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Advanced method development strategy for derivatization LC/ESI/MS

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In memory of my grandmother
Dr. Lehte Rebane

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LIST OF ORIGINAL PUBLICATIONS

I. **Rebane, R.**; Herodes, K. Evaluation of the Botanical Origin of Estonian Uni- and Polyfloral Honeys by Amino Acid Content. Journal of Agricultural and Food Chemistry, 56(22), 2008, 10716–10720.

II. **Rebane, R.**; Herodes, K. A sensitive method for free amino acids analysis by liquid chromatography with ultraviolet and mass spectrometric detection using precolumn derivatization with diethyl ethoxymethylenemalonate: Application to the honey analysis. Analytica Chimica Acta, 672 (1–2), 2010, 79–84.

III. **Rebane, R.**; Herodes, K.; Leito, I. Analysis of Selenomethylselenocysteine and Selenomethionine by LC-ESI-MS/MS with diethyl ethoxymethylenemalonate derivatization. Analyst, 136(24), 2011, 5241–5246.

IV. **Rebane, R.**; Herodes, K. Influence of boric acid on ESI ionization efficiency. European Journal of Mass Spectrometry, 18(1), 2012, 71–75.

V. **Rebane, R.**; Herodes, K. Comparison of three buffer solutions for amino acid derivatization and following analysis by liquid chromatography electrospray mass spectrometry. Journal of Chromatography A, 1245, 2012, 134–142.

VI. **Rebane, R.**; Oldekop, M-L.; Herodes, K. Comparison of amino acid derivatization reagents for LC-ESI-MS analysis. Introducing a novel phosphazene-based derivatization reagent. (submitted to Journal of Chromatography B).

Author's contribution

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ABBREVIATIONS

%ME	Matrix effect
2-NA	2-nitroaniline
Deemm	Diethyl ethoxymethylenemalonate
DMG	Dimethyl glutarate
DNS	1-dimethyaminonaphthalene-5-sulfonyl chloride, dansyl chloride
DPA	Diphenylamine
DPhP	Diphenyl phthalate
ESI	Electrospray ionization
$EtGlyP1Pyrr_3$	Carbethoxymethylimino-tri(pyrrolidino)phosphazene
FL	Flouresence
Fmoc-Cl	9-fluorenylmethyl chloroformate
FOSF	2,5-dioxopyrrolidin-1-yl *N*-tri(pyrrolidino)phosphoranylideneamino carbamate
HFIP	Hexafluoroisopropanol, 1,1,1,3,3,3-hexafluoro-2-propanol
HPLC	High performance liquid chromatography
IE	Ionization efficiency
MeCN	Acetonitrile
MeOH	Methanol
MS	Mass spectrometry
RIE	Relative ionization efficiency
RPLC	Reversed phase liquid chromatography
SCX	Strong cation exchange
SeMet	Selenomethionine
Se-MeSeCys	Se-methylselenocysteine
SPE	Solid phase extraction
TAHS	p-*N,N,N*-trimethylammonioanilyl *N′*-hydroxysuccinimidyl carbamate iodide
TEA	Tetraethylammonium perchlorate
TFA	Trifluoroacetic acid
UV-Vis	Ultraviolet visible

1. INTRODUCTION

Amino acids are among analytes that are analyzed in the wide range of disciplinaries, from investigation of brain activity to the discrimination of the origin of coffee. From the viewpoint of an analytical chemist, the main objective is to develop and validate an analytical method that is suitable for its purpose and in most cases allows very sensitive detection.

Due to the constant pursuit for more sensitive analysis, liquid chromatography mass spectrometry (LC/MS) with electrospray ionization (ESI) has become one of the most popular analysis techniques for many analytes, including amino acids. Analysis of amino acid has been around for a long time and there has been a continuous progress from classical analytical methods towards more sensitive methods such as LC/ESI/MS. Still, even more sensitive methods are constantly sought for.

In majority of cases, amino acid analysis entails derivatization. Depending on the analysis method, reasons vary, but the use of derivatization reagents has been proposed as one approach to signal enhancement in the LC/ESI/MS analysis. Derivatization reagents are often those, which have been designed for classical LC detection methods such as ultraviolet and fluorescence. In recent years there has been a course towards the design of the special derivatization reagents for amino acids that have been designed for detection at trace levels. In most cases, these are based on the reagents designed for UV and FL detection. But there are other aspects to consider than just changing the derivatization reagent. Aspects such as how to carry out a complete method development that would be compatible with ESI/MS, becomes the underlying problem.

This work is a first systematic approach for development of the amino acid analysis with LC/ESI/MS. It entails aspects such as sample preparation, derivatization buffers, chromatographic analysis (eluent suitability) and mass spectrometric detection (possibilities for signal suppression and enhancement).

In order to get an overview, all aspects are discussed together for five derivatization reagents: commercially available amino acid derivatization reagents (dansyl chloride, 9-fluorenylmethyl chloroformate, and diethyl ethoxymethylenemalonate), a new reagent presented in literature (p-*N,N,N*-trimethylammonioanilyl *N*′-hydroxysuccinimidyl carbamate iodide) and a phosphazene based reagent developed in this work.

There are various tools used in the work. A test set of seven naturally occuring amino acids is used for the comparison of derivatization reagents and their properties. Standard amino acid derivatives of Deemm and Fmoc-Cl are used to evaluate the ionization efficiencies and possibilities for signal enhancement with boric acid. Compatibilities with sample preparation, derivatization buffers and eluents are also under interest. Moreover, different possible signal enhancement techniques are tested. Finally, two applicatons have been developed, one for amino acid analysis in honey and a second one for a sensitive analysis of selenoamino acids.

2. REVIEW OF LITERATURE

2.1. Method development

Analyzing various compounds in different matrices is one of the main doings of analytical chemistry. There is a constant need for more sensitive techniques to analyze lower and lower concentrations for various applications. Over the time the possibilities for method development are growing and the design of an experiment is a difficult task for any analytical chemist. Often the time is limited in order to give an in depth approach to every step of the method. For complex matrices and low concentrations of analytes, the LC/ESI/MS is often the method of choice. In addition to low limits of detection, it enables to confirm the identity of analytes and identify unknown compounds [1]. However, in most cases, new methods are designed in a way that they are modifications of previously developed methods. Rarely are methods developed from the very beginning keeping in mind all the aspects of that certain sample matrix and the analyte – sample preparation, derivatization, chromatographic separation, mass spectrometric analysis and aspects related to these topics.

The following work is considering all these aspects and LC/ESI/MS analysis of amino acids is taken as a model case.

Method comparability

To evaluate the properties of a method and compare methods to each other, LoD and LoQ values are often used in addition to other parameters such as recovery, reproducibility, selectivity etc.

One of the aspects when considering LoD/LoQ topic is the representation of the results. In addition to different ways of determining the values, representation can be one of the following: ng/mL, fmol, pg, mg/kg, nmol/mL or modification of these [2,3]. This often complicates the understanding of the real sensitivity of the methods. In many cases, it is not stated whether the values are for standards or samples or even if the concentrations are denoting the concentration before or after the derivatization in the case of methods that use derivatization.

Moreover, in many cases, if very low LoD and LoQ values are desired, modifications are added to the system and the lowest possible values are sought for. On the other hand, there are methods that are applied to samples with quite high analyte content and the lowest possible LoD/LoQ values are probably not obtained since there is no need. However, this may show that the method is not as sensitive. Therefore, it is important that the comparison of analytical methods, in this case amino acid analysis with derivatization, should be compared in the equal grounds in order to get the objective results.

2.2. Amino acid analysis

2.2.1. Sample matrix

Quantification of all or certain amino acid(s) is done in all types of biological samples ranging from human bodily fluids and tissues to various foods. For example, in clinical biochemistry, the change of amino acid concentrations in human serum can be correlated to certain diseases [4]. As important constituents of food, amino acids supply the required building blocks for protein biosynthesis and directly contribute to the flavor of food and are precursors for aroma compounds [5]. The change of amino acid concentrations is also used to follow fermentation and ripening processes [6]. For different foods and drinks, amino acid profiles vary among the same type of products of different origin [7]. Therefore, they can be useful to identify origin of some type of foods or drinks, as well as provide information about possible adulteration. Amino acid analysis has been applied to honey samples to identify their origin [Paper I, 8–12]. It can be concluded that analysis of amino acids can be applied to large variety of matrices.

2.2.2. Sample preparation

When dealing with complex matrices, it is necessary to carry out sample preparation. Reasons for sample preparation vary and many aspects are to be considered when choosing a suitable method. LC/ESI/MS is very sensitive towards the compounds in sample matrix that are not of interest (see Section 2.2.8) and therefore, in most cases, it is desirable to remove as much of the matrix as possible from the analyte and for this, sample preparation is applied. It has been emphasized, that in order to gain lower limits of detection and better accuracy, an optimized extraction and cleanup protocol becomes crucial [13]. Moreover, sample preparation helps to concentrate the analyte and this is of interest for samples where the analyte concentration is very low compared to the sample size. In most cases sample preparation has to be given a careful look depending on the sample, further analysis etc.

There are many types of sample preparation techniques available, extraction being one of the most popular ones, including liquid-liquid extraction (LLE) and solid phase extraction (SPE). For biological samples, protein precipitation (PPT) is also common. It has been found, for phospholipids with LC/ESI/MS analysis, that PPT is the least effective sample preparation technique, often resulting in significant matrix effects due to the presence of many residual matrix components. Reversed-phase and pure cation exchange SPE methods resulted clearer extracts and reduced matrix effects compared to PPT. LLE also provided clean final extracts, however, analyte recovery, particularly for polar analytes, was very low.[14]

Amino acids are zwitterions and at low pH they are positively charged. Meaning that they are bound to the resin by their attraction to the negatively charged ion-exchange sites and strong cation exchange (SCX) resins can be applied to separate them from rest of the sample matrix [15]. Strong cation exchange resins have been used to separate amino acids and selenoamino acids from honey and serum, respectively [Paper I, Paper II, Paper III,11,12,16].

Method development for SPE is rigorous, accompanying wide range of parameters that need to be optimized, the main being the acquisition of the highest recovery rate possible. In order to obtain the highest recovery rate, all the steps of SPE have to be optimized: solvent for the activation of the sorbent, solvent for sample applying, washing solution and one of the crucial steps is the optimization of the elution solvent composition. For all these steps, the compositions, concentrations, quantities and applying speeds have to be under close investigation. Once all aspects are carefully optimized, for most methods, including for amino acid analysis in honey, high recovery rates can be obtained with SCX [Paper II].

One of the things to keep in mind when choosing a sample preparation technique is that in what state/solvent is the analyte after the sample preparation. It is desirable that there would be no solvent present in order to dissolve the sample extract with the analyte in a solvent that is suitable for the next step in the analysis. For example, if the next step is chromatographic analysis, then chromatographic solvent is preferred as a solvent. However, in the case of derivatization, solvent compatible with derivatization procedure is preferred. This requirement is satisfied by SCX (and other SPE techniques) where the eluate can be evaporated to dryness using nitrogen flow and then the residue can be dissolved in a desirable solvent [Paper I, Paper III].

Once amino acids are separated from the sample matrix with SCX, these can be directly analyzed with LC/ESI/MS [17]. Analysis of free amino acids with LC/ESI/MS^2 is possible, but the additional improvement in sensitivity of detection is needed and has been obtained with the use of an acidic mobile phase. Proposed LoD values are between 0.1 and 40 pmol [17]. Moreover, free amino acids in various foods have also been analyzed with LC/APCI/MS. LoD values of 0.01–0.17 μg/ml in apple juice have been reported [18].

However, for amino acids, for most optimal analysis, it is necessary to carry out the derivatization procedure.

2.2.3. Amino acid derivatization

Even though analysis of underivatized analytes is possible, derivatization reagents for the chromatographic determination of primary and secondary amines as well as tertiary amino groups are available. When these analytes are analyzed by HPLC with direct ultraviolet-visible (UV-Vis) detection, there are two main resons to use derivatization. First, when analytes have weak UV-Vis absorption, in order to enhance the sensitivity, derivatization of amines with a reagent

having a strong UV-Vis absorbing structure is one of the considerable methods to choose from. Moreover, the use of derivatization in separation sciences is to improve the chromatographic properties.[19]

Derivatization in LC/ESI/MS^2 is employed for various reasons: to increase detection sensitivity and selectivity, improve chromatographic retention or peak shape, eliminate sample carryover, facilitate sample cleanup, and to form a stable derivative for unstable analytes [20]. In addition, for mass spectrometric analysis, derivatization brings about an increase in the molecular weight and this means that the background noise from the matrix is reduced (since background is generally lower in the higher mass range) [21].

Derivatization reagents

If a derivativatization reaction is applied, it should: (1) react fast and quantitatively to completion; (2) be specific to a certain functional group with few by-products; and (3) form relatively stable products [22].

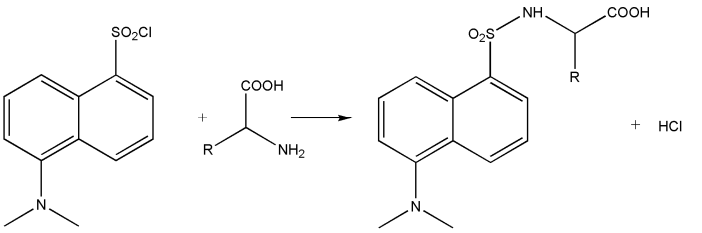
Structure of amino acid contains both a carboxylic group and an amino group, which could serve as the functional groups in derivatization. Derivatization from the amino group is predominant and for carboxylic group one of the few examples available is derivatization with butanolic HCl for analysis with ESI/MS/MS without chromatographic separation [3] and with chromatographic separation [23]. There is also a less common derivatization reagent called propyl chloroformate that reacts with both functional groups and that has been used for analysis of amino acids in rat brain microdialysates [23]. However, the most popular reagents for amino acids are for derivatizations of the amino group and therefore the rest of the work is focused on these.

For the analysis of amino acids with LC/ESI/MS, one of the approaches for choosing a derivatization reagent is to use commercially available UV absorbance or fluorescence (FL) detection tags such as dansyl chloride (DNS) [24], 9-fluorenylmethyl chloroformate (Fmoc-Cl), and diethyl ethoxymethylenemalonate (Deemm). These have been used for amino acid analysis with LC/ESI/MS [Paper III, 23]. In recent years there has been a rapid growth in designing and developing amino acid derivatization reagents that are specially meant for LC/ESI/MS applications: (5-N-succinimidoxy-5-oxopentyl)triphenylphosphonium bromide (SPTPP) [25], 3-aminopyridyl-N-hydroxysuccinimidyl carbamate (APDS) [26], *N*-hydroxysuccinimide ester of *N*-alkylnicotinic acid (C_n-NA-NHS) [27], p-*N,N,N*-trimethylammonioanilyl *N′*-hydroxysuccinimidyl carbamate iodide (TAHS) [28] and *N,N*-dimethyl-2,4-dinitro-5-fluorobenzylamine (DMDNFB) [29].

Derivatization reagents also differ by their properties that are independent of the separation technique or detector used. Such parameters are for example the speed of the derivatization reaction and also the stability of the derivatives.

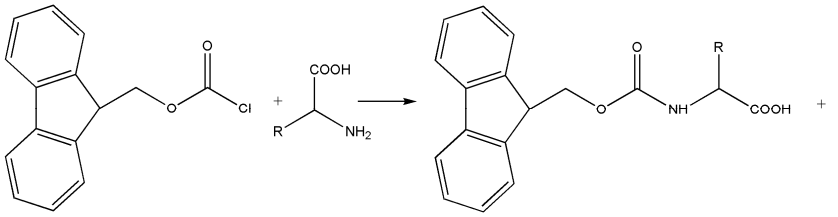
DNS (Equation 1) is a sulfonyl chloride type of derivatization reagent [22] and is used for the derivatization of primary and secondary amines [19].

Derivatization schemes for DNS vary largely. For example, in some cases, heating is applied [24,30]. However, reaction does proceed at the room temperature also [31,32]. Large excess of DNS should be avoided since it can react with the carboxylic acid group of the already labeled amino acid, giving rise to an unstable mixed anhydride, which decomposes into DNS-NH_2 and an aldehyde. If DNS-derivatives are stored in the dark and below –0°C, they can be stable for several weeks.[30]



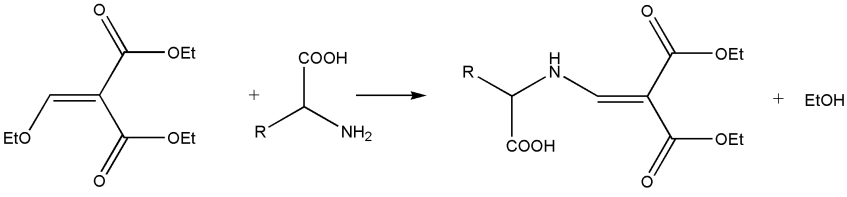
(1)

Fmoc-Cl (Equation 2) is a chloroformate-containing reagent and it reacts with primary and secondary amines and derivatives of amino acids are produced within 30 s (except acidic amino acids, which require a longer reaction time) [22]. The amino acids derivatized with Fmoc-Cl are reported to be stable at room temperature for at least 3 days. The drawback of Fmoc-Cl is the excess of unreacted reagent, which disturbs the analysis of some amino acid derivatives with FL detection.[23]

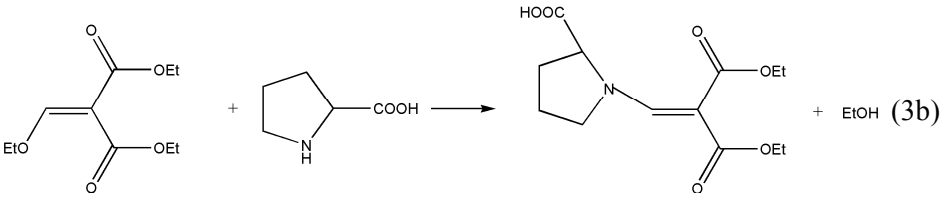


(2)

Deemm (Equation 3) can also derivatize both primary and secondary amines and has a fast reaction in the beginning (except for Pro (Equation 3b)) and then the reaction continues at a lower rate. Therefore, further analysis is recommended in 24–48h. The stability of amino acid derivatives other than Proline is up to 7 days or more when kept at –20°C.[Paper II,33]

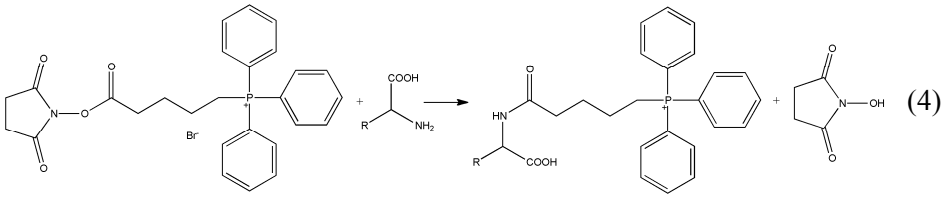


(3a)



