Injection volume
- Wavelength

An artificial mixture of impurities 1-GMN, 2-GMN, 1,2-GDN, 1,3-GDN with a concentration of 0.50 µg/ml (0.10 %) and glyceryl trinitrate was injected three times for each change of method conditions. The average for area, time and resolution between the peaks

due to 2-GMN and 1-GMN, and 1,3-GDN and 1,2-GDN were calculated. Furthermore, signal-to-noise ratio and peak symmetry were evaluated. The following limits were considered as acceptable:

Signal-to-noise ratio: > 100 for all peaks. Since the concentration of the impurities is 0.10 %, a signal-to-noise ratio of 100 is required to achieve a LOQ of 0.01 %.

Resolution: At least 1.5 between the peaks due to 1-GMN and 2-GMN and at least 2.0 between the peaks due to 1,2-GDN and 1,3-GDN.

Peak symmetry (as calculated by the ChemStation software): 0.7 – 1.3

### *3.10.1. Effect of Aqueous/Organic Ratio*

The amount of acetonitrile was changed ± 2 % absolute throughout the gradient (see Table 3-11 for the new gradient profile).

**Table 3-11:** Changes in amount of acetonitrile and the new gradient profile

| | **Percentage Acetonitrile [V/V]** | | |
|---|---|---|---|
| **Time [min]** | **-2 %** | **Normal** | **+2 %** |
| **0 → 2** | 8 | 10 | 12 |
| **2 → 12** | 8 → 38 | 10 → 40 | 12 → 42 |
| **12 → 20** | 38 → 78 | 40 → 80 | 42 → 82 |

A decrease in amount of acetonitrile resulted in an increase of retention time and vice versa. Peak areas stayed roughly the same. Signal-to-noise ratio, peak symmetry and resolution are within specified limits. Changes are shown in Table 3-12.

### *3.10.2. Effect of Flow Rate*

The flow rate of the method was changed ± 0.1 ml/min to 1.4 ml/min and 1.6 ml/min respectively. A decrease in flow rate resulted in an increase of areas and retention time and vice versa. Signal-to-noise ratio, peak symmetry and resolution are within specified limits. Changes are shown in Table 3-12.

**Table 3-12:** Effect of changes in flow rate and the acetonitrile proportion in the mobile phase (see Table 3-11) on peak area, retention time, and the resolution between the peaks due to impurities 1-GMN + 2-GMN (Mono) and 1,2-GDN + 1,3-GDN (Di).

| | | Normal | Flow rate 1.4 ml/min | Flow rate 1.6 ml/min | Mobile - 2 % | Mobile + 2 % |
|---|---|---|---|---|---|---|
| Peak Area [mAUs] | 1-GMN | 7.265 | 7.827 | 6.836 | 7.040 | 6.947 |
| | 2-GMN | 8.195 | 8.702 | 7.619 | 7.575 | 7.322 |
| | 1,2-GDN | 17.193 | 18.644 | 16.286 | 17.305 | 17.437 |
| | 1,3-GDN | 12.596 | 13.559 | 11.798 | 12.705 | 12.791 |
| | GTN | 7233.3 | 7656.2 | 6819.2 | 7206.8 | 7227.2 |
| | | | | | | |
| Retention Time [min] | 2-GMN | 2.864 | 3.070 | 2.682 | 3.053 | 2.714 |
| | 1-GMN | 3.271 | 3.506 | 3.063 | 3.542 | 3.048 |
| | 1,3-GDN | 11.846 | 12.337 | 11.404 | 12.455 | 11.245 |
| | 1,2-GDN | 12.253 | 12.711 | 11.840 | 12.884 | 11.625 |
| | GTN | 18.359 | 18.694 | 18.056 | 18.796 | 17.911 |
| | | | | | | |
| Rs | Mono | 4.7 | 4.7 | 4.6 | 5.1 | 4.3 |
| | Di | 3.1 | 2.8 | 3.3 | 3.2 | 2.9 |

### *3.10.3. Effect of Injection Volume*

The injection volume of the method was changed ± 5 µL to 15 µl and 25 µl respectively. The signal-to-noise ratio of the smaller peaks obtained by the run with a lower injection volume, was still sufficient for quantification. Increasing the injecting volume did not impair the separation of the impurities. The effect of injection volume on other parameters was negligible. The values for resolution and peak symmetry were within the specified limits. Changes in peak area, retention time, and resolution are shown in Table 3-13. With respect to injection volume, the method is robust.

**Table 3-13:** Effect of injection volume, detection wavelength and temperature on peak area, retention time, and the resolution between the peaks due to impurities 1-GMN + 2-GMN (Mono) and 1,2-GDN + 1,3-GDN (Di).