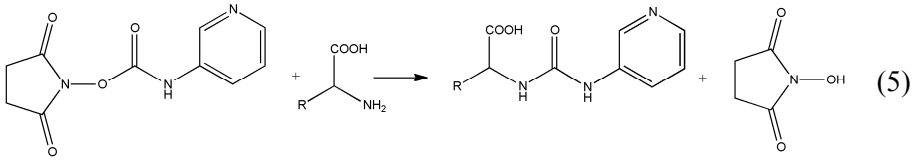
(3b)

SPTPP (Equation 4) reacts with amino acids smoothly and with high efficiency. The optimized reaction temperature is 40°C and the reaction period of 10 min. The derivatization yields for amines and amino acids are ca. 80% at least. In addition, SPTPP-derivatized amines and amino acids are stable at least 2 days when the reaction mixtures are stored at 5–10°C.[25]



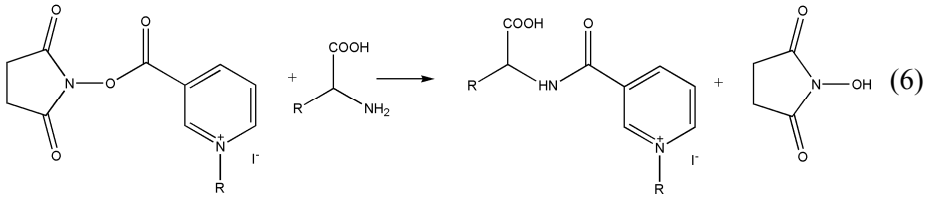
(4)

The reactivity of APDS (Equation 5) is very high and is reported to react with amino groups within a minute even at room temperature. An additional 5 min of heating is needed for the decomposition of the excess reagent solution, which reacts also with the phenolic hydroxyl group of tyrosine (Tyr-APDS). The APDS-derivatives are stable when kept in the autosampler tray at least overnight at 10°C. The reagent solution is stable for at least 1 week at 4°C.[26]



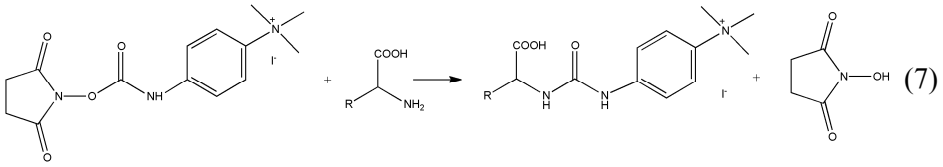
(5)

N-Acylation of amino acids with the C_n-NA-NHS (Equation 6) reagents in water produces a stable product in roughly 1 min. C_n-NA-NHS solution is stable for 5 days at ambient temperature or at least one month at 4 °C.[27]



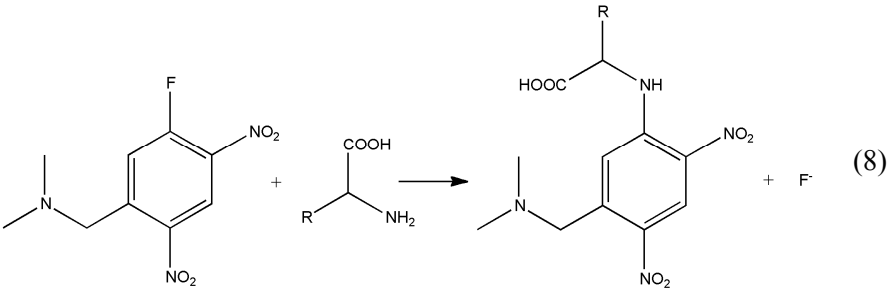
(6)

Since TAHS (Equation 7) is a carbamate-containing reagent such as APDS and C_n-NA-NHS and these are known for for their rapid and selective reactions with amino groups under mild conditions, the reaction between TAHS and amino acids is complete in a minute at room temperature. Since phenolic hydroxyl group also reacts with TAHS reagent, further heating (more than 5 min at 55°C) is needed for hydrolysis of the phenolic hydroxyl group of tyrosine. [28]



(7)

Reaction between amino acids and DMDNFB (Equation 8) can be as long as 2h and the unreacted DMDNFB is removed by extraction with diethyl ether. Derivatization procedure is not yet fully researched. However, results show that there is a considerable competition among the various amino acids for reaction with DMDNFB under conditions of evidently non-stoichiometric derivatization and a larger excess of reagent and elevated temperatures would be needed for complete conversion of the amino acid mixture. [29]



(8)

Out of these described reagents, APDS is targeted towards better chromatographic separation [26], but SPTPP [25] and TAHS [28] for sensitive analysis in positive ion mode. Moreover, TAHS, reputedly by the authors, is the most sensitive out of these novel derivatization reagents [28].

Depending on the analysis method and derivatization reagent, derivatization can increase sensitivity up to 500-fold compared to those of underivatized analytes, in the case of SPTPP [25] or 200-fold in the case of DNS-glutamic acid [24]. However, there are reports where underivatized analytes offer better signal than DNS derivatized analytes [31,34]. Therefore, a yield of derivatization procedure is strongly dependent on each individual case.

Optimization of the derivatization procedure

There are various aspects that have to be addressed when using derivatization reactions. Often times derivatization reactions require well-optimized conditions for the derivatization reaction to proceed at the highest rate and also in order to obtain cleaner chromatograms. Aspects such as solvent (concentration), buffers (pH, concentration), time of reaction before termination, temperature of reaction and the choice of derivatization reaction termination reagent are under close interest [35,36]. Optimization of commercially available derivatization reagents is widely publicized since there are tens (hundreds) of articles that employ derivatization for amino acid analysis and therefore a considerable amount of information is available about the most optimal conditions for certain sample matrices and also amino acids under interest.

Commercially available derivatization reagents have been designed for UV-Vis or FL detectors and most new reagents are also based on similar mechanisms. For example, TAHS is designed from disuccinimido carbonate (DCS) which is a derivatization reagent designed for UV-Vis. TAHS is a structural modification of that reagent [28]. Newly designed derivatization reagents have gotten less attention from the derivatization optimization point of view since often the only information about a reagent of interest is available in the article where it is first mentioned and wide optimization of derivatization procedure is not carried out. Moreover, since ESI/MS is much more sensitive towards other compounds present in the ESI source [37], in addition to classical above-mentioned parameters that can be optimized, new aspects related to the compatibility of the derivatization mixture to the LC/ESI/MS are added to list.

For various amino acid derivatization reactions pH 9 is needed. This could be provided by ammonium buffers, but since these would react with amino acids, they are not suitable and in majority of cases, borate buffer is the choice for most reagents [Paper II, Paper III,23,28,33,35,38–46]. Buffers that can provide pH around 9, are carbonate and hexafluoroisopropanol (HFIP) buffers. Carbonate buffer has been used before in Fmoc-Cl derivatization and FL detection [47–50]. HFIP has not been used for amino acid derivatization but it has been used in MS analysis as an eluent buffer component since it is volatile [51,52]. This makes it different from carbonate and borate and possibly more suitable for LC/ESI/MS applications.

2.2.4. Chromatographic analysis

Liquid chromatographic systems with various stationary phases (type and size), column internal diameter and length, and mobile-phase compositions can be used in a derivatization-based LC/MS analytical platform [22]. However, when analyzing complex mixtures, chromatographic separation of amino acids is necessary. Reversed phase liquid chromatography (RPLC) constitutes a powerful tool for the separation of very heterogeneous samples and has been widely

used in combination with mass spectrometric detection. RPLC is the chromatographic mode which best matches ESI/MS since the used mobile phases present low ionic strength and contain organic modifiers.[53]

One of the problems with LC/ESI/MS analysis is matrix effect and therefore ESI/MS detection needs chromatographic separation in order to provide the best outcome and sensitivity by decreasing the suppression of ionization related to the co-elution of sample matrix components.[17,54]

Hydrophilic amino acids do not interact with the hydrophobic stationary phase of a reversed phase column, typically eluting in the column void volume, meaning, that a better chromatographic separation is sought for [27].

Chromatographic separation depends on many aspects of analysis. One of the main aspects is the structure of an analyte. Analysis of underivatized amino acids is possible but in addition to improvement of UV-Vis absorbance, derivatization is also used to enhance separation. All previously discussed derivatization reagents react with amino group and therefore, following the derivatization, this group is lost, polarity is changed and carboxylic acid group becomes more significant and responsible for chromatographic separation [55]. Depending on their structure, derivatization reagents differ by how easily are the amino acid derivatives separated by RPLC. New derivatization reagents such as TAHS have high hydrophilicity and it is known that therefore TAHS derivates are more difficult to separate [28] as opposed to APDS, which has better separation properties due to the comparatively higher hydrophobicity of the pyridine moiety in the reagent [26]. However, compared to the underivatized amino acids, TAHS is designed to contain a hydrophobic phenyl naphthyl and combined with the activated carbamate, derivatives have increased retention in RPLC [28].

For reagents such as Deemm, Fmoc-Cl and DNS, chromatographic separation is dependent on various parameters such as column type (chemistry, size, length), choice of eluents (buffers and organic phase) and also on the gradient used for separation. Therefore, due to all different modifications of these parameters, as well as the matrix used, separation strongly varies case by case. But in most cases, it is known that the chromatographic separation of 23 amino acids is most likely to be difficult [19]. However, Deemm provides separation for all 23 amino acids [Paper II,45]. For Fmoc-Cl and DNS full separation can be achieved with well-optimized conditions [35,36,47,56].

In general, there are recent developments in chromatographic supports and instrumentation for LC that enable rapid and highly efficient separations. Various analytical strategies have been proposed, such as the use of silica-based monolithic supports, elevated mobile phase temperatures, and columns packed with sub-3 μm superficially porous particles or with sub-2 μm porous particles for use in ultra-high-pressure LC [57]. However, all these strategies have advantages and drawbacks and are not widely used for analysis of amino acid derivatives.

In conclusion, chromatographic separation of amino acid derivates depends by large on the structure of a derivate and on the extent that the separation is optimized.

2.3. Derivatization LC/ESI/MS

LC/ESI/MS has become the analytical tool of choice for identification and quantification of low molecular weight compounds. LC/MS analysis combines the separation capacities of the LC system with the sensitivity and (especially with MS/MS) the specificity of detection provided by MS systems. However, two major problems are associated with LC/MS analysis. First, no universal column packing material can be used for all possible kinds of analytes. Second, no eluent system is compatible with both all possible analytes and ESI. A compromise has to be made at some level regarding the packing material, eluent system, or analyte response. For analysis of bases, ribosides and intact nucleotides, it has been found that positive ESI is an excellent interface for RPLC. However, many biologically important compounds do not separate readily on reversed-phase packing material due to their high polarity.[58]

Derivatization chemistry has an important history in separation science, and with that knowledge in mind, an alternative would be to make the analytes more hydrophobic, thus improving both the RPLC separation and the ESI process. For LC/ESI/MS, derivatization has been used for signal enhancement for various types of analytes – phenols, thiols, carboxylic acids, amines and amides, etc.[2,3,22,54,58]

Another aspect with derivatization reagents designed for ESI/MS is that when amino acids are derivatized and therefore have a better retention with RPLC, they are eluting at higher organic solvent content. This, however, is suitable for generation of charged droplets by electrospray and therefore gives better ESI response.[59]

Derivatization for LC/MS analysis could be an alternative strategy to tackle some difficult analytical problems. Some of the advantages of derivatization include: (1) increase analyte stability during sampling, storage, preparation, and analysis; (2) improve extraction efficiency and selectivity; (3) increase retention time of polar compounds on commonly used reverse-phase columns; (4) increase HPLC separation selectivity; (5) enhance analyte nebulization ionization efficiency in an MS interface; (6) increase analyte molecular weight and improve MS selectivity; (7) facilitate structure elucidation of a specific chemical group; (8) aid fragmentation for compounds that are difficult to fragment especially in a multiple reaction monitoring mode; (9) enable protein and peptides analysis with a stable isotope-labeling and/or fluorescent-labeling reagent; and (10) expand the linear range of a calibration graph, etc.[22]

2.3.1. Ionization Efficiency Scale

In ESI source, ionization of some analytes may be highly efficient, but other analytes are not ionizable at all. In most cases, only a fraction of the analyte molecules (or ions) in the liquid phase that is sprayed into the ESI source, are eventually converted to gas-phase ions (via protonation, adduct formation, deprotonation, etc.). The term ionization efficiency (IE) is used to express the extent to which analyte molecules in liquid phase are converted to gas phase ions and eventually detected in detector. In literature, by IE, the efficiency of generating gas-phase ions from analyte molecules or ions in the ESI source, efficiency of ion transport and detection efficiency is meant. An approach for quantifying ESI efficiencies (as logIE values) and setting up a self-consistent quantitative experimental ESI efficiency scale of organic compounds under predefined ionization conditions (ionization by monoprotonation) has been developed. Using this approach, a logIE scale containing 62 compounds of different chemical nature and ranging for 6 orders of magnitude has been established. The scale is based on over 400 relative IE (**Δ**logIE) measurements between more than 250 different pairs of compounds.[60,61] This scale gives information about how different analytes have different ionization efficiencies.

This scale can be used for prediction of IE of molecules based on their structure. In the scope of amino acid analysis by derivatization, this scale can be used as a reference when designing new derivatization reagents.

2.3.2. Design of new derivatization reagents

Even though UV/FL-detector designed derivatization reagents have been used for amino acid analysis with LC/ESI/MS [Paper III,23,31], their suitability for that type of analysis has not been under close investigation. Suitability of Fmoc-Cl for LC/ESI/MS has been evaluated and concluded that it is less suitable for the quantitative analysis of amino acids due to the method's poorer repeatability, linearity and higher limits of detections than those achieved by butanol and propyl chloroformate derivatives [23]. DNS has been commonly used to improve the detection in the positive-ion ESI mode, because it contains a basic secondary amine moiety, which is helpful to pre-formation of ions with an acidic mobile phase [20,22,54].

As previously discussed, for MS detection oriented derivatization, the aim is to increase evaporation of the solvent and nebulization efficiency or introduce chargeable or easily ionizable moieties. Less frequent reasons for MS derivatization include the fragmentation aid in the multiple reaction monitoring mode, identification of specific chemical groups and the correction and extension of linear dynamic range of calibration graph.[22]

There have been specially designed derivatization reagents for amino acid LC/ESI/MS analysis (DMDNFB, TAHS, SPTPP, C_n-NA-NHS, APDS) and their design principles are discussed below.

One of the derivatization reagents is DMDNFB, that is a redesign of DNFB. Although 19 amino acids can be examined and derivatized with DNFB and are acceptably resolved with chromatography, only three of the amino acids were observed in the positive ion mode of electrospray ionization. However, when DNFB was redesigned into novel reagent called DMDNFB, the DMDNFB-derivatized amino acids exhibited larger positive ESI response, which was attributed to the introduction of the *N,N*-dimethylaminomethyl protonatable site. Moreover, DMDNP derivatization is superior in chromatographic separation. Detection limit for DMDNP-Leu was 5 ng/mL.[29]

The reportedly most sensitive derivatization reagent designed for ESI/MS is TAHS, which provides detection at subfemtomole to attomole levels. Since TAHS was designed for triple-stage quadrupole mass spectrometer, the idea was to have a characteristic and selective cleavage at the bonding site between the reagent moiety and the amino acid in the collision cell. Therefore, ureide bond (R-NH-(C=O)-NH-R'), in which R is the component of the amino compounds and R' is the reagent moiety, was found to facilitate analysis. To form the ureide bond with the amino groups of the analytes, activated carbamate-containing reagent was chosen.[28] To achieve effective ionization, TAHS is designed to have a phenyl group as hydrophobic moiety and cationic group as ionic moiety (trimethylammonium group), meaning that ESI positive ion mode response is enhanced via a charged quaternary ammonia that aids the formation of an intense product ion for multiple reaction monitoring.[22,28]

Another reagent designed for MS analysis is SPTPP. SPTPP has a permanently positively charged quaternary phosphonium functional group. For synthesis, (4-Carboxybutyl)-triphenylphosphonium bromide was reacted with N-hydroxysuccinimide and esterized to form SPTPP. Materials used for SPTPP are mentioned to be quite inexpensive and the synthetis procedure is not complicated. Detection limits were in the range of 0.015–0.43 fmol.[25]

New reagent C_n-NA-NHS has been designed by combining the effect of having both a hydrophobic and quaternary amino groups in proximity. Lengthening the alkyl chain in the hydrophobic quaternary amine portion of derivatized amino acids increases their surface-active properties and directs them to the surface of electrospray droplets facilitating ionization.[27]

In the case of APDS, by making it more hydrophobic compared to the corresponding TAHS derivatives, the design of a derivatization reagent is towards better chromatographic separation [26].

Design of a new derivatization reagent can also take into account the fragmentation patterns of derivatized analytes if a certain scan mode is preferred: neutral loss scan, selected/multiple reaction monitoring, precursor ion scan or fragment ion scan. Two kinds of fragmentations are possible. First, it is possible that the leaving group has a constant mass like in the case of Deemm and all analytes will have a different *m/z* after fragmentation [Paper III]. The second case is where all analytes will result with fragments with the same *m/z* like in the case TAHS, APDS, and SPTPP [25,26,28]. TAHS has been

knowingly designed for tandem mass spectrometry with a fragmentation that could allow the use of precursor ion scan [28].