| | | Normal | Injection 15 µL | Injection 25 µl | UV 208 nm | UV 212 nm | Temp. 35 °C | Temp. 45 °C |
|---|---|---|---|---|---|---|---|---|
| Peak Area [mAUs] | 2-GMN | 7.265 | 5.468 | 9.103 | 8.107 | 6.565 | 7.362 | 7.354 |
| | 1-GMN | 8.195 | 6.171 | 10.155 | 9.358 | 7.192 | 8.130 | 8.345 |
| | 1,3-GDN | 17.193 | 12.837 | 21.411 | 19.843 | 14.993 | 17.356 | 17.649 |
| | 1,2-GDN | 12.596 | 9.447 | 15.680 | 14.697 | 10.956 | 12.706 | 12.733 |
| | GTN | 7233.3 | 5611.9 | 8611.4 | 8576.3 | 6084.9 | 7185.8 | 7231.2 |
| | | | | | | | | |
| Retention Time [min] | 2-GMN | 2.864 | 2.860 | 2.868 | 2.862 | 2.864 | 2.904 | 2.823 |
| | 1-GMN | 3.271 | 3.267 | 3.275 | 3.267 | 3.271 | 3.324 | 3.216 |
| | 1,3-GDN | 11.846 | 11.850 | 11.855 | 11.852 | 11.851 | 12.173 | 11.514 |
| | 1,2-GDN | 12.253 | 12.256 | 12.261 | 12.257 | 12.257 | 12.556 | 11.941 |
| | GTN | 18.359 | 18.356 | 18.359 | 18.358 | 18.356 | 18.544 | 18.155 |
| | | | | | | | | |
| Rs | Mono | 4.7 | 4.7 | 4.6 | 4.7 | 4.7 | 4.7 | 4.7 |
| | Di | 3.1 | 3.1 | 3.1 | 3.1 | 3.1 | 2.9 | 3.2 |

**Table 3-14:** New gradient profiles to simulate for different dwell volumes

| Initial Hold Time [min] | | | Water [% V/V] | Acetonitrile [% V/V] |
|---|---|---|---|---|
| 1 min | 2 min | 3 min | | |
| 0 → 1 | 0 → 2 | 0 → 3 | 90 | 10 |
| 1 → 11 | 2 → 12 | 3 → 13 | 90 → 60 | 10 → 40 |
| 11 → 20 | 12 → 20 | 13 → 20 | 60 → 20 | 40 → 80 |

### *3.10.4. Effect of Detection Wavelength*

The detection wavelength of the method was changed ± 2 nm to 208 nm and 212 nm respectively. A detection wavelength of 208 nm resulted in an increase of peak area due to a higher absorption of UV light of the compounds. The opposite is the case for a detection

wavelength of 212 nm. In none of the cases, the resolution or the signal-to-noise ratio was outside the specified limits. Other parameters were not affected by these changes. See Table 3-13 for the effect of detection wavelength on peak area, retention time, and resolution.

### *3.10.5. Effect of Temperature*

The temperature of the method was changed ± 5 °C to 35 °C and 45 °C respectively. A lower temperature lead to higher retention times due to the increased interaction of the compounds with the stationary phase. A higher temperature reduces these interactions. Hence, shorter retention times were obtained. These changes had only minor impact on other parameters which stayed within specified limits (see Table 3-13).

### *3.10.6. Effect of Initial Hold Time*

The initial hold time of the gradient for the method was changed ± 1 min from 2 min to 1 min and 3 min respectively to simulate for different dwell volumes on various HPLC instruments. See Table 3-14 for the changed gradient program.

Besides the expected changes in retention time, only the resolution between the peaks due to the impurities 1,2-GDN and 1,3-GDN were affected (see Table 3-15). However, all parameters were within the specified limits.

### *3.10.7. Effect of Column*

A Knauer Eurosphere II 18H (5 µm; 250x4.6 mm, Batch: KU01215) was used for the validation study. To test whether the method works satisfying with similar columns, various columns were tested (see Table 3-16).

Column A is the same as the normal one apart from the smaller particle size (3 µm). Slight differences in resolution and a better signal-to-noise ratio were observed. Backpressure was significantly higher and the peaks were fronting. It has to be noted that in case of gradient elution, the European Pharmacopoeia does not allow the adjustment of the particle size [5].

**Table 3-15:** Effect of Initial hold time of gradient on peak area, retention time, and the resolution between the peaks due to impurities 1-GMN + 2-GMN (Mono) and 1,2-GDN + 1,3-GDN (Di).

| | | Normal | Initial Hold 1 min | Initial Hold 3 min |
|---|---|---|---|---|
| Area [mAUs] | 2-GMN | 11.681 | 11.689 | 11.706 |
| | 1-GMN | 13.685 | 13.849 | 13.907 |
| | 1,3-GDN | 23.179 | 23.424 | 23.581 |
| | 1,2-GDN | 19.485 | 19.617 | 19.658 |
| | GTN | 4612.1 | 4665.8 | 4607.5 |
| | | | | |
| Time [min] | 2-GMN | 2.867 | 2.865 | 2.863 |
| | 1-GMN | 3.275 | 3.271 | 3.269 |
| | 1,3-GDN | 11.856 | 11.069 | 12.621 |
| | 1,2-GDN | 12.261 | 11.413 | 13.098 |
| | GTN | 18.368 | 17.638 | 19.070 |
| | | | | |
| Rs | Mono | 4.7 | 4.7 | 4.7 |
| | Di | 3.0 | 2.7 | 3.5 |

**Table 3-16:** Effects of different columns on range of S/N for all impurities, peak symmetry, and the resolution between the peaks due to impurities 1-GMN + 2-GMN (Mono) and 1,2-GDN + 1,3-GDN (Di).

| Column | Resolution | | Signal-to-noise ratio | Peak Symmetry |
|---|---|---|---|---|
| | Mono | Di | | |
| **Normal** | 4.7 | 3.1 | 128 – 157 | 0.87 – 0.96 |
| **A** | 4.4 | 3.6 | 188 – 220 | 1.16 – 1.29 |
| **B** | 2.3 | 5.2 | 238 – 261 | 0.74 – 1.17 |
| **C** | 3.9 | 3.7 | 115 – 320 | 0.72 – 0.86 |
| **D** | 1.7 | 2.0 | 55 – 75 | 0.75 – 0.88 |
| **E** | 1.5 | 3.6 | 100 – 125 | 0.72 – 0.95 |