In conclusion, when designing new derivatization reagents, in addition to the aspects that must be fulfilled no matter the detection used, the following conditions should be satisfied when derivatization reagents are used in LC/MS/MS: (1) the derivatized analytes should have high ionization efficiency and they should be detected with high sensitivity; (2) it should react with the target functional group under mild conditions; (3) it should have a hydrophobicity that is appropriate for the separation of the derivatized analytes by a reversed-phase system; (4) it should be less susceptible to ion suppression; (5) fragmentation of the derivatized target analytes could be accomplished more easily by collision-induced dissociation and efficiently generate a particular product ion for sensitive MS/MS detection; and (6) it should be inexpensive and easily obtainable compared to the numerous conventional derivatization reagents that are commercially available.[25]

2.3.3. Practical aspects of LC/ESI/MS

Matrix effect and signal suppression

Efficiency of ESI source in generating gas phase ions from a compound in solution depends mainly on the properties of the compound [60]. On the other hand, compounds other than analytes present in the ESI source can have considerable effect on the ionization of the analyte. Trifluoroacetic acid is perhaps the most well-known eluent additive to cause analyte signal suppression in ESI [62]. If the compounds causing suppression or enhancement of an analyte signal, originate from the sample matrix, the effect is called the matrix effect [37,63]. Matrix effects occur when molecules coeluting with the compound/s of interest alter the ionization efficiency of the electrospray interface. Matrix effects are compound dependent and chemical nature of compounds has a significant effect on the degree of matrix effects.[37]

The matrix effect (%*ME*) can be quantitatively expressed by Equation 9, where $A_{standard}$ is the area of chromatographic peak of the analyte in conditions considered as standard, and A_{matrix} is the measured analyte peak area when (possibly) interfering compound is present. As analyte concentrations are equal in those two measurements, the %*ME* value of 100% indicates that compound under question does not affect the ionization of an analyte. %*ME* values below and over 100% indicate ionization suppression and enhancement, respectively.[64]

$$\%ME = \frac{A_{matrix}}{A_{standard}} \cdot 100\% \tag{9}$$

The same formalism can be used in order to evaluate the effect of different aspects (splitter use, mobile phase additives etc.) on the ESI/MS analysis.

When discussing matrix effects, it is useful to discriminate between ion suppression (or enhancement) by the matrix, on the one hand, and different matrix effects exerted by different sample lots, on the other hand. Xu et al. have suggested that the difference in response between a neat solution sample and the postextraction spiked sample is called absolute matrix effect, while the difference in response between various lots of postextraction spiked samples is called the relative matrix effect [65]. If no counteraction is taken, an absolute matrix effect will primarily affect the accuracy of the methods, while a relative matrix effect will primarily affect the precision of the method. In the current work, absolute matrix effect is under discussion and the term matrix effect is applied.

In ESI, droplets with a surface excess charge are created. Enke introduced a predictive model based on competition among the ions in the solution for the limited number of excess charge sites. Thus, at low concentrations of the analyte, the response-concentration relationship is linear. However, at higher concentrations, the response becomes independent of the analyte concentration but highly affected by the presence of other analytes.[66] King et al. investigated the mechanism of ion suppression in ESI and demonstrated that the gas phase reaction leading to the loss of net charge on the analyte, is not the main process that causes ion suppression [67]. In addition, the presence of non-volatile solute is much more important since this changes the droplet solution properties [20].

King et al. analyzed biological samples and concluded that the ionization suppression typically observed in sample extracts from biological samples is not likely to be caused by reactions occurring in the gas phase. It is most likely that ionization suppression is resulted by the high concentrations of nonvolatile materials present in the spray with the analyte. The exact mechanism by which the nonvolatile materials inhibit release of analyte into the gas phase, has not been clearly demonstrated, although a likely list of effects relating to the attractive force holding the drop together and hindering formation of smaller droplets should account for a large portion of the ionization suppression observed with ESI.[67]

The two main techniques used to determine the degree of matrix effects on a LC/ESI/MS method are postextraction addition and postcolumn infusion. The postextraction addition technique requires sample extracts with the analyte of interest added postextraction compared with pure solutions prepared in mobile phase containing equivalent amounts of the analyte of interest.[37,68] For quantitative assessment, Equation 9 can be used if A_{matrix} is replaced with $A_{postextraction}$, the area of the chromatographic peak of the analyte added postextraction. Postcolumn infusion is a method where with a syringe pump continuous infusion of an analyte with a tee-piece is added to the chromatographic effluent. Because the compound being tested is introduced into the mass

detector at a constant rate, a constant ESI response should ideally be observed. In actuality it is common to see suppression of the signal at the time point that corresponds to the void volume of the column. Moreover, during the chromatographic run, an ion suppression can occur. The degree of ion suppression and the recovery time to full response can vary from compound to compound and from sample to sample, and can also be dependent on the sample preparation method.[68] However, this kind of approach does not give a quantitative info about the matrix effect [69].

Matrix effect is usually caused by the compounds of sample injected together with the analyte. In general, matrix effects may be caused by the compounds of previous injections, either as late-eluting peaks (bands) or as impurities depositing on the internal surfaces of ESI source [68]. The observed degree of ion suppression can also be dependent on the concentration of the analyte being monitored [70], which is related to the matrix/analyte ratio [68].

ESI/MS optimization

For the achievement of the best sensitivity and selectivity of the LC/ESI/MS instrument, complete instrument optimization is needed. In addition to parameters of the mass spectrometer and detector, the optimization of both the ionization process and ion transport in the mass spectrometer is of crucial importance in order to achieve high sensitivity, low detection limits and acceptable accuracy in LC/ESI/MS.[71]

In most cases when ESI/MS is used, MS^2 is preferred since compared to other detection techniques for liquid chromatography, it increases sensitivity and selectivity. For analysis of underivatized amino acids, comparison has been made between different detectors, including evaporative light scattering (ELSD), UV, conductivity (CD), refractive index (RID), chemiluminescent nitrogen (CLND), ESI/MS, ESI/MS^2 and NMR detector and results show that ESI/MS^2 analysis is the most sensitive [72].

The optimization of MS^2 is relatively important since using MS^2 detection signal-to-noise ratio is improved and lower LoD and LoQ are achieved [21]. A good article describes the procedure of how the instrument's parameters are optimized or if the default parameters are used. However, it is a topic that is not always very widely covered and rarely is more detailed info about the optimization procedure available. There have been few works that have discussed some of the aspects of ESI/MS optimization in our workgroup [71].

For ESI/MS optimization, different types of procedures are proposed. First and the simplest one is the straight infusion of a standard solution and neither the composition nor the flow rate of the solvent matches the actual chromatographic elution conditions [17,71,73]. During the infusion, ESI and MS parameters are ramped one after another. For each parameter the analyte signal vs. parameter value plot is recorded and parameter value at the maximum of the plot is regarded as the optimized value. Another one is similar to the methods

recommended by the manufacturer and it takes into account the mobile phase composition at the retention time of an analyte. Meaning, that the composition of the solvent of a standard solution corresponds to the composition of an eluent at the time of the elution.[71,74,75]

There is also a method for optimization that is a modification of the standard solution infusion and it simulates the conditions when the analyte reaches the ionization source during the chromatographic analysis. Therefore, the tee-piece is used to mix the chromatographic solvent to an infused analyte. The composition of the chromatographic solvent corresponds to the solvent composition at the time each analyte elutes and the flow rate corresponds to the flow rate of an analysis.[71]

Results show that for pesticides, the methodology of optimization strongly influences the effectiveness of finding true optima of the operating parameters. Both eluent flow rate and composition during optimization have to mimic the situation during real analysis as closely as possible in order to achieve parameters giving the highest sensitivity.[71] However, when underivatized amino acids are analyzed, results show that all amino acids have quite similar optimums for parameters [17].

Mobile phase modifiers for ESI/MS

The use of additives and buffers in HPLC mobile phase serves two purposes. First, they are primarily used to add buffering capacity in order to achieve reproducible retention of acidic and basic compounds during a typical chromatographic run (isocratic or gradient). Second, depending on the nature of an additive, they can also be used as an ion-pairing agent to create a pseudoneutral species with the target analyte and thus produce a sharper peak shape and longer retention times (increase in separation power and theoretical plates).[13] For pH range of 2–3, which is suitable for protein chromatographic separation, suitable buffers would be formic acid, trifluoroacetic acid (TFA), acetic acid and ammonium formate [53].

However, the use of additives with LC-MS system can serve an additional purpose, which is to protonate basic molecules when operating the ESI source in positive mode, and vice versa for acidic molecules.[13] Ammonium hydroxide, acetic acid, formic acid, ammonium acetate and ammonium formate are commonly used mobile phase additives for ESI/MS [53]. Nonvolatile compounds are not suitable for ESI/MS since they can deposit on the ion source. This would result in capillary obstruction affecting the sensitivity and the accuracy of the quantitative analysis [54]. Nevertheless, volatility is not the only limitation; the buffer added to the mobile phase also has a significant effect on the signal suppression commonly observed in complex matrices. Widely used volatile reagent such as TFA is also not suitable for sensitive mass spectrometric detection, since it acts as a signal suppressor by forming very strong ion pairs with analytes that cannot be broken apart in the conditions used in the

electrospray ionization interface. Moreover, TFA also results in spray instability and signal reduction due to the high conductivity and high surface tension of the eluent.[54,53] For ESI/MS analysis, formic acid concentration of 0.2% (v/v) has been found to be optimal for applications.[53] It can be concluded, that for analysis of small peptides and other organic molecules, formic acid and acetic acid could be the best candidates for LC/ESI/MS analysis [54].

Methods for signal enhancement

In order to enhance ESI signal, there are several approaches. Many interrelated parameters can affect the generation of ions. Nebulization and ionization of analytes in the ion source are determined by the instrument parameters, such as the flow rate of nebulizer gas, temperature, and electrospray capillary voltage among others. Secondly, solution phase factors play an important role in ionization, such as pH, mobile-phase composition, surface tension, and the concentration of electrolytes and analytes. The chemical and physical properties of an analyte are perhaps the most critical parameters for superior sensitivity in the various modes of ionization. Derivatization changes the structure of the analyte and therefore changing its physical and chemical properties, which can result in high ionization efficiency.[54,67] Several research groups have used derivatization to introduce functional groups into analytes in order to increase the detection sensitivity for ESI [22,27,28,54]. For example, dansylation reaction introduces ionizable basic nitrogen that enhances the ESI response [20].

Compared to derivatization, the adjustments of the mobile phase in many cases are easier. There have been reports where substituting methanol for acetonitrile can significantly improve the signal in positive ion mode [20,54]. On the other hand, for negative ion mode, no enhancement was observed when substitution was made [20]. Depending on the analytes, acetonitrile might provide better chromatographic separation, which may overweigh the small ionization advantage achieved with methanol [76]. The pH of the mobile phase or the presence of mobile phase additives has also been shown to have a significant effect on the formation of protonated or adduct ions [54]. Acidification of the mobile phase with formic acid can enhance the signal as well as maintain a reasonably high retention factor for the analyte even at a relatively high acetonitrile concentration. Formic acid and acetic acid could increase the response at lower concentrations than ion-pairing agents [53]. A concentration of 1 mM formic acid in the mobile phase has been found to be optimal for negative ESI analysis of a carboxylic acid compound [20]. For protein analysis, it has been found that the highest responses were always obtained with formic acid in which case the signal increases up to a concentration around 50 mM [53].

Another possible way to enhance signal is to use post-column addition of signal-enhancing modifier [54]. In the case of TAHS, 0.2% acetic acid in acetonitrile is added after the column outlet as the sheath solution and up to 2.2-fold signal increase is reported [28]. For ibuprofen, in negative ion ESI,

2-(2-methoxyethoxy)ethanol has been since it prevents or weakens ion competition in the droplet surface. Moreover, compared to conventional modifiers such as methanol or acetonitrile, sensitivity was 109 times higher.[77] The major advantage of post-column infusion is that ionization conditions for the analytes could be optimized without changing the chromatographic separation. The application of post-column infusion has involved pH adjustment, reducing of ion-suppression and improvement of ionization efficiency through the formation of an adduct ion, or improving nebulization and desolvatation.[54]

ESI/MS is a concentration-sensitive detector, which means that the signal intensities are not dependent on the flow-rate, at least in the range from 100 nL/min up to 1 mL/min [53]. However, it has been found that the flow rate of LC effluent introduction can affect the sensitivity of ESI/MS. For LC/ESI/MS, the dimensions of the chromatographic column used for separation will determine the flow rate of sample introduction. However, post-column splitting could also be utilized to reduce the amount of effluent reaching the ESI/MS.[76,78] For a regular ESI interface, usually a setup is used where a effluent flow after column is splitted with a tee-piece and then a flow is adjusted by selecting tubing of appropriate internal diameter and length for the waste line [79].

It has been shown that reducing the ESI flow rate to the nanoliter per minute range, leads to increased desolvatation, ionization, and ion-transfer efficiency compared to ESI conducted at higher flow rates [80]. Results show that for LC/ESI/MS, exceptional mass sensitivity is achieved via the use of nano-ESI, which is compatible with nanobore chromatographic columns. There are results showing that flow rates down to 20–50 μl/min could be used with a conventional ESI interface but lower flow rates resulted in an unstable spray, peak broadening and retention time shifts [79]. However, there are devices that allow conducting LC/ESI/MS analysis under nano-ESI (split ratio about 2000:1) conditions while utilizing conventional chromatographic equipment and columns, meaning improvement in sensitivity and reduction of signal suppression [81]. Contrary, there are reports where significant decreases in sensitivity are detected when splitting is applied [76]. In these cases, however, when real samples are used, there could be a possibility of smaller matrix effects since smaller nano-ESI droplets are more resistant to ionization suppression effects.[76,78,79,81]

Sodium adduct formation

For ESI/MS analysis, either negative ($[M-H]^-$) or positive ions ($[M+H]^+$) could be detected. But since sodium and potassium ions are ubiquitous in the experimental environment, $[M+Na]^+$and $[M+K]^+$ are therefore observed adducts using the ESI/MS detection mode [22,54,62]. Moreover, the formation of metal adduct ions has been used to improve sensitivity of detection. It has been proposed that the sodium ion is bound to the oxygen atoms of the analyte. Good

candidates for formation of the sodium ion adduct would be compounds with functional groups such as a methyl ester, a carboxylic acid, a thioether, a carboxylic acid amide, alcohols and lactones [54]. For analysis of steroids, methanol has demonstrated better signal for sodium adducts than acetonitrile as the organic component of the mobile phase. Moreover, it has been found that the addition of ammonium acetate will suppress the response of the $[M+Na]^+$ adduct.[82].

Sodium adduct formation in ESI source has not been under close investigation. However, some work has been done on the peptide sodium adduct formation when acetic acid content is changed on the mobile phase. Results show that in case of ion trap mass analyzer, sodium adduct formation correlates with the lower solution pH, more than with any other variable of the experimental setup. No way was found how to avoid the formation of the sodium adducts. When a triple quadrupole instrument was used, sodium adducts were absent. Meaning that the formation of adducts could be also related to the design of an ion source (ion trap was with a sprayer orthogonal to the inlet and triple quadrupole was with a "off-axis" design). It is proposed that one of the reasons for different adduct formation is related to the differences in the actual flow rate into the two sources.[83] In conclusion, competition between protons and sodium ions as charge carriers in ESI/MS is a highly complex process and needs further studies.

2.3.4. Boric acid

For many derivatization reactions, pH 8–10 is needed for the derivatization to occur. Therefore, borate buffer is by far the most popular buffer for these applications. In addition to using it for commercially available derivatization reagents (Fmoc-Cl, Deemm), it is also a popular buffer for novel derivatization reagents (SPTPP, TAHS, APDS) [25,26,28]. In most cases borate buffer contains nonvolatile boric acid, which might not be compatible with LC/ESI/MS system [Paper IV].

Boric acid is a compound with long history and the complexation of boric acid and boronic acid with polyhydroxy compounds is a phenomenon that has been studied for more than a century and has found widespread use in various fields of science and technology. At a pH in the range from 8.0 to 12.0, aqueous borate solutions contain tetrahydroxyborate ions and also more highly condensed polyanions such as triborate and tetraborate. Equilibrium between the different species depends on the pH and the total borate concentration. Boric acid has been used as a mobile phase additive for HPLC for separating ribose, arabinose and ribulose (ion-moderated partitioning).[84] However, it is more widely used in amino acid derivatization for providing pH in the range of 8 to 11.

Aqueous borate solutions at low concentrations are subject to equilibrium of boric acid and borate anions, reflecting the capacity of boric acid as a Lewis

acid (pK_a = 9.2 at 25°C) to bind hydroxyl ion [85]. In addition, boric acid is known to form complexes [85,86]. Different complexes of boric acid with various analytes, such as caffeic acid, NADH and nucleotides, have been investigated in negative ion ESI and results indicate vast complexation ability for boric acid [85,87,88].

Properties of boric acid have been widely explored. For HPLC systems, pH variation in mobile phase containing boric acid as buffering system and acetonitrile as organic modifier, have been researched. Dissociation constants for boric acid and acetonitrile mixtures were determined and developed model proposed how to evaluate the pH of the mobile phase when boric acid is mixed with acetonitrile. Results show that the pH rises when the content of acetonitrile gets higher [89].

In addition to determining various properties of boric acid, it has had many different applications in fields other than chromatography. Due to its most fundamental properties, namely it produces a Brønsted acid from its reaction with water: $B(OH)_3 + H_2O \rightarrow H^+ + B(OH)_4^-$, it has been used as a catalyst [90,91]. An efficient method is reported for the preparation of Mannich products (β-amino carbonyl compounds) in water from aldehydes, aromatic amines and cycloalkanones under very mild conditions using boric acid and glycerol. Although boric acid is a weak acid, its complex with polyhydroxy compounds such as glycerol is stronger due to the chelate formation and release of H^+ ions in the aqueous medium. This increases both the yield and diastereoselectivity of the Mannich products in water [90]. Moreover, boric acid has been used as a novel and safe catalyst for aza-Michael reactions in water. It was discovered that boric acid efficiently catalyzes the conjugate addition of aliphatic amines to α,β-unsaturated compounds to produce β-amino compounds, with great alacrity and excellent yields. Aromatic amines do not participate effectively in the reaction [91].

3. EXPERIMENTAL

3.1. Materials

Chemicals

HPLC-grade methanol and acetonitrile were obtained from J.T. Baker. Amino acid standards (L-glutamine, L-cysteine, L-proline, L-trypthophan, L-tyrosine, L-histidine, L-leucine, L-isoleucine, L-phenylalanine, L-alanine, L-aspartic acid, L-arginine, L-asparagine, glycine, L-serine, L-valine, L-methionine, L-threonine, L-lysine, L-glutamic acid) were purchased from Sigma and except β-alanine and ornithine, which were purchased from Fluka. Se-MeSeCys and SeMet were kindly donated by LGC. Derivatization reagents diethyl ethoxymethylenmalonate and dansyl chloride (DNS) were purchased from Fluka, 9-fluorenylmethyl chloroformate (Fmoc-Cl) was from Aldrich.

Sodium hydroxide (Chemapol, Former Soviet Union); Fmoc-phenylalanine (Sigma-Aldrich); 2-mercaptoethanol (Sigma); dichloromethane (J.T. Baker); tetrahydrofuran (Rathburn); diphenyl phthalate (Riedel de-Haën); sodium dihydrogensulphate and dimethyl glutarate (Merck); acetic acid, magnesium sulphate (Lach-Ner); ethyl acetate (Fisher); iodomethane, hydrogen chloride, orthophosphoric acid, boric acid, sodiumtetraborate, ammonium hydroxide, potassium hydroxide, diphenylamine, phosphorus pentachloride and 2-nitroaniline (Reakhim, Former Soviet Union); formic acid, *N,N*-Dimethylamino-*p*-phenylenediamine (DPD), *N,N*′-dihydroxysuccinimidyl carbonate (DSC), triethylamine, sodium tetrafluoroborate, glycine ethyl ester hydrochloride, 4-chloro-2-nitroaniline and ammoniumbicarbonate (Aldrich); and tetraethylammonium perchlorate, ammonium acetate, Fmoc-β-alanine and Fmoc-glycine (Fluka). All reagents were of analytical grade unless otherwise stated.

Synthesis, purification and identification of noncommercial $((CH_3)_2N)_3$-P=N-C_6H_5 and hydrazinotripyrrolidinophosphonium hexafluorophosphate are described in described in ref. [108] and [93], respectively.

All aqueous solutions were prepared with ultrapure water purified in-house by Millipore Milli-Q Advantage A10 (Millipore, USA). Amino acid standards were dissolved in 0.1 M hydrochloric acid, and diluted with ultrapure water to obtain different concentrations.

SPE eluent was 2.5 M ammonium hydroxide with 10% acetonitrile.

Honey samples were bought over the period of 2004–2011 from the various Estonian markets and supermarkets. Onion samples containing SeMet and Se-MeSeCys were obtained from the Estonian University of Life Sciences. Blood serum samples were collected from the Tartu University Hospital Blood Centre.

Equipment

HPLC system Agilent Series 1100 LC/MSD Trap XCT (Agilent Technologies, Santa-Clara, USA) was equipped with an in line degasser, a binary pump, an

autosampler and a column thermostat. For detection photodiode array detector (PDA) with 6 mm path length flow cell and electrospray interface mass spectrometer (ESI/MS) were used. The system was controlled with Chemstation (Rev.A.10.02) and LCMSD Trap Control (Version 5.2) software. Chemstation (Rev.A.10.02) and DataAnalysis (Version 3.2) were used for UV and MS chromatograms analysis and peak integration.

Chromatographic analysis of Deemm and TAHS derivatives was performed using an analytical column Synergi Hydro-RP 80A (4.60 mm × 250 mm, 4 μm) (Phenomenex, USA) with guard cartridge (4.0 mm x 2.0 mm), polar endcapped C18 (Phenomenex). For Fmoc-Cl and DNS derivatives Eclipse XDB-C18 (4.6 × 250 mm, 5 μm) analytical column with guard column (4.6 × 12.5 mm, 5 μm) was used (Agilent). And for 2,5-dioxopyrrolidin-1-yl N-tri(pyrrolidino)-phosphoranylideneamino carbamate (FOSF) derivatives Eclipse XDB-C18 (4.6 × 150 mm, 5 μm) analytical column with guard column (4.6 × 12.5 mm, 5 μm) was used (Agilent).

Synthesis of carbethoxymethylimino-tri(pyrrolidino)phosphazene (EtGlyP1Pyrr$_3$) (Figure 1)

The instructions were based on a synthesis published by Schwesinger et al. [92]. Two-neck round bottom flask is placed on the bath of octane and liquid nitrogen in order to keep the temperature around –50°C. 2 g of phosphorus pentachloride is added to the flask and then 2.5 mL of pyrrolidine and 4 mL of triethylamine (Et_3N) are added with the syringe. Reaction mixture is kept at the –48°C for about one hour and then warmed to the room temperature. At room temperature, 10 mL of dichloromethane is added to the mixture and heated to 50°C. Then the formed precipitate (Et_3N•HCl) is filtered out. The rest is dried under a vacuum for solvent change. To the dried residue, 30 mL of tetrahydrofuran is added and the solution is cooled down to –50°C. Then 1.36 g of glycine ethyl ester hydrochloride and 1.32 g of Et_3N are added to the mixture. Temperature is raised to the room temperature and then heated to 50°C and then again cooled down to the room temperature. A formed precipitate (Et_3N•HCl) is filtered out and the solvent dried under vacuum. To the residue 20 mL of MilliQ water is added and then 0.7 g of $NaBF_4$ and then 20 mL of methylene chloride. Mixture is transferred to the separation funnel. Methylene chloride fraction is collected and dried with anhydrous magnesium sulphate ($MgSO_4$). Solution is filtered through cellulose filter paper and concentrated by rotary evaporation. Recrystallization is carried out with the 1:10 mixture of ethyl acetate : methanol.

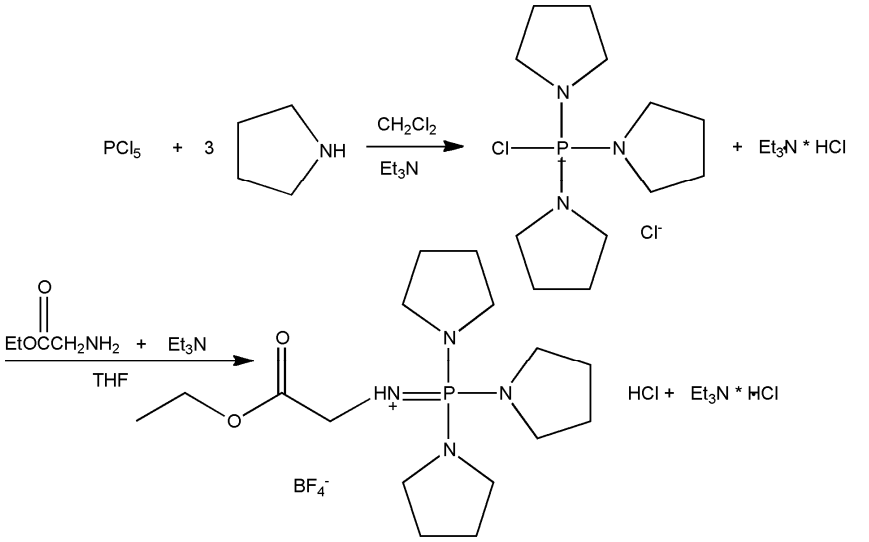


Figure 1. Synthesis of carbethoxymethylimino-tri(pyrrolidino)phosphazene.

Synthesis of Deemm-derivatives (Phe, Gly, β-Ala, Leu, Ser)

Deemm derivatives were prepared by following the instructions by Alaix et al. [97]. 10 mmol of an amino acid and 12 mmol of potassium hydroxide were dissolved in 15 mL of methanol. 2 mL of Deemm (10 mmol) was added to the mixture. Reaction mixture was stirred for 15 min on the magnetic stirrer and then the reaction mixture was concentrated by rotary evaporation. Residue was dissolved in 10 mL of MilliQ water. With 0.1 M HCl, the pH of the mixture was set to 4.25. Separated oil is extracted with ethylacetate (3 x 10 mL). Extract is dried with anhydrous magnesium sulphate. Solution is filtered through cellulose filterpaper and concentrated by rotary evaporation. Obtained reagent was either viscous oil (Deemm-Ser and Deemm-Phe) or white crystalline solid (Deemm-Gly, -β-Ala and -Leu).

Synthesis of TAHS (Figure 2)

TAHS was synthesized by following the instructions by Shimbo et al. with minor modifications (Figure 2) [28]. DSC (600 mg) was dissolved in 25 mL of acetonitrile at room temperature. DPD (300 mg), dissolved in 25 mL of acetonitrile, was added dropwise to the DSC solution over a period of approximately 2 h. Then the reaction mixture was concentrated by rotary evaporation. The residue was resuspended in 5 mL of acetonitrile and then filtered, to obtain p-*N,N*-dimethylaminoanilyl *N*′-hydroxysuccinimidyl carbamate which was dissolved in 10 mL of acetonitrile/dichloromethane (4:1) at room temperature. Iodomethane (0.4 mL, 8 equiv) was added to the solution, which was then stirred for 23 h at room temperature. After the reaction mixture was filtered, TAHS was obtained.

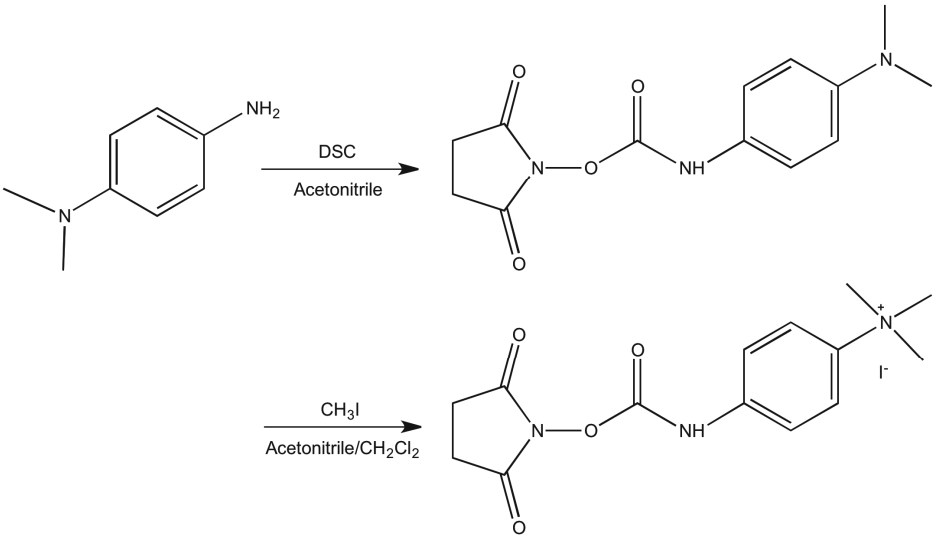


Figure 2. Synthesis of TAHS.

Synthesis of FOSF (Figure 3)

Synthesis of a FOSF reagent is as follows (Figure 3): DSC (80 mg) was dissolved in 4 mL of acetonitrile at room temperature. Hydrazinotripyrrolidinophosphonium hexafluorophosphate (80 mg) dissolved in 4 mL of acetonitrile, was added dropwise to the DSC solution over a period of approximately 2 h. Then the reaction mixture was concentrated by rotary evaporation. The residue was resuspended in 2 mL of water. Formed white crystals were filtered from the water.

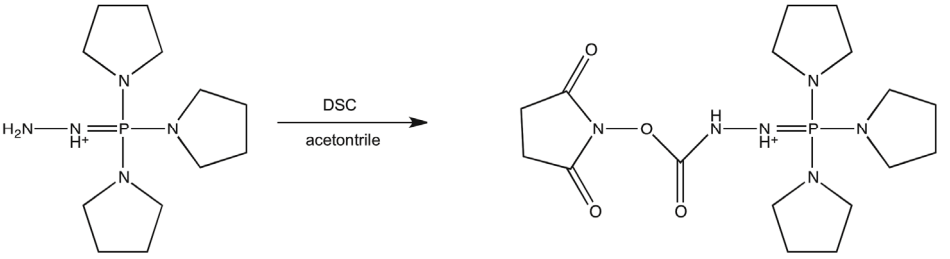


Figure 3. Synthesis of FOSF.

Sample preparation

Approximately 1 g of honey (or 2 g of onion or 1 mL of blood serum) was weighted and diluted with 25 mL of phosphate buffer (0.03 M, pH 2.12). The solution was filtered through a wide-pore paper filter.

For amino acids, solid phase extraction (SPE) cartridges with styrene − divinylbenzene polymeric strong cation exchange sorbent, 500 mg (Alltech, USA) were used. SPE cartridge was first conditioned with 10 mL of HCl (0.1 M) at flow rate 4 mL/min. The buffered honey sample was applied to the cartridge at ~1.5 mL/min flow rate. The analytes were eluted with 15 mL of 2.5 M ammonium hydroxide containing 10% of acetonitrile. The eluate was evaporated to dryness using nitrogen flow and redissolved in 1 mL of ultrapure water.

Preparation of standard solutions

Amino acid stock solutions (1–20 mg/g) were prepared by dissolving respective substances in 0.1 M HCl with 30% MeOH. Stock solutions were prepared once and stored at –20ºC. All dilutions (0.5 ng/g–3000 ng/g) were made with ultrapure MilliQ water. Working standard solutions were prepared daily.

Derivatization

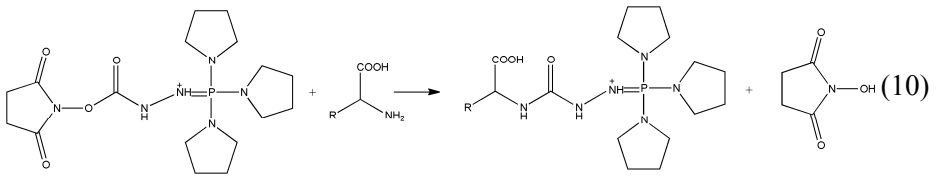
Deemm derivatization: to 1 mL of sample 30 µL of Deemm, 1.5 mL methanol, and 3.5 mL of 0.75 M sodium borate buffer (pH 9.0) was added [94]. The derivatized mixture was kept at room temperature protected from direct light. LC-MS analysis has to be carried out at least 24 h but not more than 48 h after the derivatization [Paper II]. Prior to LC-MS analysis, the sample solutions were filtered through 0.45 µm cellulose acetate syringe filter (Whatman) as in Paper III.

Fmoc-Cl derivatization: to 300 µL of amino acid solution 300 µL of 0.75 M sodium borate buffer (pH 9.0) and 300 µL of Fmoc-Cl (5 mM in acetonitrile) was added and vigorously mixed. Then the mixture was kept at room temperature for 5 min and then 300 µL of His (8 mg/g) was added and vigorously mixed again [46]. Prior to LC-MS analysis, the sample solutions were filtered throught 0.45 µm regenerated cellulose syringe filter (Agilent).

DNS derivatization: to 100 µL of amino acid solution 20 µL of 2 M NaOH and 30 µL of concentrated $NaHCO_3$ and 500 µL of DNS solution (10 mg/ml in acetone) is added. Reaction mixture is placed into 4°C in the dark for 45 min. Reaction is stopped with 10 µL of 25% NH_4OH.[32] Prior to LC-MS analysis, the sample solutions were filtered throught 0.45 µm regenerated cellulose syringe filter (Agilent).

TAHS derivatization: with little modifications from the ref. 28. To 10 µL of amino acid solution, 30 µL of 0.2 M sodium borate buffer (pH 9.0) and 20 µL of TAHS solution (approximately 20 mg/ml) was added. Reaction was carried out at room temperature and stopped after 10 min with 200 µL of 0.2% acetic acid. (Heating was not necessary since tyrosine is not analyzed)

FOSF derivatization (Equation 10): same as above described for TAHS.



(10)

LC/UV/MS analysis

HPLC conditions for finalized methods for analysis of 7 amino acid mixtures.

For Deemm derivatives: mobile phase A: buffer solution (pH = 3.2; 1 mM ammonium acetate in 0.1% formic acid); mobile phase B: acetonitrile. Gradient program was as follows: 0–12 min, 20–25%; 12–20 min 25%; 20–50 min 25–60% B. The eluent flow rate was 0.9 mL/min and the column was maintained at 40°C and 5 µL of the sample was injected. The UV detection wavelength was 280 nm (full spectra were acquired for additional confirmation). [Paper II]

For Fmoc-Cl derivatives: mobile phase A: 0.1% formic acid; mobile phase B: acetonitrile. Gradient program was as follows: 0–45 min, 30–100% B. The eluent flow rate was 0.8 mL/min and the column was maintained at 30°C and 10 µL of the sample was injected. The UV detection wavelength was 280 nm (full spectra were acquired for additional confirmation). [Paper VI]

For DNS derivatives: mobile phase A: 0.1% formic acid; mobile phase B: acetonitrile. Gradient program was as follows: 0–45 min, 10–100% B. The eluent flow rate was 0.8 mL/min and the column was maintained at 30°C and 5 µL of the sample was injected. The UV detection wavelength was 280 nm (full spectra were acquired for additional confirmation).[Paper VI]

For TAHS derivatives: mobile phase A: (pH = 3.2; 1 mM ammonium acetate in 0.1% formic acid; mobile phase B: acetonitrile. Gradient program was as follows: 0–30 min, 5–70% B. The eluent flow rate was 0.8 mL/min and the column was maintained at 30°C and 5 µL of the sample was injected. The UV detection wavelength was 280 nm (full spectra were acquired for additional confirmation).[Paper VI]

For FOSF derivatives: mobile phase A : 1 mM ammoniumbicarbonate pH = 7; mobile phase B: acetonitrile. Gradient program was as follows: 0–20 min, 20–40% B. The eluent flow rate was 0.8 mL/min and the column was maintained at 30°C and 5 µL of the sample was injected. The UV detection wavelength was 280 nm (full spectra were acquired for additional confirmation).[Paper VI]

ESI source parameters that were same for all derivatization reagents: Nebulizer gas (nitrogen) 50 psi (345 kPa), Drying gas (nitrogen) 12 L/min and Drying gas temperature 350°C. Other MS parameters were optimized for all the reagents and all amino acids.

HPLC conditions for evaluation of the boric acid influence on the ionization [Paper IV]:

For Deemm derivatives (Deemm-Ser, Deemm-β-Ala, Deemm-Leu): mobile phase component A – buffer solution (pH = 3.2; 1 mM ammonium acetate in 0.1% formic acid); mobile phase component B – acetonitrile. Gradient program was as follows: 0–12 min, 20–25%; 12–20 min 25%; 20–50 min 25–60% B. The eluent flow rate was 0.9 mL/min, the column was maintained at 40°C and 5 μL of the sample was injected.

For Fmoc-Cl derivatives (Fmoc-β-Ala, Fmoc-Gly, Fmoc-Phe): mobile phase component A – 0.1% formic acid; mobile phase component B – acetonitrile. Gradient program was as follows: 0–45 min, 30–100% B. The eluent flow rate was 0.8 mL/min, the column was maintained at 30°C and 10 μL of the sample was injected.

For analysis of mixture of other compounds: mobile phase component A – buffer solution (pH = 3.2; 1 mM ammonium acetate in 0.1% formic acid); mobile phase component B – acetonitrile. Gradient program was as follows: 0–30 min, 10–100 % B. The eluent flow rate was 0.8 mL/min, the column was maintained at 30°C and 5 μL of the sample was injected.

MS optimization

Optimized MS parameters were used for measurements. Optimization was carried out by manufacturer instructions infusing an analyte in the ESI source and using software tools.

In the current work more MS optimization is under close interest and discussed in more detail (see Section 4.5).