A: Knauer Eurosphere 18H; 3 µm; 250x4.6 mm;
B: Knauer Eurosphere II 18H; 5 µm; 150x4.6 mm;
C: Phenomenex Luna 5u C18(2) 100A; 5 µm; 250x4.6mm;
D: Machery Nagel Nucleosil 100-3 C18 HD; 3 µm; 250x4.6 mm;
E: Thermo ODS Hypersil; 5 µm; 250x4.6 mm

The length of the column may be adjusted ±70 % to meet the system suitability criteria [5]. Column B has shorter dimensions of 150x4.6 mm. Resolution of the early eluting impurities 1-GMN and 2-GMN was lower whereas the resolution of the later eluting peaks was increased. Column C, D, and E have the same dimensions as the one used for development and validation. While the use of C gave good results, resolution of the impurities 1-GMN and 2-GMN was borderline with E. Due to the poor peak shape that was obtained with D, the performance of this column stayed well behind the others and was found not suitable for the method.

### *3.10.8. Conclusion*

Taken all together, the method is robust to changes in flow rate, aequous/organic ratio, injection volume, detection wavelength, temperature, initial hold time of gradient and column.

## *3.11. Assay*

In the assay of the current monograph [3], glyceryl trinitrate is hydrolyzed with sodium hydroxide and nitrite is released. This enables the diazotation of sulfanilic acid which is then diazo coupled with naphthylethylenediamine dihydrochloride. The absorption of this product is measured by means of UV/Vis spectroscopy. The procedure has also to be done with a reference and a blind solution. This quantification method requires several steps and the compliance with a timetable for accurate results.

A HPLC-method would be simpler and faster compared to the UV-VIS method and would also allow an automation. Therefore, a method of quantifying the content of glyceryl trinitrate with the same method used for related substances was validated. However, the sample for assay has to be more diluted than in related substances in order to fit into the linear range of the detector. Quantification is done by means of an external standard. Precision, robustness, selectivity, and limit of quantification have already been shown above. Since the assay of glyceryl trinitrate solution was not subject of the revision of the monograph, the new method by means of HPLC is not included in it.

### *3.11.1. Linearity*

Five solutions of glyceryl trinitrate at levels in the range of 0.40 to 0.60 mg/ml (corresponding to 80 % - 120 % of the nominal concentration of the sample required by the method) were prepared. 50 µL of every solution was diluted to 1.0 ml with water to obtain a final concentration of glyceryl trinitrate in the range of 20 – 30 µg/ml. Each of these solutions was injected three times. The average of areas was plotted against the concentration and a linear regression was made (Figure 3-8). The ratio of the y-intercept of the area at 100 % is -0.4 %. The Residual sum of squares is 1.2279. $R^2$ is over 0.999 and the y-Intercept is below 5 %. The method is linear in the range from 20 – 30 µg/ml (80 – 120 %).

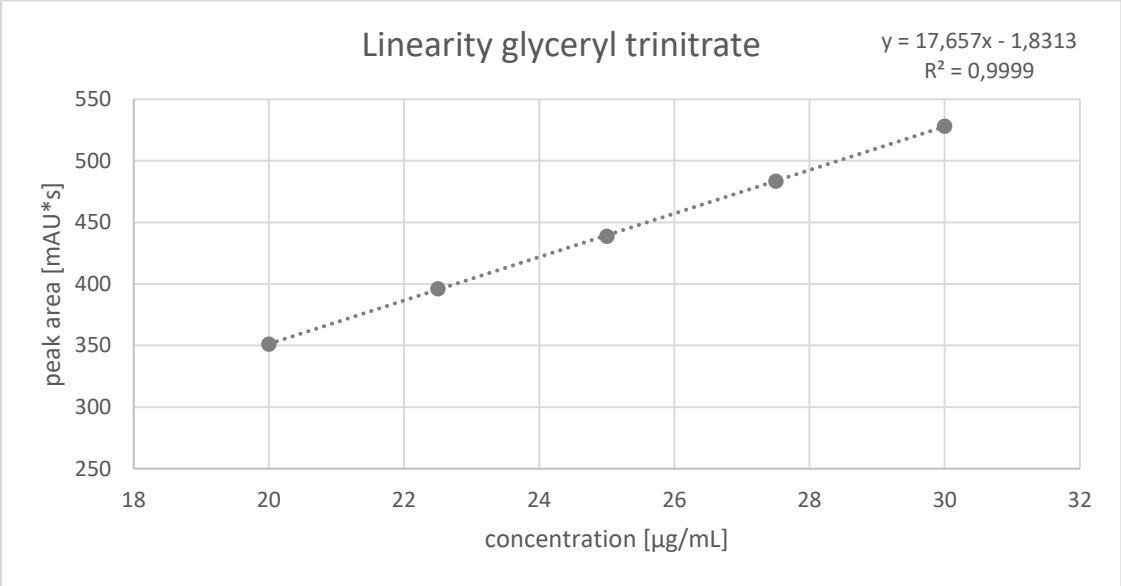



**Figure 3-8:** Linearity curve for the assay of glyceryl trinitrate

### *3.11.2. Accuracy*

For the determination of the accuracy, three solutions of glyceryl trinitrate with a concentration of 20, 25 and 30 µg/ml (corresponding to 80, 100 and 120 % of the nominal concentration of the method) were prepared. Each solution was injected three times. The content of glyceryl trinitrate was calculated with a separately prepared reference solution with a concentration of 25 µg/ml (100 %, see Table 3-17). The accuracy at 100 % was also determined with the formula obtained from linear regression (see Table 3-18). The deviation from the true value is lower than 5 %. The method is accurate.

**Table 3-17:** Accuracy of the assay calculated with a reference solution

| | Calculated Content [%] | | |
|---|---|---|---|
| | 80 % | 100 % | 120 % |
| **1.** | 77.4 | 98.3 | 117.6 |
| **2.** | 77.3 | 98.3 | 117.5 |
| **3.** | 77.4 | 98.1 | 117.5 |
| | | | |
| **Average [%]** | 77.4 | 98.2 | 117.5 |
| **SD** | 0.0 | 0.1 | 0.1 |
| **RSD** | 0.0 | 0.1 | 0.1 |
| | | | |
| **Recovery [%]** | 96.8 | 98.2 | 97.9 |

**Table 3-18:** Accuracy of the assay calculated with the formula from linear regression

| | Area [mAU*s] | Calculated Content [%] |
|---|---|---|
| **1.** | 441.694 | 100.5 |
| **2.** | 441.828 | 100.5 |
| **3.** | 441.750 | 100.5 |
| | | |
| **Average** | 441.757 | 100.5 |
| **SD** | 0.067 | 0.015 |
| **RSD** | 0.0 | 0.0 |

## *3.12. Stability*

### *3.12.1. Stability of the sample solution*

A sample of glyceryl trinitrate solution with a concentration of 0.50 mg/ml was prepared freshly and injected immediately and after one, three, five and seven days. The sample was stored at room temperature (25 °C). Changes in the content of impurities and GTN over time were evaluated (see Table 3-22).

**Table 3-22:** Content of impurities and GTN in percent

| | Day 0 | Day 1 | Day 3 | Day 5 | Day 7 |
|---|---|---|---|---|---|
| **1-GMN** | NQ[1] | NQ[1] | NQ[1] | NQ[1] | NQ[1] |
| **2-GMN** | ND[2] | ND[2] | ND[2] | ND[2] | ND[2] |
| **1,2-GDN** | 0.041 | 0.040 | 0.041 | 0.041 | 0.042 |
| **1,3-GDN** | 0.085 | 0.085 | 0.086 | 0.085 | 0.089 |
| **GTN** | 100.0 | 98.8 | 101.6 | 102.4 | 98.1 |

[1] NQ: not quantifiable; [2] ND: not detectable

No changes in content of impurities were detected over five days. A slight increase of impurity 1,3-GDN was observed after seven days. With respect to different conditions under which the solutions might be stored, they are considered to be stable for three days at room temperature. Nevertheless, it is recommended that solutions are prepared freshly and stored in the refrigerator.

### *3.12.2. Stress Studies:*

To further investigate the stability of glyceryl trinitrate in an aqueous solution, samples of diluted glyceryl trinitrate were stressed with acid, basic and heat conditions.

Acid:

A solution of glyceryl trinitrate with a concentration of 25 µg/ml in 0.1 M hydrochloride acid was prepared and the stability of glyceryl trinitrate was examined. The sample was analyzed according to the method with one change: A corresponding amount of sodium hydroxide was also drawn up by the needle of the injector to neutralize the hydrochloride acid in the sample before injection. No notable change in peak area was observed within 24 hours.

Basic:

Solutions of glyceryl trinitrate with a concentration of 25 µg/ml in 0.1, 0.01, 0.001 and 0.0001 M sodium hydroxide were prepared. The sample were analyzed according to the method with one change: A corresponding amount of hydrochloride acid was also drawn up by the needle of the injector to neutralize the sodium hydroxide in the sample before

injection. In Figure 3-9, the residual peak area of glyceryl trinitrate over time in percent is given.

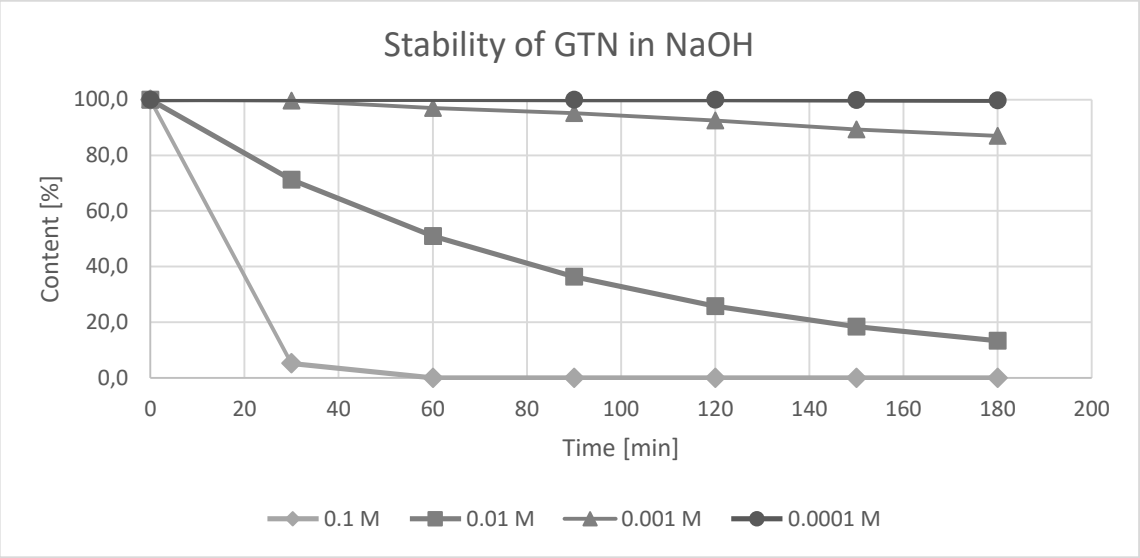



**Figure 3-9:** Residual peak area of glyceryl trinitrate in percent after treatment with sodium hydroxide

The residual peak area for glyceryl trinitrate after 24 hours is 1.6 % in 0.01 M NaOH, 39.6 % for 0.001 M NaOH and 75.7 % for 0.0001 M NaOH.