3.2. Methods

3.2.1. Measuring ionization efficiencies

Procedure proposed by Oss et al. [60] was used for the ionization efficiency (IE) measurements. The solvent composition acetonitrile/0.1% aqueous formic acid in volume ratio 80:20 was used. For every relative ionization efficiency (RIE) measurement solutions of two compounds in the solvent were made and these were infused using two syringe pumps connected with a tee-piece with around 1 mm^3 of dead volume (for mixing the solutions). The concentration ratio of the compounds was varied by varying the ratio of infusion rates of the two pumps. Concentrations of compounds in the sprayed solutions were in the range of $n \bullet 10^{-7}$ to $n \bullet 10^{-4}$ mol/L depending on the two compounds and their ratio in the mixture. With all compounds the concentration in the spray was varied by at least a factor of 4 (leading to the variation of the concentration ratio of the two compounds by a factor of 16). The RIE measurements were carried out at the overall solution flow rate of 8.3 μL/min (0.5 mL/h). The mass spectra were registered over a time period of ca. 100 s (ca. 250 spectra) and were averaged.

The RIE values were found according to Equation 11 and expressed as logRIE values.

The RIE of a compound B_1 relative to B_2 is:

$$\mathrm{RIE}(B_1/B_2) = \frac{\mathrm{IE}(B_1)}{\mathrm{IE}(B_2)} = \frac{K_1'}{K_2'} = \frac{R_1 C_2}{R_2 C_1} \tag{11}$$

where IE are the individual ionization efficiencies of the analytes, R are responses of ions B_1H^+ and B_2H^+ in the mass spectra, *C* is the concentration of the neutrals B_1 and B_2, and $K' = K \cdot \alpha$, where α are the protonation ratios of the compounds B_1 and B_2: $\alpha_1 = [B_1H^+]/C_1$ and $\alpha_2 = [B_2H^+]/C_2$ and K are the partition coefficient of the ions B_1H^+ and B_2H^+.

The MS and ESI parameters were not changed or optimized but the factory defaults were used: nebulizer gas pressure 15 psi, drying gas flow rate 7 L/min, drying gas temperature 300°C. Only the MS parameter target mass (TM) was modified in order to assess the discrimination during ion transport. All RIE measurements were carried out using three different TM values: M+1 of the first compound, M+1 of the second compound and *m/z* ratio 500. The logRIE value was found as average of the values obtained with the three target masses. For measurements of compounds with large *m/z* ratio, logRIE was calculated from the TM of 500.

3.2.2. Optimization of user-adjustable parameters of the ESI source

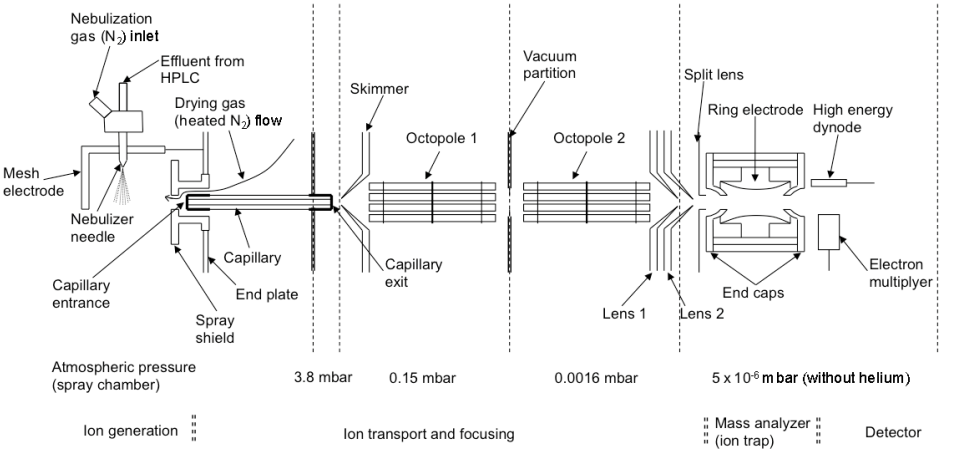


Figure 4. Design of the ion trap mass spectrometer with an ESI source.

For Agilent XCT (Figure 4), the following parameters can be adjusted:

Capillary voltage. The potential, which brings about the electrospraying process, is maintained between the capillary entrance and the sprayer needle, which is referred as a capillary voltage.

Capillary exit. Ions are carried into the vacuum region of the mass spectrometer through a glass capillary. The end of the capillary is maintained at 0 or 360 V.

Skimmer voltage. Gas molecules are prevented from entering the system and this done by a skimmer. In order to help the ions pass the small hole inside the skimmer, its potential is set relative to ground and maintained at lower potential than the capillary exit.

Focusing and guidance of the ions to the lenses are done by the first and the second octopole. The energy distribution of the ions is also unified by the octopoles. Both radio frequency (*octopole RF*) and direct current (*Octopole 1 DC* and *Octopole 2 DC*) can be optimized for the two octopoles.

Lens 1 and *Lens 2 voltage.* The final guidance of the ions to the ion trap is controlled by the lenses. The voltages, more negative compared to the octopoles, are applied to the two lenses given relative to the ground.

Trap Drive. The radio-frequency potential applied to the ring electrode, which is responsible for trapping ions. The higher is the *m/z* of the ions trapped, the higher trap drive is used.

According to the manufacturer's instructions and recommendations, the optimization of the parameters should be carried out in the order they were mentioned above. The parameters are optimized via parameter ramping one after the other; after the parameter has been optimized, its value is fixed before the optimization of the next parameter. Therefore, it is crucial to restore the default parameters before starting the optimization of a new analyte. The *default* values and the range of the values for parameters are presented in Table 1.

Another parameters important for MS^2 analysis, are *Fragmentation Amplitude* and *Fragmentation CutOff*.

In order to obtain the solutions for amino acid derivatives for MS optimization, high concentration (at mg/g levels) standards were derivatized and injected to the chromatographic system. At the corresponding retention time of an amino acid derivative, effluent was collected and used for the MS optimization procedures.

Table 1. Table presents default values (starting conditions) and the optimization range used in this work for the optimization of the MS parameters.

Parameter	Default value	*range*	
		min	**max**
Capillary (V)	–3500	–5000	0
Skimmer (V)	40	0	150
Cap Exit (V)	200	0	360
Oct 1DC (V)	12	2.5	100
Oct 2DC (V)	2.5	0	12
Trap Drive	78	–60	60
Oct RF (Vpp)	200	0	300
Lens 1 (V)	–5	–25	5
Lens 2 (V)	–60	–100	0
FragAmp	1	0%[a]	1000%[a]
FragCutOff	100	10%[a]	100%[a]

[a] – relative to the mass to be fragmented

For optimization, two procedures were applied:

Procedure A: Recommended by the manufacturer. Effluent was infused to the ESI source at the rate of 0.3 mL/h (0.005 mL/min). Composition of the solvent used for optimization corresponds to the solvent composition of the amino acid derivative when it reaches the ionization source after the chromatographic separation (solvent composition at the retention time), but the eluent flow rate is considerably lower.

Procedure B [71]: The tee-piece is used to mix the chromatographic solvent (0.8 mL/min or 0.9 mL/min for Deemm-derivatives) with the infused amino acid effluent. The composition of the chromatographic solvent corresponds to the solvent composition at the time each amino acid derivative elutes.

The parameters that remained constant for all amino acids, were the drying gas temperature (350°C), nebulizer gas pressure (50 psi = 345 kPa) and drying gas flow (12 L/min).

For the optimization, the starting point for all amino acid derivatives were the default parameters that the software provided for the target mass *m/z* 300.

3.2.3. Experimental setup for investigation of boric acid influence on ionization

Influence on the standard amino acid derivatives and other compounds

To test the influence of boric acid on chromatographic peaks in ESI source and mass spectra of the analytes, boric acid solution or ultrapure water was added to the chromatographic effluent before the ESI inlet via a tee-piece. The addition was carried out using a syringe pump at 0.5 mL/h (8.3 μl/min) flow rate. Concentration of boric acid in the ESI source was 1.8 mM for Deemm derivatives and 2 mM for other compounds. For each analyte, matrix effect (%*ME*) was calculated from the peak areas with boric acid (A_{matrix}) and water ($A_{standard}$) infused (Equation 9).

Influence on the amino acid analysis

Amino acid derivatization and LC/ESI/MS^2 analysis was carried out by the procedures developed previously. For comparisons, three different types of experiments were conducted: 1) regular analysis by LC/ESI/MS^2; 2) to the eluent flow with tee-piece at the flow rate of 0.5 mL/h, mobile phase was added to the source; and 3) to the eluent flow with tee-piece at the flow rate of 0.5 mL/h, 0.2 M boric acid in the mobile phase was added.

In addition to post-column addition experiments, 4 mM boric acid was added to the chromatographic eluent (aqueous component) in order to evaluate the influence of boric acid on the ionization and LoD and LoQ values.

3.2.4. Other method modifications setups

Diverting boric acid away from the ESI source

The used instrument has a motor-driven valve, which enables to divert the chromatographic effluent can be diverted away from the ESI source during the chromatographic run for any given time period.

For brevity, the experiment where initial 5 minutes of the chromatographic run are diverted into waste, is called the waste run. And the term regular run is used for any unmodified analysis.

Post-column splitting device

Flow splitting was achieved by means of a tee-piece after the column and before the ESI interface. Splitting ratio was adjusted by selecting PEEK tubings of appropriate internal diameter and length for the waste line. Splitting ratios used were 65%, 75% and 85%, denoting the percentage that goes to waste.

4. RESULTS AND DISCUSSION

The main results obtained in this study are described in the following sections. Section 4.1 is based on the [Paper I] and [Paper II], section 4.10.2 is based on [Paper II], section 4.1 and 4.10.3 contain information from [Paper III], section 4.6.1 is based in [Paper IV], 4.8.1 is based on [Paper V], and section 4.3 is based in the [Paper VI].

Data presented and discussed in sections 4.2, 4.4, 4.5, 4.6.2, 4.7, 4.8.2–4.8.4, and 4.9 have not been previously published.

4.1. Solid phase extraction (SPE) [Paper II]

For current work, amino acid analysis was considered for four types of matrices: honey, human serum, onion and garlic, the main being honey where all amino acids were analyzed [Papers I and II]. In human serum, onion and garlic samples, selenoamino acids were tested [Paper III].

Isolation of amino acids from various matrices is essential for ESI/MS detection. Therefore, simple dissolution of samples is often not suitable if MS-amendable method is targeted. Amino acid isolation was first attempted with selg-prepared Dowex ion exchange resin. However, using self-prepared columns was time consuming and reproducibility was problematic. Solid phase extraction in cartridge format (SPE) was more convenient and faster method for sample preparation [15]. Moreover, SPE will use less solvents and extract will be more concentrated and will easily allow analysis of multiple samples simultaneously. Moreover, generally smaller sample sizes can be used. In the current work, SPE was first recorded use for amino acid isolation from honey and onion samples. Though, SPE has been previously used to isolate amino acids from, for example, human serum [95].

The performance of the solid phase extractions largely depends on the choice of the cartridge filling as well as the elution procedure. During method optimization, various types of cartridge fillings were tested. Anion exchange cartridges were not considered since extraction of amino acids as anions was not fully successful [15]. Both, strong and weak cation exchange SPE cartridges were tested. Weak cation exchange SPE was not suitable for amino acid isolation because at the suitable loading pH (around 7) not all amino acids were trapped on the column. Strong cation exchange SPE cartridges proved to be better suited for this type of analysis.

Two types of strong cation exhange cartridges were tested, Phenomenex Strata and Alltech SCX cartridges. Phenomenex Strata has silica based sorbent and Alltech SCX cartridges are polymer based (polystyrene divinylbenzene). The silica based sorbent of Phenomenex Strata appeared to be incompatible with the method due to the high pH required to elute amino acids. Therefore, Alltech SCX cartridges were selected for SPE.

Considering the recommended use of SCX cartridges, conditioning of the SPE cartridge was carried out with 10 mL of 0.1 M HCl.

For loading amino acids into SPE cartridge, pH of the solvent must be low in order to convert amino acids into cationic form. Constant extraction recoveries were observed if phosphate buffer with pH in the range 2.7–3.3 was used as the sample diluent. The ionic strength of the buffer used for dissolving honey played an important role in ion exchange processes. Buffer concentrations used in literature with Dowex ion exchange resin [8,11] appeared to be too high for SPE. Phosphate buffer concentrations between 1 M and 0.01 M were tested and the most effective concentration was 0.03 M. While applying buffered sample to the cartridge, it was important to maintain flow rate smaller than 1.3 mL/min since at higher flow rates, decrease in the recovery rate was observed.

After the sample loading step, washing is usually carried out before the elution step. For washing, 3 mL of phospate buffer (the same as used for sample dissolution) was used. After the washing step and before the elution, the cartridge was dryed to remove all the phosphate from the system (about 20 seconds). This kind of action can only be taken when polymeric solid extraction phase is used. This is another advantage of using polymer phased SPE cartridges.

Another important step of the SPE is the elution of analytes from the sorbent. High solvent pH is needed to elute amino acids from cation exchange SPE cartridge. Borate buffer with pH 11 did not give good results neither alone nor with organic modifier (acetonitrile, methanol, isopropanol were tested). Moreover, it would not be compatible for the application where the idea is to concentrate the eluate by evaporating it to dryness since borate buffer is not volatile. Moreover, there is a possibility that the concentrated boric acid can interfere with the derivatization procedure. Therefore, the use of ammonium hydroxide was considered and results showed that the diluted ammonium hydroxide (2.5 M) gave better recoveries than concentrated ammonium hydroxide (14 M). The best results were obtained with 15 mL of 2.5 M ammonium hydroxide containing 10% of acetonitrile (small change in that precentage did not affect recovery). Percentage of an organic solvent shall not be higher due to the swelling of the sorbent. In published procedure where polymer-based cation exchange process was used for amino acid extraction aqueous buffer solutions are employed [23]. In case of aqueous eluting buffer, lower recoveries were observed for amino acids with aromatic groups (Phe, Trp, Tyr). This was apparently due to the secondary retention of aromatic moieties on polystyrene-divinylbenzene base. In preventing this secondary retention, acetonitrile appeared to be more efficient eluent modifier than methanol or isopropanol. The flow rate of elution up to 5 mL/min did not affect the recovery.

The eluate is evaporated to dryness under nitrogen flow to concentrate amino acids in samples as well as to volatilize ammonia, which would interfere with amino acid derivatization since it is able to react with derivatization

reagents designed for amino acid derivatization through an amino functional group. Moreover, the evaporation to dryness is advantageous before applying derivatization to samples since it is better to have sample redissolved in ultra-pure water in order to carry out derivatization. If amino acids would be in a solvent other than water, the proceeding of a derivatization reaction would have to be under close investigation.

High price of the cartridges is often the reason for not considering SPE. Therefore, SPE cartridges were tested for repeated use. It appeared that the cartridges can be reused. Up to 7 honey samples can be safely analyzed with a single SPE cartridge [Paper II]. For more complex matrices such as onion samples and human serum, fewer number of samples can be analyzed with the same cartridge.

Recoveries of SPE extraction were also tested for SCX cartridge. For recovery determination, guidlines usually advise the use of blank samples [96]. Since blank samples are not available for amino acid samples, spiked and unspiked samples were analyzed for recovery determination. Equation 12 was used for calculation.

$$R = \left(c_{spiked\_sample} - c_{sample}\right) \Big/ c_{spiked} \cdot 100\% \qquad (12)$$

Where R is recovery, c_{spiked_sample} and c_{sample} are analyzed concentrations of the amino acid under study in spiked and unspiked sample, respectively and c_{spiked} is spiking level. As a rule, for honey samples, recoveries between 80 and 100% were obtained with the exception of Pro, Met and Trp. Recoveries are comparable or slightly better than reported in literature. In literature, recoveries for amino acid analysis have been between 76% and 106% [10] or 78.8% and higher [9].

The developed sample preparation procedure was used for analysis of selenoamino acids in onion, garlic and human plasma samples. Recoveries were calculated with the Equation 12. Selenoamino acids are not detected in respective matrices unless supplemented to the living organisms. Therefore, blank matrices were obtained and used for recovery studies. For Seleno-methionine (SeMet) recoveries were 116% and 84.6% and for Seleno-methylselenocysteine (Se-MeSeCys) 93% and 101.7% (in serum and onion respectively). However, for more oilier garlic samples, recoveries were below 50%, meaning that the current SCX is not suitable for oily samples. Removal of oil prior to SPE could increase the recovery of selenoamino acids from garlic matrix.

4.2. Synthesis of a new derivatization reagent

The new direction in the amino acid analysis with LC/ESI/MS^2 is the design of new derivatization reagents, which would provide better ionization in the ESI source than the traditional reagents. Therefore, in addition to commercially available reagents, Deemm, Fmoc-Cl and DNS, one novel reagent from the literature was chosen to be part of the comparison – TAHS [28]. TAHS was chosen since it provides very low limits of detections and has been designed for sensitive LC/ESI/MS analysis. Since TAHS was not commercially available, it was synthesized in-house with some minor modifications to the original procedure. (See Experimental)

For the development of a new reagent, at first, a molecule called carbethoxymethylimino-tri(pyrrolidino)phosphazene (EtGlyP1$Pyrr_3$) was designed (Figure 1). I was designed to have a phosphazene in its structure since on the electrospray ionization efficiency scale these are among the best ionizable analytes [60]. The advantage of this compound was that since it existed without any derivatization procedure, it was possible to use it for ionization efficiency scale measurements to compare against Deemm and Fmoc-Cl amino acid derivatives. Results showed good ionization efficiency of the compounds compared to Deemm and Fmoc-Cl derivatives (these results are more thoroughly discussed in Section 4.4). Therefore, the structure of a phosphazene was chosen for the further design of an amino acid derivatization reagent that would be with good LC/ESI/MS sensitivity.

The new reagent designed and synthesized in-house was 2,5-dioxopyrrolidin-1-yl N-tri(pyrrolidino)phosphoranylideneamino carbamate (Equation 10) and is from now on called FOSF in further discussions. In addition to good ionization, phosphazenes have been previously synthesized in our workgroup [93]. One of them – hydrazinotripyrrolidinophosphobium hexafluorophosphate – was used as the basis for the synthesis of the derivatization reagent. It was converted into a derivatization reagent via reaction with disuccinimido carbonate, similarly to the previously published TAHS synthesis [28]. However, since FOSF is a strong base, there is no need for additional second step to create a charged group as in the case of TAHS [28]. This is also preferred since the use of harmful iodomethane from the TAHS synthesis is not needed. Synthesis of phosphazene based derivatization reagent was successful and a novel derivatization reagent was created. Due to the similarity of the reaction centers of FOSF and TAHS, the derivatization procedure was chosen the same as for TAHS. Separation for chromatographic analysis was developed with shorter column and ammoniumbicarbonate as a buffer with pH = 7 since these conditions provided better chromatographic separation and peak shapes compared to more acidic eluent buffers. Other aspects of the novel reagents are discussed in the following Section 4.3.