According to Halasz et all [6], glyceryl trinitrate is hydrolyzed to nitrate, nitrite, formic acid, oxalic acid, 2-hydroxy-propanedial and others (Figure 3-10). In the chromatogram of the stressed solutions (Figure 3-11) new peaks could be observed. The peaks due to retention time 1.465 min and 1.526 min could possibly be nitrite and nitrate by their retention times. The peak at 3.230 min was identified as impurity 1-GMN. The peaks at retention time 1.848 min and 2.131 min are unidentified. Formic acid and oxalic acid could not be detected. 2-hydroxy-propanedial was not available as substance and could not be evaluated.

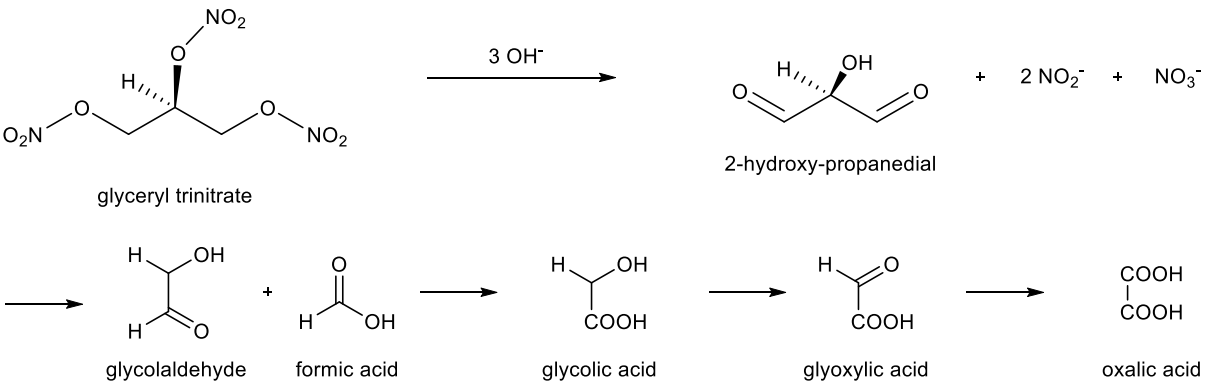



**Figure 3-10:** Hydrolysis pathway of glyceryl trinitrate[6]

Heat:

A sample was exposed to a temperature of 60 °C for 24 hours. The content of three impurities increased (1-GMN 0.005 → 0.054 %; 1,2-GDN 0.041 → 0.078 %; 1,3-GDN 0.085 → 0.110 %). Impurity 2-GMN was still not detectable. Two additional peaks at 1.444 min (identified as nitrite) and 1.513 min (identified as nitrate) minutes occurred. The area of the peak due glyceryl trinitrate was reduced to 92.0 %.

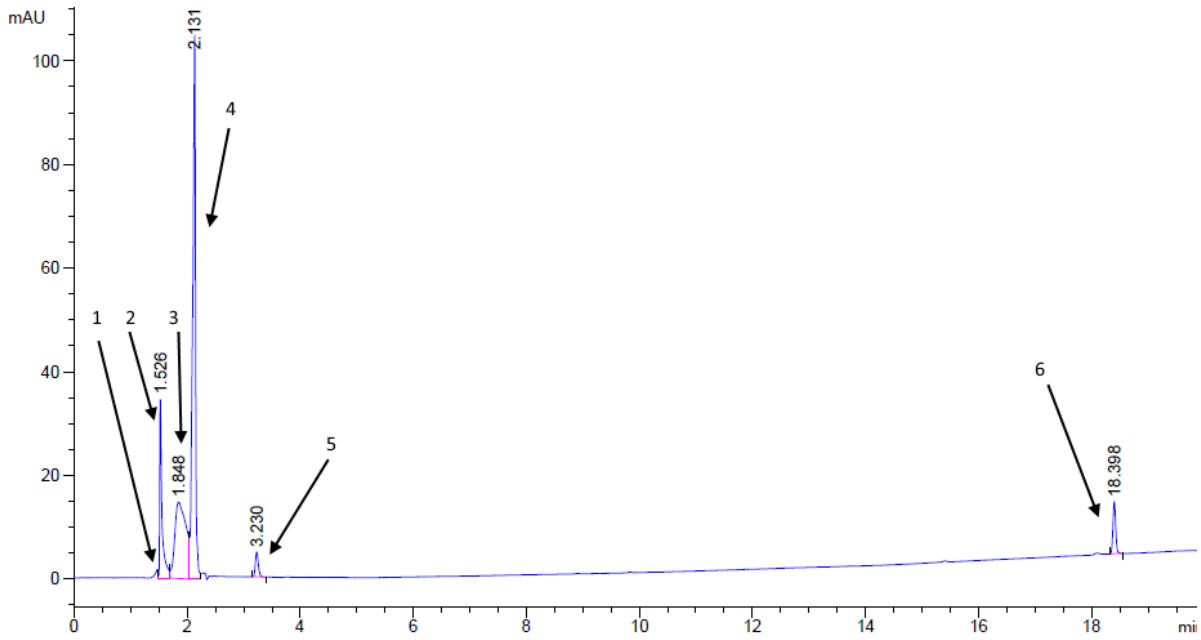



**Figure 3-11:** 25 µg/ml glyceryl trinitrate stressed in 0.01 M sodium hydroxide for 3 hours; 1: nitrite; 2: nitrate, 3: unidentified; 4: unidentified, 5: 1-GMN, 6: GTN

## 4. Conclusion

A method for the determination of related substances in glyceryl trinitrate solution and an assay was successfully developed and validated. The method is simple, robust, precise, accurate, and provides a good sensitivity for all impurities.

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IV

# IV Final Discussion

The purpose of the studies that were performed, was to elaborate a method for impurity profiling of methionine and particularly the evaluation of enantiomeric purity of amino acids. The two important challenges that have to be faced in connection with the analysis of amino acids are the detection and the choice of an appropriate chromatography principle for separation.

# 1. Impurity Profiling of L-Methionine

In contrast to most other amino acids, methionine can be detected with UV spectrophotometric methods at low wavelengths. In the current monograph of L-methionine in the European Pharmacopoeia [1], the test for related substances is performed by means of reversed phase chromatography and a common UV/Vis detector is used. While the sensitivity with this method is satisfactory and the separation of the hydrophobic impurities is very good, the retention of the hydrophilic impurity L-methionine sulfoxide is poor and the substance more or less coeludes with the columns dead time peak. For this compound reversed phase chromatography is not appropriate. For such cases, where a single stationary phase does not yield the desired results, mixed-mode chromatography has been successfully utilized [2, 3]. Here, the impurities of L-methionine could be separated with a mixed-mode column consisting of cation exchanger and reversed phase. By this, all substances were well separated in isocratic elution mode with good resolution and the method runtime could be reduced. The use of mixed-mode chromatography was found to be very suitable for the impurity profiling of L-methionine. Several batches of methionine were evaluated and the content of impurities was found to be very low (lower than 0.05 %).

Due In general, by applying more than one separation technique at once, the separation of mixtures of substances with a broad chemical diversity is feasible. Method development is more difficult in comparison to normal reversed phase columns, since the effect of chromatographic parameters is not well predictable. The increased costs and the low number of manufactures are other clear downsides of these mixed-mode columns. However, due to its good suitability for challenging separations, several methods that use mixed-mode chromatography have already been described in literature. It is expected that over time more of these methods will find its way into monographs and the use of these columns will be

established for routine analysis. Since many of the ionic and hydrophilic compounds lack a chromophore, mixed-mode chromatography is used in combination with CAD, ELSD/CNLSD or mass spectrometry. However, due to different production processes and impurity profiles of amino acids, analytical methods with various separation techniques and detection methods have been established [4].

## 2. Enantioseparation of Amino Acids

The separation of chiral compounds like amino acids and other compounds is based on the formation of diastereomers. This can take place by the interaction of chiral substance with a chiral stationary phase (CSP) or a chiral selector. By this principle, true enantiomers are separated and hence this approach is called direct enantioseparation. This still applies, when the compounds are derivatized prior analysis with an achiral derivatization reagent, since the derivatives remain enantiomeric. The indirect enantioseparation requires the derivatization with a chiral derivatization reagent (CDA), which generally entails many disadvantages. Because of that, the direct separation is the preferred choice. In the past, this approach was not always feasible due to the lack of a suitable CSP and the difficult detection of substances with poor to nonexistent chromophores as it is the case for amino acids. This has changed during the last decades by the greatly increased selection of CSPs and the advent of universal like detectors such as CAD and ELSD/CNLSD. However, during the last decades, many methods with an indirect approach have been developed which have some advantages a direct approach cannot offer. Due to the higher plate number of reverse phase columns, an indirect approach is often superior with regard to resolution and selectivity between the two enantiomers of AAs [5, 6]. However, this cannot be generalized. Hence, derivatization can be a way to achieve higher resolutions. This is in particular important for the separation of complex mixtures of substances. Method development with chiral columns is arduous and often involves a screening of different chiral stationary phases, since changing the chromatographic parameters is typically more limited. On the other hand, issues with regard to chiral purity of the derivatization reagent, kinetic resolution, and racemization effects have to be considered if an indirect approach is desired. Hence, the development of chiral methods does always require extra work in comparison to achiral methods and it is hard to predict which approach is more promising. The direct enantioseparation of AAs can only be achieved

with a significant higher cost of instrumentation whereas the indirect approach can be accomplished on existing instruments. While modern detectors like CAD do not require a chromophore, the sensitivity of the method for the analytes can be an issue. Low temperatures and low flow rates are sometimes used in conjunction with CSPs since it can be beneficial for chiral selectivity, but it leads to broad and small peaks. Derivatization reagents introduce strong fluorophores or chromophores and with reversed phase columns and optionally gradient elution narrow and high peaks of the derivatives are obtained. Therefore, the limit of quantification is typically better with the indirect approach. To enable the separation of all kinds of enantiomers, new types of CDAs are developed [7].

In this work, it could be demonstrated, that the indirect enantioseparation of AAs can easily be achieved with common equipment and chemicals. With the use of OPA/NIBLC, a sensitive method for the evaluation of enantiomeric purity by means of HPLC was developed. The sensitivity for the minor enantiomer is sufficient when UV spectrophotometric detection is used. An orthogonal method by means of capillary electrophoresis with OPA/NAC and γ-cyclodextrin was also established. Due to the use of OPA, proline and cysteine cannot be analyzed. This is a compromise that has to be made, when an automated analysis is desired.

Taken all together, neither the indirect nor the direct analysis is superior choice of chiral analysis for AAs or other substances. Depending on factors like analytes, the desired limit of quantification, and the available instruments for analysis, a decision has to be made which approach is more suitable. Nevertheless, some disadvantages usually have to be taken into account.