In addition to EtGlyP1Pyrr$_3$, pure Deemm amino acid derivatives were prepared for various ionization efficiency and boric acid influence measurements. These were prepared by following the protocol published by Alaiz et al. [97].

All the products from various syntheses were tested for their purity with chromatographic analysis with both MS and UV detection. Results showed high level of purity for Deemm amino acid derivatives and EtGlyP1Pyrr$_3$. However, both TAHS and FOSF contained low levels of impurities due to the more complex synthesis procedure. Since the derivatization of amino acids was not compromised (linear calibration graphs etc), extra steps for purification of the reagents were not carried out.

4.3. Comparison of derivatization reagents [Paper VI]

For amino acid LC/ESI/MS analysis, many derivatization reagents have been used. Deemm, Fmoc-Cl and DNS are amino acid derivatization reagents, which have been widely used for various applications over the years and are commercially available through various chemical suppliers. Moreover, these reagents are suitable for analysis of primary and secondary amino acids and also provide stable derivatives as opposed to reagents such as *o*-phthaldialdehyde (OPA) [106].

However, for LC/ESI/MS^2 analysis there has not been a comparison of all the reagents on the one system. In order to choose a derivatization method for a particular analytical task, it is necessary to compare properties of various amino acid derivatization reagents. Comparison of analytical performance of derivatization methods using results published by different groups may be inconclusive – aims, instruments and presentation of performance criteria are different. For example, different calculation and presentation methods for LoD/LoQ values are used. Another aspect to consider is that the mass spectrometry systems can have different instrument setups. In some cases, modifications are made in order to further enhance the signal such as using a sheath solution after the column outlet in the case of TAHS analysis [28]. Therefore, the straight comparison would provide useful information for choosing the derivatization reagent, which would allow the lowest detection and quantitation limits.

For comparison, 7 amino acids were chosen (Arg, Asp, Gly, β-Ala, Pro, Trp, Phe) so that their properties would represent the variability of amino acid structures. Comparisons are done in positive ESI mode using MS^2. For all amino acids and reagents MS parameters were optimized (elaborate discussion of MS optimization is provided in the Section 4.5). Various aspects are compared in order to get widespread information about five amino acid derivatization reagents.

4.3.1. General comparison

When comparing derivatization properties of Deemm, Fmoc-Cl, DNS, TAHS and FOSF, Deemm is poor for analysis of Pro. Deemm-Pro derivative is not stable, therefore resulting in high LoQ of the analysis [33, Paper II]. In this work, no LC-MS signal could be registered for DNS-Asp even at elevated Asp concentrations. However, previously DNS has been used for Asp analysis [36,103,104]. Fmoc-Cl proved to be suitable for derivatization and analysis of all the tested amino acids. TAHS provided very good signal for all amino acids, similarly to Fmoc-Cl. Elevated concentration of Arg solution was needed for FOSF derivatization – FOSF-Arg signal could not be obtained at normal concentration. This is most probably related to high basicity of Arg side chain.

4.3.2. Derivatization procedure

One aspect of the derivatization procedure is the preparation of a derivatization reagent solution. For Fmoc-Cl and DNS it is a general practice that a fresh solution is made before each measurement. For FOSF and TAHS no such info is available. However, experiments showed that there were no problems with FOSF and TAHS derivatization reagent solution for about a 9-month period (12 for TAHS). After that time the ability to derivatize amino acids decreased significantly and it was not possible to use the same solution again. It could be concluded that for all amino acid reagents, except Deemm, it is preferable that a freshly prepared derivatization reagent solution is made before each measurement. For Deemm, this practice is not necessary since Deemm is used for derivatization without any dissolution and over the course of approximately 8 years of usage, no problems were observed with derivatization.

For most amino acid derivatization reagents, the derivatization procedures are quite similar: derivatization reaction is carried out at high pH for some relatively short time and then the reaction is ended with a compound that would react with the excess reagent or the pH of the medium is changed in order to stop the reaction.

Out of these 5 derivatization procedures, for Fmoc-Cl and DNS, the reaction was stopped with using up the excess reagent: His for Fmoc-Cl and ammonia for DNS. For TAHS and FOSF, the pH of the derivatization mixture was changed with acetic acid and excess reagent is not removed since it does not interfere with the chromatographic analysis and MS detection. The simplest derivatization reaction is for Deemm – excess Deemm does not need to be removed or the pH changed. However, the downside is that, in order for the reaction to proceed to the end, analysis should be carried out 24–48 h after the derivatization [Paper II].

It is also important to discuss the possibilities for method automatization, which has been addressed by Shimbo et al. [98]. As for derivatization reagents under comparison, Deemm, TAHS and FOSF can in principle be used with an

automated system. Moreover, Fmoc-Cl has been used with automated derivatizations [99,100], as well as DNS [101].

4.3.3. Repeatability

With MS, the signal stability can be very different depending on the analyte, solvents and also the cleanliness of the ESI-source. Stability of the signal was evaluated at higher (amino acid concentrations around 3000 ng/g) and lower concentrations (LoQ concentration levels). Relative standard deviations of peak areas of six consecutive injections were calculated. Relative standard deviations at higher concentrations were all under 10%, which is acceptable in MS analysis and comparable to other LC/ESI/MS methods for derivatization reagents [23,25]. However, differences between the reagents emerged when signal stability at lower concentrations were assessed (Table 2). Results showed that for most reagents, even at low analyte concentration, relative standard deviation is below 10% and therefore very good for measurements at LoQ levels. However, for DNS, signals for different amino acids at low concentrations provided poor stability. Even though the absolute signals at low concentration levels were high compared to Deemm and Fmoc-Cl, the repeatability was poor. Reasons for that are unknown but might be related to the composition of derivatization mixture, which might produce signal modifiers, which are influential at low concentrations.

Table 2. Signal stability as relative standard deviation (n = 6) for 5 derivatization reagents at LoQ concentration levels.

	Deemm	**Fmoc-Cl**	**DNS**	**TAHS**	**FOSF**
Arg	8%	4%	9%	6%	6%
Asp	9%	8%	[a]	8%	14%
Gly	4%	6%	24%	9%	3%
β-Ala	5%	7%	20%	7%	5%
Pro	9%	0%	18%	4%	9%
Trp	2%	4%	25%	4%	6%
Phe	5%	7%	24%	4%	8%

[a] – the signal of Asp was not obtained for DNS analysis.

4.3.4. Chromatographic separation

For all derivatization reagents, chromatographic separation was optimized for 7 amino acids (23 for Deemm in Paper II). Various reagents are very different by their chromatographic separation properties. Differently from traditional derivatization reagents, novel reagents are charged at chromatographic conditions,

which is unfavorable for their reversed phase separation. Separation is more easily obtained for DNS, Fmoc-Cl and Deemm. For all amino acid derivatives, the retention times are presented in Table 3. Comparison shows that for commercial derivatization reagents, the elution order of amino acid derivatives is the same, but for TAHS and FOSF, the order of elution is slightly different.

Table 3. Retention times in minutes and percentage of MeCN content at the time of the elution for amino acid derivatives.

	Deemm	**Fmoc-Cl**	**DNS**	**TAHS**	**FOSF**
Arg	14.2 (25%)	13.0 (46%)	15.2 (35%)	14.8 (17%)	14.0 (27%)
Asp	23.0 (29%)	19.8 (57%)	[a]	15.6 (18%)	9.0 (19%)
Gly	27.5 (34%)	22.1 (60%)	22.0 (49%)	15.2 (17.5%)	13.5 (26%)
β-Ala	31.4 (38%)	22.5 (61%)	23.1 (51%)	17.3 (20%)	12.8 (25%)
Pro	35.0 (43%)	25.8 (66%)	27.4 (60%)	19.1 (21%)	15.8 (30%)
Trp	45.6 (55%)	28.7 (70%)	28.7 (62%)	31.6 (34%)	19.7 (35.5%)
Phe	47.0 (57%)	30.2 (73%)	30.4 (65%)	29.9 (32%)	20.3 (36%)

[a] – the signal of Asp was not obtained for DNS analysis.

For Deemm, in previous works, complete separation was obtained for 23 amino acids [Paper II]. Moreover, Se-MeSeCys and SeMet can be separated from 23 amino acids with Deemm also, providing chromatographic separation for 25 amino acids in total [Paper III]. This is significant since for many derivatization reagents, separation of that many amino acids could be a challenge. For the seven amino acids under interest, Deemm provides very good separation (Figure 5), Fmoc-Cl (Figure 6) and DNS (Figure 7) provide relatively good separation also. However, in case of Fmoc-derivatives, separation of Fmoc-Gly and Fmoc-β-Ala was problematic.

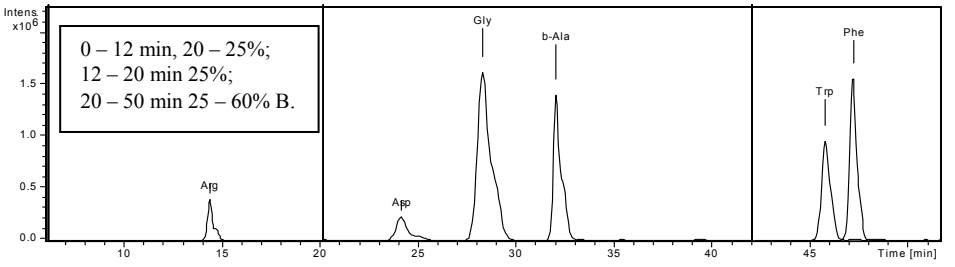


Figure 5. Representative chromatogram of Deemm derivatives (A: pH = 3.2, 1 mM ammonium acetate in 0.1% formic acid and B: acetonitrile).

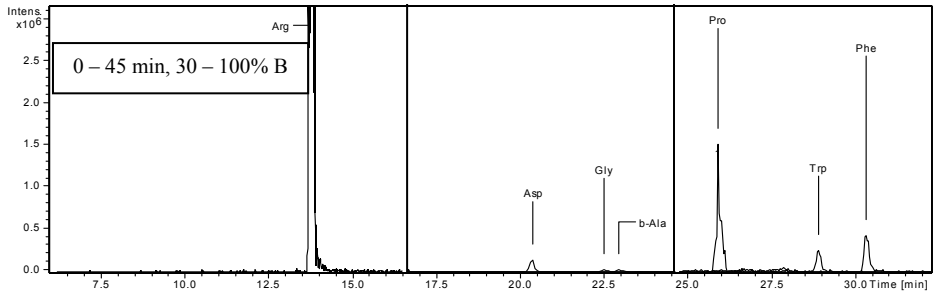


Figure 6. Representative chromatogram of Fmoc-Cl derivatives (A: 0.1% formic acid and B: acetonitrile).

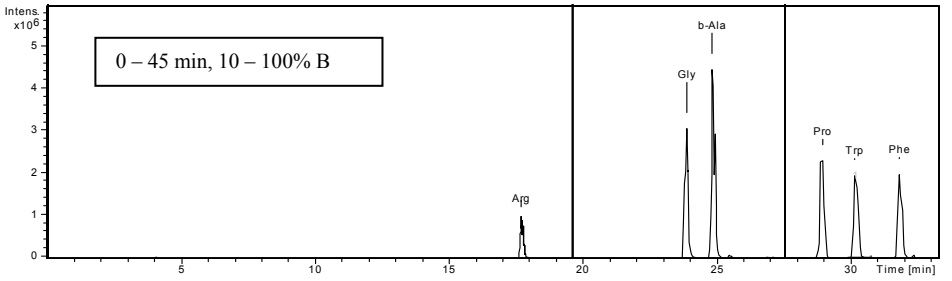


Figure 7. Representative chromatogram of DNS derivatives (A: 0.1% formic acid and B: acetonitrile).

For FOSF, several eluent compositions and components, pH values up to 7 and gradient programs were tested. The best chromatographic separation of FOSF derivatives was achieved with shorter column and ammoniumbicarbonate buffer (pH = 7) since these conditions provided better chromatographic separation. Moreover, peak shapes were better with these conditions compared to longer columns and more acidic eluents. However, getting all amino acids separated would be a challenge for the charged derivatization reagents – FOSF (Figure 8) and TAHS (Figure 9) [28].

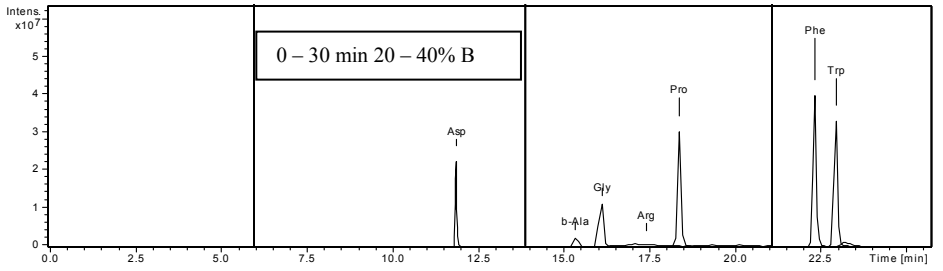


Figure 8. Representative chromatogram of FOSF derivatives (A: 1 mM ammonium-bicarbonate pH = 7; mobile phase B: acetonitrile).

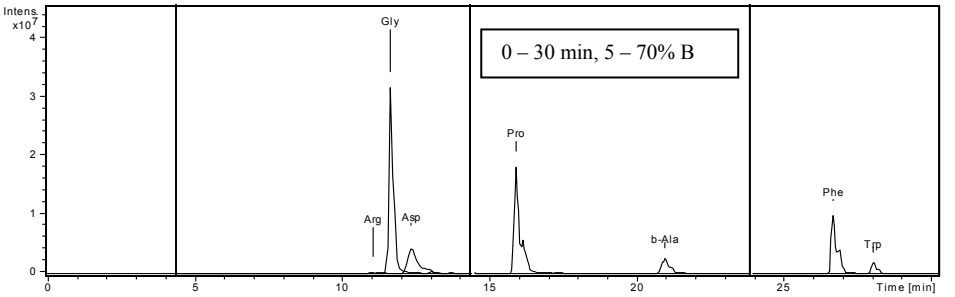


Figure 9. Representative chromatogram of TAHS derivatives (A: pH = 3.2, 1 mM ammonium acetate in 0.1% formic acid and B: acetonitrile).

Retention times of the derivatives of different amino acids vary since the content of MeCN needed for elution depends on the amino acid and reagent used. Fmoc-Cl and DNS need significantly higher content of MeCN for elution. In case of Deemm, the difference in the content of MeCN in the eluent between the fastest eluting peaks and the latest eluting peak is the largest. This results a longer run and better chromatographic separation. In the case of TAHS and FOSF, peaks start to elute at significantly lower MeCN content and peaks are eluting at quite similar eluent compositions resulting a poor chromatographic separation and also shorter run times. Moreover, with careful optimization, there is a possibility for isocratic analysis.

4.3.5. Linearity

Linearity of calibration graphs for amino acid derivatives were assessed. It was observed that for different derivatization reagents, the linear dynamic ranges differed significantly (Table 4). Therefore, it was briefly investigated by visual inspection of residual plots and squared correlation coefficients.

By its nature, LC/ESI/MS^2 does not have a very wide linear range and for certain analytes, linear range can be even narrower [102]. For Deemm, Fmoc-Cl and DNS, linear dynamic ranges are quite wide and this is their advantage over novel reagents, which seem to have quite limited linear dynamic range. This could be due to their very sensitive nature in ESI-source and moreover due to the fact that TAHS carries a permanent charge. For Deemm, the linear dynamic range is very large and this means that it is easy to apply for samples with wide range of amino acid concentrations.

In the case of novel reagents, these could be used for especially sensitive analysis and also in the very narrow amino acid concentration ranges. This is not desirable for applications where the analyte concentration in the sample can vary a large extent.

Table 4. Linear dynamic range for amino acid derivatization reagents in fmol.

	Deemm	Fmoc-Cl	DNS	TAHS	FOSF
Arg	108–27871	222–5502	142–14126	26–230	[c]
Asp	156–40295	488–8025	[b]	37–335	60–333
Gly	266–68863	1662–14114	364–36238	24–589	98–492
β-Ala	212–54790	1288–10933	282–28071	52–456	30–141
Pro	[a]	363–9543	246–24501	16–398	53–254
Trp	52–13541	108–2618	67–6722	13–109	4–47
Phe	50–13004	103–2737	70–7026	12–114	9–239

[a] – Pro was unstable for Deemm analysis and not added to the comparison.
[b] – the signal of Asp was not obtained for DNS analysis.
[c] – the signal of Arg for FOSF was not stable and not considered for linear range.

4.3.6. LoD and LoQ values

The limits of detection (LoD) were calculated from amino acid standards and expressed as three times the standard deviation (n = 6) and the limits of quantitation (LoQ) were calculated from the injections of amino acid standard solutions and are expressed as ten times the standard deviation (n = 6). Since all methods were equally optimized, results can be compared. LoD and LoQ are expressed in femtomoles on the column (Table 5).

Table 5. LoQ (n = 6) values for the studies amino acid derivatives and expressed in fmol on column.

fmol LoQ	Deemm	Fmoc-Cl	DNS	TAHS	FOSF
Arg	84	259	365	81	[c]
Asp	154	943	[b]	117	96
Gly	384	3615	3887	61	168
β-Ala	227	1687	377	101	54
Pro	[a]	174	1381	31	130
Trp	53	164	55	92	7
Phe	26	193	252	22	41

[a] – Pro was unstable for Deemm analysis and not added to the comparison.
[b] – the signal of Asp was not obtained for DNS analysis.
[c] – the signal of FOSF-Arg was not stable and LoQ values obtained not reliable.

Firstly it is observed that for some derivatives, the LoD/LoQ values differ from the lowest point in the linear range. This is due to the method that is used for LoQ calculations what takes into account the repeatability of the signal and at LoQ level, repeatabilities differ significantly case by case.

Comparison of overall results shows that different reagents do provide different LoQ values. It can be concluded from the results that the difference in the LoD/LoQ values is mostly due to the differences in the molecular structure of the reagents. It has been proposed that with higher organic content, the ionization efficiency is better [23]. However, in the current case, the LoD/LoQ values are lower for reagents that elute with lower MeCN content (TAHS and FOSF) and LoD/LoQ values are higher for those derivatives that elute at the higher MeCN content. Confirming even further that the differences in ionization are related to the structures of the derivatization reagents.