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# V

# V Summary

The requirements for the impurity profiling of substances for pharmaceutical use have become greater over time. They can be accomplished by the use of modern instrumental analysis techniques, which have been evolved in the last decades. New types of columns with HILIC, mixed-mode and chiral stationary phases are suitable for the separation of all kinds of substances mixtures, that were previously hardly possible with the use of common reversed phase columns. Modern, almost universal detectors like CAD, ELSD and CNLSD can be applied for a sensitive detection of substances without a chromophore. However, in addition to some small individual disadvantages to these methods, the costs are high and applications are still kind of rare. Thus, the introduction of these devices at a broader level has not yet taken place. While this presumably will change over time, there is a need for methods that enable the impurity profiling of challenging substances with widespread analytics devices.

Methionine is a substance with hydrophobic and hydrophilic impurities. With the help of a mixed-mode stationary phase, which is a combination of a reversed phase and a strong cationic exchanger, the separation of all putative impurities was found possible with good sensitivity and selectivity. The method requires apart from the column only standard isocratic HPLC equipment and was successfully validated.

The evaluation of the enantiomeric purity of amino acids is challenging. Two approaches were made. The first method utilizes CE by means of in-capillary derivation with OPA and the subsequent separation with a cyclodextrin. With the use of OPA/NAC and γ-cyclodextrin, a simple and cost-effective method for the indirect enantioseparation of 16 amino acids was developed. With the second approach, racemic amino acids can be analyzed with HPLC and in-needle derivatization. For this, different columns and chiral thiols were evaluated and the chromatographic parameters were optimized. A method with OPA/NIBLC, a pentafluorophenyl column made the enantioseparation of 17 amino acids feasible. A LOQ of the minor enantiomer down to 0.04 % can be achieved with UV spectrophotometric detection. A similar method was developed for impurity profiling of L-amino acids. This can be used alternatively for the amino acid analysis performed by the European Pharmacopoeia.

A simple, robust, precise and accurate method for the evaluation of impurities in glyceryl trinitrate solution was developed and validated. The four impurities of glyceryl trinitrate are separated by means of an acetonitrile-water gradient and the assay for this substance is also possible.

# VI

# VI Zusammenfassung

Die Anforderungen an die Reinheitsanalytik von Substanzen für pharmazeutische Zwecke sind mit der Zeit größer geworden. Diese können durch die Verwendung von modernen instrumentellen Analysetechniken, die sich in den letzten Jahrzehnten entwickelt haben, erfüllt werden. Neue Arten von Säulen mit HILIC, mixed-mode und chiralen Phasen sind geeignet für die Trennung von allen möglichen Substanzgemischen, die vorher mit der Verwendung von gewöhnlichen Umkehrphasensäulen kaum möglich waren. Moderne, fast universelle Detektoren wie CAD, ELSD und CNLSD können für eine sensitive Detektion von Substanzen ohne Chromophor verwendet werden. Allerdings, neben ein paar individuellen Nachteilen von diesen Methoden, sind die Kosten sehr hoch und die Anwendungen noch eher selten. Daher haben sich diese Geräte noch nicht auf breiter Ebene durchgesetzt. Auch wenn sich das mit der Zeit ändern wird, gibt es die Notwendigkeit für Analysemethoden mit denen die Reinheitsanalytik von herausfordernden Substanzen auf verbreiteten analytischen Geräten ermöglicht wird.

Methionin ist eine Substanz mit hydrophilen und hydrophoben Verunreinigungen. Mit der Hilfe einer „mixed-mode“-Phase, welche eine Kombination aus Umkehrphase und starker Kationenaustauscher ist, wurde die Trennung von allen mutmaßlichen Verunreinigungen mit guter Sensitivität und Selektivität ermöglicht. Die Methode benötigt abgesehen von der Säule nur eine normale isokratische HPLC und wurde erfolgreich validiert.

Die Untersuchung der chiralen Reinheit von Aminosäuren ist anspruchsvoll. Zwei Ansätze wurden durchgeführt. Die erste Methode verwendet CE mittels In-Capillary-Derivatisierung durch OPA und der anschließenden Trennung mit Hilfe von einem Cyclodextrin. Durch den Gebrauch von OPA/NAC und γ-Cyclodextrin, wurde eine einfache und kosteneffektive Methode für die indirekte Enantioseparation von 16 Aminosäuren entwickelt. Mit dem zweiten Ansatz können Aminosäuren mittels HPLC und einer In-Needle-Derivatisierung analysiert werden. Dafür wurden verschiedene Säulen und chirale Thiole getestet und die chromatographischen Parameter optimiert. Eine Methode mit OPA/NIBLC, einer Pentafluorophenyl-Säule ermöglichte die Trennung on 17 Aminosäuren. Ein LOQ des kleinen Enantiomers von 0,04 % kann mit UV-spektroskopischer Detektion erreicht werden. Eine ähnliche Methode wurde für die Reinheitsanalytik von L-Aminosäuren entwickelt. Diese kann alternativ zur Aminosäurenanalyse im Europäischen Arzneibuch verwendet werden.

Eine einfache, robuste, präzise und richtige Methode zur Untersuchung der Verunreinigungen in Glyceryltrinitratlösung wurde entwickelt und validiert. Die vier Verunreinigungen von Glyceryltrinitrat werden mit Hilfe eines Acetonitril-Wasser-Gradienten getrennt und die Gehaltsbestimmung dieser Substanz ist ebenfalls möglich.

# VII

# VII Appendix

# 1. List of Publications and Documentation of Authorship

**1** **Impurity Profiling of L-Methionine by HPLC on a Mixed Mode Column**

Kühnreich, Raphael; Holzgrabe, Ulrike

*Journal of Pharmaceutical and Biomedical Analysis*, Volume 122, 15 April 2016, Pages 118-125

doi: 10.1016/j.jpba.2016.01.057

**2** **Indirect Enantioseparation of Amino Acids by CE Using Automated In-Capillary Derivatization with *ortho*-Phthalaldehyde and *N*-Acetyl-L-Cysteine**

Kühnreich, Raphael; Holzgrabe, Ulrike

*Chromatographia*, Volume 79, 17 June 2016, Pages 1013-1022

doi: 10.1007/s10337-016-3122-0

**3** **High Performance Liquid Chromatography Evaluation of the Enantiomeric Purity of Amino Acids by Means of Automated Precolumn Derivatization with *ortho*-Phthalaldehyde and Chiral Thiols**

Kühnreich, Raphael; Holzgrabe, Ulrike

*Chirality*, Volume 28, 29 November 2016, Pages 795-804

doi: 10.1002/chir.22660

This section contains a list of the individual contribution for each author to the publications reprinted in this thesis.

P1 **Kühnreich R, Holzgrabe U,** Impurity profiling of l-methionine by HPLC on a mixed mode column. *Journal of Pharmaceutical and Biomedical Analysis* 122 (2016), 118-125.

| **Author** | 1 | 2 |
|---|---|---|
| Study Design and concept development | x | x |
| Experimental work | x | |
| Data analysis and interpretation | x | |
| Manuscript planning | x | x |
| Manuscript writing | x | |
| Correction of manuscript | x | x |
| Supervision of Raphael Kühnreich | | x |

P2 **Kühnreich R, Holzgrabe U,** Indirect Enantioseparation of Amino Acids by CE Using Automated In-Capillary Derivatization with *Ortho*-Phthalaldehyde and N-Acetyl-L-Cysteine. *Chromatographia* 79.15-16 (2016), 1013-1022.

| **Author** | 1 | 2 |
|---|---|---|
| Study Design concept development | x | x |
| Experimental work | x | |
| Data analysis and interpretation | x | |
| Manuscript planning | x | x |
| Manuscript writing | x | |
| Correction of manuscript | x | x |
| Supervision of Raphael Kühnreich | | x |

P3 **Kühnreich R, Holzgrabe U,** High Performance Liquid Chromatography Evaluation of the Enantiomeric Purity of Amino Acids by Means of Automated Precolumn Derivatization with *Ortho*-Phthalaldehyde and Chiral Thiols. *Chirality* 28 (2016), 795-804.

| **Author** | 1 | 2 |
|---|---|---|
| Study Design concept development | x | x |
| Experimental work | x | |
| Data analysis and interpretation | x | |
| Manuscript planning | x | x |
| Manuscript writing | x | |
| Correction of manuscript | x | x |
| Supervision of Raphael Kühnreich | | x |

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Die Beiträge der Mitautorin an den Publikationen ist in den vorausgehenden Tabellen aufgeführt.

| | | |
|---|---|---|
| Prof. Dr. Ulrike Holzgrabe | _____ | _____ |
| | Ort, Datum | Unterschrift |
| Raphael Kühnreich | _____ | _____ |
| | Ort, Datum | Unterschrift |

# 2. Conference Contributions

Kühnreich R., Holzgrabe U.

*Chiral separation of amino acids by HPLC and CE using online derivatization with ortho-phthalaldehyde and a chiral thiol*

DPhG Jahrestagung, 2014, Frankfurt am Main

# 3. Abbreviations

| | |
|---|---|
| AA | amino acid |
| AAA | amino acid analysis |
| ADA | anthracene-2,3-dialdehyde |
| BGE | background electrolyte |
| CBQCA | 3-(4-carboxybenzoyl)-2-quinolinecarboxaldehyde |
| CD | cyclodextrin |
| CDA | chiral derivatization reagent |
| CE | capillary electrophoresis |
| CNLD | chemiluminescent nitrogen detector |
| CNLSD | condensation nucleation light scattering detector |
| CSP | chiral stationary phase |
| Dabsyl-Cl | 4-(4-dimethylaminophenylazo)benzenesulfonyl |
| Dansyl-Cl | 5-(dimethylamino)naphthalene-1-sulfonyl chloride |
| ELSD | evaporative light scattering detector |
| FDAA | 1-fluoro-2-4-dinitrophenyl-5-L-alanine amide |
| FLD | fluorescence spectrophotometric detection |
| FMOC-Cl | fluorenylmethyloxycarbonyl chloride |
| HPLC | high performance liquid chromatography |
| IS | internal standard |
| LE | ligand exchange |
| LOD | limit of detection |
| LOQ | limit of quantification |
| ME | 2-mercaptoethanol |
| MPA | 3-mercaptopropionic acid |
| N.D. | non-detectable |
| N.Q. | non-quantifiable |
| NAC | *N*-acetyl-L-cysteine |
| NDA | naphthalene-2,3-dicarboxaldehyde |
| NIBLC | *N*-isobutyryl-L-cysteine |
| OPA | *ortho*-phthalaldehyde |
| Ph.Eur. | European Pharmacopoeia |
| PITC | phenylisothiocyanate |
| Rs | resolution |
| RSD | relative standard deviation |
| S/N | signal-to-noise ratio |
| SD | standard deviation |
| UV | ultraviolet spectrophotometric detection |
| $\lambda_{em}$ | emission wavelength |
| $\lambda_{ex}$ | excitation wavelength |