Moreover, amino acids differ from each other also, meaning that with the same reagent, LoD/LoQ values for different amino acids vary significantly. For example, for Fmoc-Cl, the LoD/LoQ values for Trp and Phe are significantly lower than for Gly and β-Ala (up to 18 times). For amino acids that elute in the end of the chromatogram, with higher organic percentage in the eluent, LoD/LoQ values are much lower than for amino acids that elute in the beginning of the chromatogram. It can be attributed to the fact that ionization is better with higher organic percentage in the eluent [54]. In addition, for all reagents, Gly derivatives provide much higher LoD/LoQ values compared to other amino acids (except for TAHS). It could be related to the fact that Gly does not have a side chain and therefore, the ionization efficiency is determined mostly by the reagent side. However, their retention on the reversed phase chromatography is poor and they elute at low organic solvent composition but with low organic content, the ionization is poorer in the ESI source. In order for a molecule to carry a charge and at the same time have a good chromatographic retention, the charge should be sterically shielded. This is taken into account with a design of FOSF.

Comparison of the reagents to each other shows that novel reagents, TAHS and FOSF, do differ from the commercially available derivatization reagents providing lower LoD and LoQ values. From commercially available reagents, Deemm shows comparable results to new reagents. Moreover, Deemm has been used for very sensitive selenoamino acid detection [Paper III] and proves that Deemm is very sensitive for LC/ESI/MS^2 analysis. Besides, it is commercially available and provides much better chromatographic separation than novel reagents because Deemm does not carry a permanent charge. This is a useful concept for novel LC/MS derivatization reagents – derivative part should be uncharged for better chromatographic separation, but easily charged in ESI. According to ionization efficiency scale by Oss et al. [60], diesters like Deemm are good candiates for derivatization reagents.

As for Fmoc-Cl and DNS, these provide higher LoD/LoQ values. It is important to note that DNS provides signal at very low concentrations but due to poor signal stability, the values for LoD/LoQ indicate that DNS is not as good as Deemm. If the stability problem with DNS is addressed, its LoD/LoQ values could be lowered.

LoD/LoQ values of novel derivatization reagents are quite similar to each other. One could not be preferred to the other by these results.

Since the results are calculated in fmol on column, LoD/LoQ could be improved by using bigger injection volume or decreasing volumes of derivatization solvents, in order to increase the amount of analyte entering the column.

In conclusion, choosing a derivatization reagent carefully can significantly influence the method sensitivity.

4.3.7. MS^2 analysis

In most cases, MS^2 analysis is targeted since it provides more sensitive analysis. Therefore, a separate discussion of the MS^2 analysis with different reagents is included.

In most cases, the $[M+H]^+$ is used as a parent ion (Table 6). However, for Fmoc-Cl and Deemm derivatives, Na-adduct is fragmented (except Arg). For Fmoc-Cl and Deemm, the Na-adducts were more abundant than $[M+H]^+$. Moreover, signal from protonated form was missing from the MS^1 spectra of Fmoc derivates. It has been proposed that the formation of Na-adducts can be related to the geometry of the instrument [83]. However, since with the used instrument, the formation of Na-adducts is dependent on the derivatization reagent, it is very likely that it is a property of a derivatization reagent. Moreover, it has been written that the use of ammonium acetate should suppress the presence of Na-adducts [82] but Na-adducts are most abundant independently of the eluent component used since in the case of Deemm ammonium acetate is present in the eluent but Na-adduct is still most abundant.

For Fmoc-Cl, all derivatives (except Arg) gave the same fragment, 263. However, Arg is different than other 6 amino acids by not giving a Na-adduct and fragmenting differently, giving a fragment with *m/z* 336. This can be explained by Fmoc-Cl reacting with more basic side-chain amino group rather than α-amino group. The same applies to Deemm-Arg that does not have a Na-adduct either. Fragmentation of Deemm derivatives differs from Fmoc-Cl derivatives, since for each amino acid, different fragment is observed and neutral 46 amu fragment is lost. This makes Deemm fragmentation different from all other derivatization reagents discussed in this work. Regarding MS analysis modes, single reaction monitoring (SRM) can be used for both types of derivatives. Additionally neutral scan can be used for Deemm derivatives and parent ion scan for other derivatives. Both modes can be utilized for analysis of complex mixtures.

It is discussed that TAHS has been designed keeping MS^2 fragmentation in mind [28]. Other derivatives included in present study proved to be also suitable for MS^2 analysis. In all cases, LoQ values were lower for MS^2 than for MS^1.

Table 6. Parent and fragment ions chosen for the MS^2 analysis.

Amino acid	Deemm		Fmoc-Cl		DNS		TAHS		FOSF	
	Parent ion (*m/z*)	Fragment (*m/z*)	Parent ion (*m/z*)	Fragment (*m/z*)	Parent ion (*m/z*)	Fragment (*m/z*)	Parent ion (*m/z*)	Fragment (*m/z*)	Parent ion (*m/z*)	Fragment (*m/z*)
Arg	345	321	397	336	408	252	351	177	472	298
Asp	326	280	378	263	–	–	310	177	431	298
Gly	268	222	320	263	309	252	252	177	373	298
β-Ala	282	236	334	263	323	252	266	177	387	298
Pro	308	262	360	263	349	252	292	177	413	298
Trp	397	351	449	263	438	252	381	177	502	298
Phe	358	312	410	263	399	252	342	177	463	298

4.4. IE scale of amino acids and their derivatization reagents

IE measurements were added to the experimentation set in order to compare the ionization of amino acids and their derivatives with other compounds on the IE scale [60,61]. Moreover, the original IE scale has been developed in our workgroup and all the setup for IE measurements freely available. For IE measurements, the same solvent composition (acetonitrile/0.1% aqueous formic acid in volume ratio 80:20) is used and therefore the influence of different organic content of eluent is eliminated. In this respect, IE expressed "true" difference of ionization efficiency.

IE was measured only for amino acids and derivatives that were available as pure compounds. For that purpose, Fmoc-Cl derivatives (β-Ala, Gly, Phe) were commercially purchased and Deemm derivatives of the same amino acids were synthesized. Same amino acids were also part of the set of 7 amino acids chosen for the comparison of the derivatization reagents. For evaluation of the phosphazene ionization properties, it was possible to have EtGlyP1Pyrr for comparison. In addition to derivatized amino acids, the set of compounds for IE measurements also included free amino acids to get an insight to the ionization properties of the compounds.

In order to tie new compounds to the already existing scale, some compounds from already constructed IE scale [60] were also included to the measurement set. In total, four compounds were chosen: $PhP1(NMe_2)_3$ (logIE = 5.18), diphenylamine (logIE = 4.18), diphenylphthalate (logIE = 4.10), and 4-chloro-2-nitroaniline (logIE = 2.32) (Figure 10). Compounds were chosen so that they would be close to predicted logIE values of the amino acids and their derivatives.

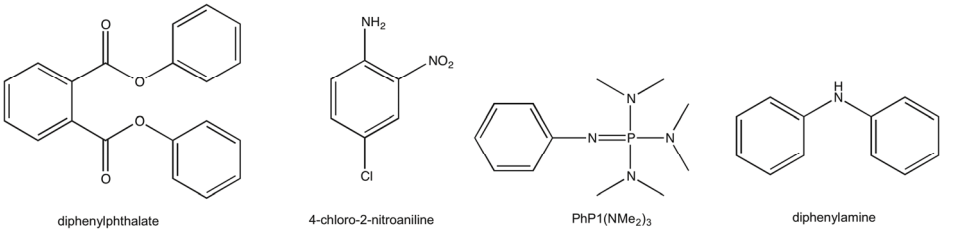


Figure 10. Stuctures of $PhP1(NMe_2)_3$, diphenylamine, diphenylphthalate and 4-chloro-2-nitroaniline.

For IE measurements, molar masses of the compounds must also be considered. Due to the nature of the measuring method, using the instrument specific target mass, it is important that the molecular masses would be similar for the compounds that are measured against each other. It is due to the fact that for a measurement, mass spectrometer is tuned to certain mass (target mass) and the

further is the *m/z* of the studied compound from the target mass, the more is its signal discriminated. For example, if an IE measurement is between an amino acid and its derivative, amino acid has significantly smaller molecular mass. Meaning that when the measurement is done at the target mass of an amino acid, the signal of a derivative is very small or even lost, even though it is superior in ionization and results can be misleading. LogRIE values that are calculated based on such experiments would lead to an apparent low logIE of the derivative. The difficulty is, however, that IE scale is mostly composed of compounds that are with lower molecular mass than amino acid derivatives. This is why diphenylphthalate (M=318 g/mol) was used instead of initially chosen diphenylamine (M=169 g/mol). However, in the end, after a thorough investigation of the calculations and measurement data, it was concluded that with some modification, it is possible to add all the measurements to the scale and the modification being that the logIE value was calculated with the data acquired from the target mass = 500 measurements (marked grey on the Figure 11).

In preliminary tests, amino acids (β-Ala, Gly and Phe) were measured against Fmoc-derivatives but the measurements were difficult to carry through due to the low molecular masses of amino acids and their poor ionization efficiency. Therefore, amino acids were not included on the scale. However, measurements clearly showed significantly poorer ionization than Fmoc-derivatives and the difference between the ionization of different amino acids, Phe having the best ionization out of three amino acids measured, probably due to its aromatic side-chain.

IE measurements were done in one-year period and a scale of logRIE values was constructed (Figure 11). The scale presented contains logIE values calculated directly from the measurement results; no further data treatment is carried out [60].

Extensive in-source fragmentation of the Fmoc-Cl derivatives appeared to be a problem for IE measurements. Since the main fragment was the amino acid itself, it was not possible to measure Fmoc-Cl amino acid derivative against corresponding amino acid, for example, it was not possible to measure Fmoc-β-Ala against β-Ala since they both would result a same *m/z*.

Another aspect of the measurements is that for Deemm and Fmoc-Cl derivatives, the most abundant ion is Na-adduct. However, the IE scale included only ionization via protonation ($[M+H]^+$) and neglects Na-adducts. Topics concerning the formation of Na-adducts deserve further study, but are out of the scope of the current work.

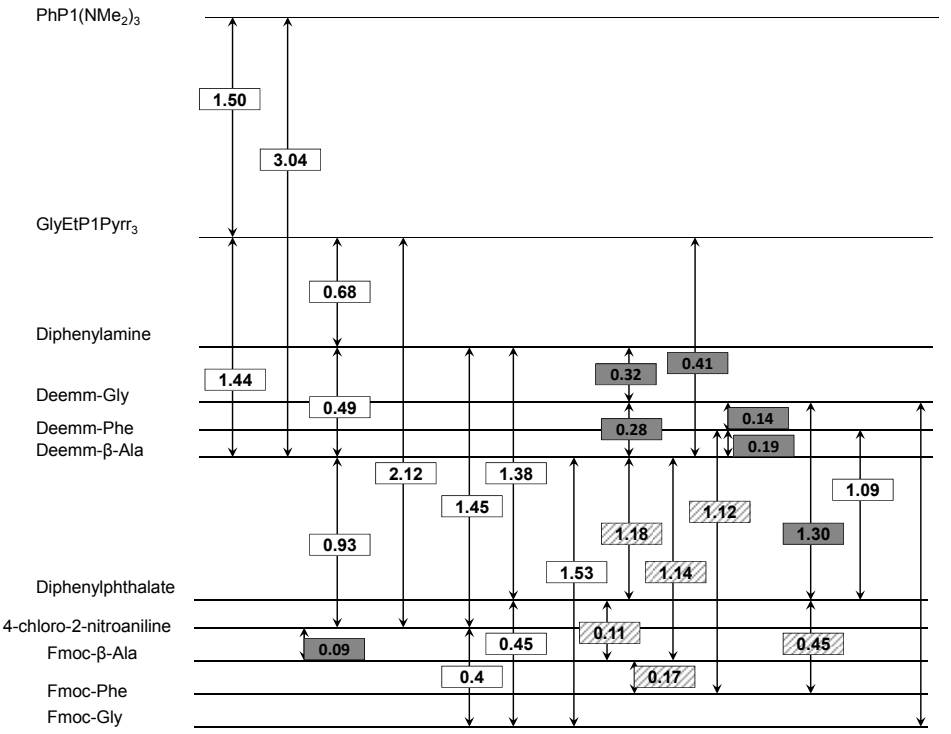


Figure 11. logRIE measurements: a) white – measurements according to Equation 11, b) darker grey – measurements at target mass 500 and c) striped – measurements not in the best agreement with other measurements but still acceptable.

The overview of the IE scale measurements shows that Deemm-derivatives are ionized more efficiently than Fmoc-Cl derivatives. The difference between the best ionizable Deemm-derivative (Deemm-Gly) and the poorest ionizable Fmoc-derivative (Fmoc-Gly) is around 2 log units meaning that Deemm-Gly has around 100 times better ionization that Fmoc-Gly. This is a significant difference when choosing a more sensitive amino acid derivative. Comparison of the same reagents in the LC/ESI/MS^2 system shows the difference between the LoD/LoQ values in the range of 10 times. One explanation is that IE scale measurements discard the Na-adduct that is most abundant for Fmoc-Cl and Deemm derivatives. This illustrates how the ionization differences in the controlled environment and real chromatographic system vary. In this case, the differences in the ionization are decreased by the LC/ESI/MS^2 analysis. Results are similar for other pairs.

The amino acid side contributes significantly to the ionization of the derivatives. For example, Deemm-Gly ionizes about 2 times better than Deemm-β-Ala. Again, the difference in chromatographic conditions is not as significant. When for Deemm derivatives, ionization efficiency increases in the order

Deemm-β-Ala, -Phe and -Gly, then for Fmoc-Cl, the order is opposite. Due to the limited number of tested compounds, generalizations are inconclusive, but the reversal IE order may be attributed to the combination of difference in pK_a and side-chain hydrophobicity. It could be emphasized that it is quite surprising that the Phe-derivatives are not the best in the comparison since it could be predicted that due to aromatic functional group it could be better ionizable than simple Gly- and β-Ala-derivatives. However, in the LC/ESI/MS^2 conditions, Phe-derivatives elute at high organic content and therefore provide lower detection limits.

Results for the novel phosphazene showed that it has even higher ionization than Deemm and Fmoc-Cl derivatives. This is expected when examining the structure of the EtGlyP1$Pyrr_3$, since phospazenes are highest on the IE scale concluding that it can be used as a tool for designing derivatization reagents and to test their efficiency.

The obtained IE scale of amino acid derivatives extends over 3 log units, showing how different are the properties that amino acid derivatives possess. Moreover, the scale is in accordance to the results obtained by the comparison of different derivatization reagents. Even though the IE scale is not very elaborate and not all amino acids are considered, it still gives an idea what to consider when designing new amino acid derivatization reagents and reagents with similar structures and functional groups should be targeted.

It can be concluded from the results that there are significant differences in ionizations of different compounds. However, this measurement takes place in strongly controlled conditions and when carrying out real analysis with derivatization procedure, chromatographic separation and MS detection, situation can be quite different. Despite that, some insight is given by these results how to improve amino acid analysis when looking for even more sensitive analysis, especially when designing new derivatization reagents.

4.5. Optimization of MS parameters

The ESI-interface available on the used system is very common when using (reversed-phase) liquid chromatography. In this type of source, the ions formed are guided from the atmospheric pressure region to the high vacuum of the mass analyzer. For achieving the highest signal, the parameters of the mass spectrometer, which are modifiable, need to be optimized.

For analysis of the amino acid derivatives, the instrument used was the Agilent XCT quadrupolar ion trap mass spectrometer with ESI. For this type of instrument, the parameters to be optimized were: Capillary voltage, Capillary exit, Skimmer Voltage, Lens 1 voltage, Lens 2 voltage, Trap Drive, Fragmentation Amplitude and Fragmentation CutOff. Detailed description of the instrument, parameters and the procedures for optimization are described in the Experimental section of this work.

In addition to parameters that can be optimized using the instrument control software, there are parameters of the source that can be manually changed and no automatic optimization is provided: nebulizer gas (nitrogen), dry gas (nitrogen) and the dry gas temperature. These parameters were firstly optimized. Parameters chosen were 50 psi (345 kPa) for nebulizer gas, 12 L/min for dry gas and the temperature 350°C. These parameters have been previously used for developed and validated methods for analysis of Deemm-derivatives. Those values are close to the maximum possible values that the instrument can provide for these parameters. For all derivatization reagents these were re-evaluated and three values for each parameter were tested: nebulizer gas (25, 35 and 50 psi), dry gas (7, 9, 12 L/min) and the temperature (325, 350 and 360°C). Results showed that previously chosen values (50 psi for nebulizer gas, 12 L/min for dry gas and the temperature 350°C) provide the highest signal for most reagents.

Representative figures are presented based on DNS-derivatives (Figure 12) and have been calculated relative to the values: nebulizer gas 50 psi, dry gas 12 L/min and temperature 350°C. Improvement could be provided only by the higher temperature, however, this would be almost the instrument's maximum and not chosen. Therefore, for the further optimizations and analysis, previously validated parameters are used and are same for all the reagents.

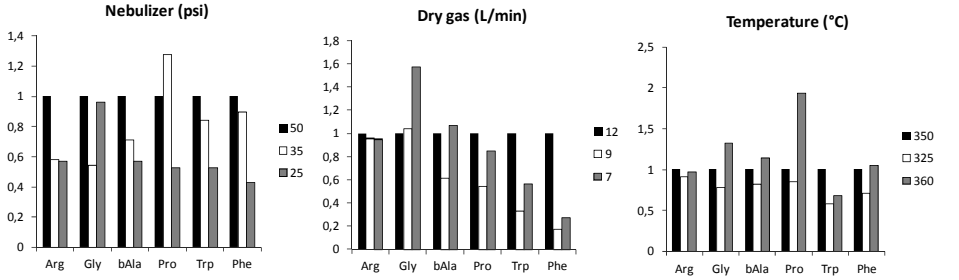


Figure 12. Optimization of nebulizer gas, dry gas and the source temperature on the example of DNS.

For all the parameters, optimization with the instrument control software was carried out for all 7 amino acid derivatives of the five amino acid derivatization reagents (6 for DNS) (Table 7). Various aspects were evaluated. In order to evaluate the impacts and changes of the parameters obtained through optimization, these were entered into the instrument controlling software for each chromatographic run. This way the influence on the signal by the combination of all the parameters can be evaluated.

Table 7. Results from the optimization with procedure A (white) and procedure B (grey).

	Capillary	Skimmer	Cap Exit	Oct 1DC	Oct 2DC	Trap Drive	Oct RF	Lens 1	Lens 2	Frag Ampl	FragCutOff
Deemm											
Arg	3106.56	41.56	156.72	13.11	2.43	66.52	78.69	–4.18	–69.02	3	179.89
	1500	40.66	100.82	14.84	1.15	43.38	108.2	–4.92	–70.49	[a]	[a]
Asp	2532.79	43.03	136.39	13.98	1.79	45.84	68.85	–3.44	–63.11	1.08	153.38
	2360.66	62.05	121.15	20	2.13	68	162.3	–4.18	–61.64	0.87	161.4
bAla	2475.41	38.28	75.41	13.4	1.44	30.1	83.61	–3.69	–70.49	1.81	129.9
	2418.03	43.03	95.74	14.84	1.05	35.02	113.11	–4.67	–60.16	1.81	141.92
Gly	2475.41	43.03	116.07	13.04	1.1	38.46	93.44	–4.92	–67.54	0.54	130.49
	2934.43	69.18	105.9	12.83	2.05	58	73.77	–4.92	–75.92	0.99	132.68
Pro	4885.24	30.33	99.18	11.11	1.31	38.87	108.2	–5.41	–66.07	1.28	154
	4540.98	101.31	360	5.37	1.34	76.17	24.59	–6.39	–10	1.73	90.38
Phe	3393.44	38.28	146.56	12.25	0.67	35.67	78.69	–4.67	–61.64	3	250.6
	3909.83	33.52	136.39	11.97	1.19	40.59	196.72	–4.67	–55.74	3	250.6
Trp	3278.69	43.03	131.31	11.39	1.42	42.23	88.52	–4.92	–51.31	0.5	183.53
	3622.95	62.05	121.15	20	0.5	39.77	186.89	–5.16	–100	0.5	183.53
Fmoc-Cl											
Arg	3450.82	40.66	151.64	12.83	1.25	43.87	290.16	–3.93	–63.11	1.69	261.63
	2532.79	59.67	146.56	11.39	1.42	43.05	167.21	–4.67	–49.84	0.5	173.77
Asp	2877.05	40.66	141.48	10.25	1.59	42.23	103.28	–5.9	–64.59	0.5	168.55
	5000	38.28	50	10.25	1.76	41.41	147.54	–5.66	–52.79	1.65	205.73
bAla	2590.16	43.03	126.23	11.68	1.31	38.13	113.11	–6.89	–69.02	2.39	233.8
	3622.95	45.41	126.23	10.82	1.36	40.59	83.61	–4.43	–64.59	0.91	168.09
Gly	3909.83	50.16	131.31	14.55	1.59	40.59	78.69	–4.92	–80.82	2.06	224
	3852.46	45.41	90.66	12.83	1.48	39.77	137.7	–5.9	–67.54	2.06	224
Pro	3049.18	43.03	105.9	11.11	1.36	41.41	98.36	–6.89	–66.07	0.87	169.38
	5000	45.41	131.31	12.54	1.3	43.05	167.21	–5.41	–67.54	0.99	178.23

	Capillary	Skimmer	Cap Exit	Oct 1DC	Oct 2DC	Trap Drive	Oct RF	Lens 1	Lens 2	Frag Ampl	FragCutOff
Phe	3221.31	47.79	126.23	11.39	1.36	43.87	211.48	–2.21	–40.98	0.5	172.74
	3221.31	33.52	116.07	12.54	1.48	45.51	152.46	–6.39	–100	1.49	166.02
Trp	3336.06	40.66	126.23	10.53	1.25	43.05	88.52	–6.39	–77.87	0.5	172
	5000	33.52	156.72	9.67	1.36	42.23	93.44	–2.95	–60.16	1.49	145
DNS											
Arg	3393.44	47.79	161.8	11.68	1.59	43.05	103.28	–5.66	–64.59	0.5	185.27
	2475.41	40.66	151.64	11.68	1.36	43.87	181.97	–4.92	–67.54	1.57	171.9
bAla	2647.54	40.66	121.15	14.55	1.65	48.6	122.95	–4.18	–74.92	2.06	173.15
	5000	40.66	110.98	13.69	1.42	41.41	137.7	–4.92	–58.69	2.02	181.09
Gly	5000	47.79	141.48	13.11	1.13	39.77	181.97	–5.16	–89.67	0.71	183.37
	4196.72	40.66	121.15	12.83	1.3	38.95	152.46	–4.92	–69.02	0.71	216.3
Pro	3336.06	16.89	50	12.54	1.42	43.87	147.54	–3.69	–73.44	2.18	124.15
	2418.03	35.9	121.15	11.39	1.59	42.23	157.38	–4.43	–60.16	0.97	181.37
Phe	3336.06	33.52	182.13	11.11	1.02	38.13	167.21	–6.64	–100	1.53	217.16
	4311.47	40.66	131.31	10.53	1.53	43.05	93.44	–5.6	–74.92	0.5	177.91
Trp	3450.82	69.18	141.48	9.67	1.48	45.51	78.69	–6.39	–61.64	1.44	159.77
	3795.08	47.79	136.39	10.53	1.25	43.87	196.22	–4.92	–74.92	1.44	134.27
TAHS											
Arg	2834.43	40.66	50	10.25	2.68	42.23	83.61	–5.66	–66.07	2.31	121.99
	1500	33.52	90.66	11.11	1.93	41.41	83.61	–4.43	–60.16	2.14	113.36
Asp	2762.29	45.41	131.31	13.11	1.88	42.23	68.85	–4.67	–70.49	0.62	133.15
	2188.52	33.52	85.57	11.68	1.3	38.95	83.61	–6.89	–83.77	0.54	130.61
bAla	5000	52.54	151.64	14.84	1.59	41.41	83.61	–6.89	–74.92	0.5	103.35
	1500	43.03	126.23	13.4	1.48	39.77	98.36	–4.91	–67.54	0.87	103.35
Gly	2303.28	43.03	110.98	14.26	1.82	41.41	73.77	–4.43	–60.16	0.5	108.24
	2188.52	40.66	55.08	13.4	1.88	48.6	98.36	–4.18	–57.21	0.5	97.91
Pro	2360.66	35.9	121.15	12.54	0.96	39.77	162.3	–5.9	–69.02	1.2	118.24
	2532.79	69.18	136.39	12.83	1.48	37.31	98.36	–4.67	–71.97	1.12	125.42

	Capillary	Skimmer	Cap Exit	Oct 1DC	Oct 2DC	Trap Drive	Oct RF	Lens 1	Lens 2	Frag Ampl	FragCutOff
Phe	5000	40.66	146.56	13.11	2.05	47.97	132.79	–3.44	–45.41	1.81	116.06
	2418.03	43.03	141.48	13.69	1.82	47.97	98.36	–5.41	–82.3	0.5	132.88
Trp	5000	54.92	156.72	15.12	1.25	45.51	221.31	–5.9	–77.87	0.5	79.32
	2934.43	50.16	156.72	13.4	1.76	44.69	93.44	–4.43	–64.59	1.57	79.32
FOSF											
Arg	3622.95	47.79	156.72	9.1	1.48	42.23	157.38	–1.48	–30.66	0.51	206.6
	3967.21	59.67	136.39	9.39	1.42	43.05	108.2	–5.16	–51.31	0.51	179.51
Asp	5000	31.15	141.48	10.82	1.53	44.69	167.21	–4.92	–42.46	0.49	142.72
	2360.66	35.9	146.56	11.11	1.48	45.51	201.64	–5.66	–73.44	0.59	227.51
bAla	3737.7	57.3	177.05	13.4	1.65	43.87	196.72	–5.16	–74.92	1.73	207.46
	2418.03	33.52	131.31	14.26	0.84	42.23	118.03	–5.16	–63.11	0.5	166.22
Gly	3106.66	47.79	131.31	11.97	1.7	42.23	73.77	–6.39	–67.54	2.31	199.95
	2016.39	35.9	131.31	13.69	1.3	42.23	152.46	–4.92	–63.11	1.03	193.84
Pro	5000	40.66	166.89	13.69	1.36	42.23	300	–7.87	–71.97	1.5	224.78
	3136.93	38.28	110.98	11.97	1.53	43.05	300	–6.15	–77.87	0.5	96.14
Phe	5000	88.2	136.39	10.82	1.53	45.51	191.8	–7.62	–100	0.54	157.12
	2418.03	33.52	141.48	9.67	1.42	43.87	98.36	–5.9	–71.97	1.94	141.94
Trp	5000	66.8	197.38	9.67	1.3	43.87	93.44	–5.16	–51.31	1.73	166.24
	2532.79	33.52	156.72	9.67	1.3	44.69	98.36	–5.66	–63.11	0.5	145.66

[a] – unable to obtain stable fragmentation during optimizatio

Firstly, the impact on MS^1 was investigated. This leaves out the influence of the Fragmentation Amplitude and the Fragmentation CutOff. Two concentration levels were used (2000 ng/g and 30 ng/g levels for DNS, Deemm and Fmoc and 400 ng/g and 10 ng/g for TAHS and FOSF). Secondly, for evaluating the influence of Fragmentation Amplitude and the Fragmentation Cutoff, the comparison of peak areas from the MS^2 analysis were also compared. This approach is different from the methods previously described, since it has been assumed that the parameters influencing fragmentation are not dependent of the procedure used for optimization or solvent composition and flow rate [71]. Moreover, the comparison was carried out between the results that procedure A and B provided (Section 3.2.2). Results were evaluated in two ways, first how similar are the values obtained and secondly, how do these parameters influence the peak area. Point by point comparison is presented followingly.

4.5.1. Optimization of the MS^1 parameters

At first, the optimization procedures A and B were evaluated. It has been suggested that the procedure with larger eluent flow rate would be more suitable for MS optimization with this type of instrument [71]. By large, it could be concluded that for the procedures A and B, Octopole 1DC and Trap Drive provide similar values, except for Deemm derivatives. All other parameters, at least for one amino acid for certain derivatization reagent, have different values when comparing values obtained by procedure A and B.

Since the final goal is to use these parameters for the chromatographic analysis, chromatographic peaks are now observed. However, for Fmoc-Cl, Deemm and TAHS, the differences between the chromatographic peak areas in majority of cases are relatively insignificant (approximately below 20%, randomly distributed between which procedure is better). For DNS, the default parameters (Section 3.2.2) give very good results, up to 20% better results than the optimized methods. This emphasizes that the combination of all the parameters have more influence on the signal than the value of each parameter alone. And for FOSF, more than 30% of the signal enhancement is obtained with procedure B optimization compared to default parameters as well as procedure A.

More thorough comparison was carried out for the cases where the signal from the procedure A and B differed largely. In the case of Deemm, signal is not in correlation to one parameter but to the combination of the parameters. It seems like the final results are dependent on the combination of all the parameters. After the evaluation of the parameter values and the peak areas, there does not seem to be a correlation between the values and the signal intensities meaning that signal can be high with low parameter value and the opposite also.

Capillary voltage is one of the values, which varies largely for different procedures and amino acids. In many cases, the software proposes the maximum value (5000 V) as the most optimal. And even for the same reagent and

amino acid derivative, the value can be different for the procedure A and B. However, this does not seem to affect the overall ionization.

It is interesting to compare optimal parameters for different amino acids with the same reagent. From the results it can be concluded that for each derivative, both the derivatization reagent side, as well as the amino acid side are similarly important while analyzing its ionization and optimization. This conclusion is drawn from the comparison of the values that the software proposed as most optimal. They vary for the different amino acid derivatives of the same reagent in addition to difference between the reagents.

By large, it could be said that the optimization of MS^1 parameters is dependent on the reagent and amino acid. Even for the same derivatization reagent, results vary and it is difficult to make overall conclusions. In Figure 13, two characteristic optimization results are shown. Relative change in peak area is calculated compared to the default parameter set. Changes both for procedure A and B are presented.

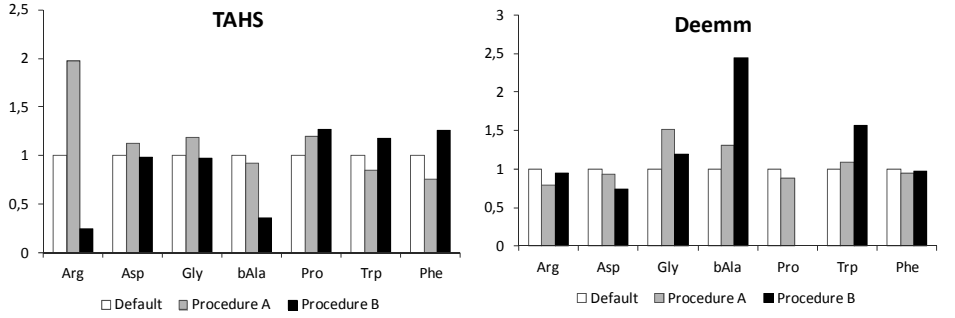


Figure 13. Results for optimization of MS^1 for TAHS and Deemm.

It can be seen that the peak areas obtained with procedure A are more similar to the default procedure. Moreover, even though the absolute values of the optimized parameters differ between the amino acids, the ionization is not as strongly affected and it is possible to optimize 2–3 compounds from various retention times and use those for other amino acids nearby also. It is advised to use procedure B for the optimization.

4.5.2. Optimization of the fragmentation parameters

Parent and fragments used for optimization of MS^2 are in Table 6. When comparing the peak areas obtained with the MS^2, it can be seen that in most cases, the areas are strongly dependent on the optimization procedure. The default values of parameters provide very poor signal (as low as 1% of signal remained compared to the optimized values in the case of Fmoc-Cl). This is a clear sign that the optimization of the fragmentation parameters is crucial. When in many

cases, the default parameters of the MS^1 provide relatively good values, the wrong choice of fragmentation parameters can lose up to 99% of the signal. On the Figure 14, results from the optimization of MS^1 and MS^2 for Fmoc-Cl are shown. Relative change in peak area is calculated compared to the default parameter set for procedure A and B, at the same concentration level. It can be seen that in the case of MS^1 optimizations, peak areas are similar for un-optimized and optimized parameter sets. However, unlike for MS^1, when for MS^2 analysis the default parameter set is used, results are significantly worse compared to optimized paramaters. Meaning that a lot of signal is lost due to the unoptimized parameters.

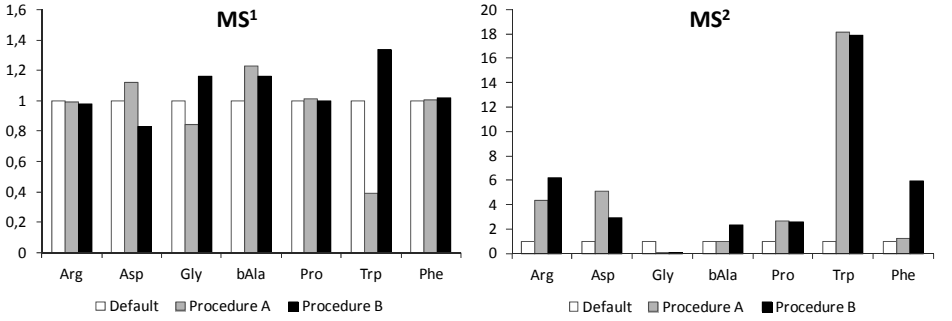


Figure 14. Results for optimization of MS^1 and MS^2 for Fmoc-Cl (note the scale).

Instrumentation used in this work has two fragmentation parameters: Fragmentation Amplitude (default value 1) and the Fragmentation CutOff (default value 100). From the close investigation of the values of these parameters, as well as the peak areas in the case of MS^2 analysis, it can be concluded that the main influence on the MS^2 analysis is from the Fragmentation Amplitude, and not from the Fragmentation CutOff. Fragmentation CutOff has very similar values for both procedures A and B, while values for the Fragmentation Amplitude (FragAmp) vary largely, up to 2–3 times.

When instrument is carrying out an optimization, it produces optimization curves that consist of points that are created by the instrument's software when scanning through the possible values for a paramater. Each point represents the intensity of the ion under interest at that parameter value. For example, on the Figure 15a, there is an optimization curve for Deemm-Arg for Octopole 1DC optimization. The curve has one maximum.

In the case of optimization of FragAmp, each point represents the signal intensity of a fragment ion at corresponding FragAmp value. It was also observed, that only in the case of FragAmp optimization curve, two maximums occur (Figure 15b). The phenomenom of the two maximums is unclear. Similar behavior has been observed earlier with different analytes with the same instrument.

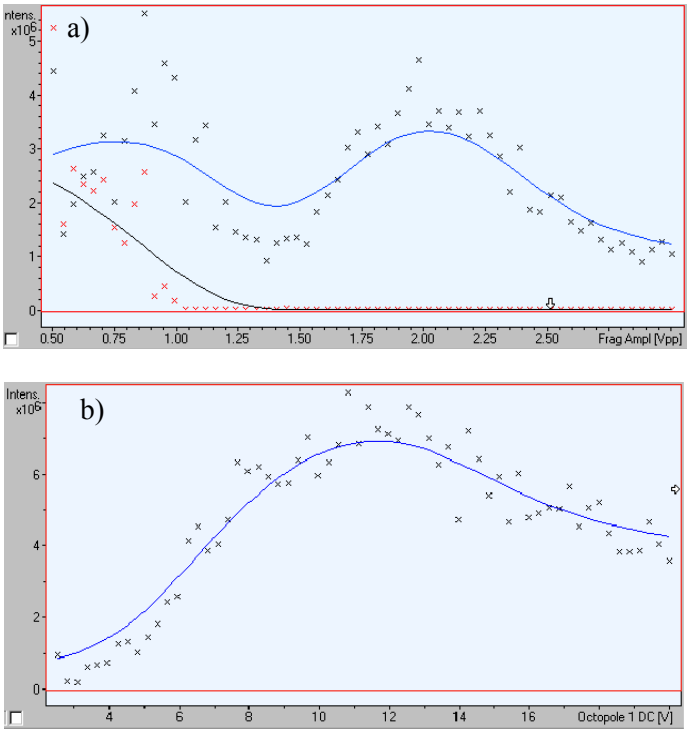


Figure 15. Example of a) an optimization curve with one maximum (Deemm-Arg, Octopole 1DC Procedure A); b) an optimization curve with two maximums which is observed when optimizing Fragmentation Amplitude for (DNS-β-Ala, Procedure A).

From the investigation of the curves for FragAmp optimization, it emerged that for all reagents, at least for 4 amino acids, the optimization curve had two clear maximums. For most cases, the first maximum was around 0.5–1 V and the second maximum higher, around 1.5–3 V, depending on the amino acid and the derivatization reagent. For some cases (Deemm (all amino acids), Fmoc-Cl (Gly), DNS (Gly, β-Ala), TAHS (Asp, Arg)), the software chose the same maximum for the procedure A and B, but for all other cases, the software chose one maximum for the procedure A and another for the procedure B. Example of this behavior is presented on the Figure 16. Only for Deemm, for all amino acids, both procedures chose the same maximum. It is interesting that for other reagents, software chose two different values for both procedure. It could be related to the fact that out of the observed five derivatization reagents, Deemm-derivatives are the only ones that when fragmenting, lose exactly the same neutral fragment while in the case of other reagents, the fragment depends on the amino acid since amino acid side is lost.

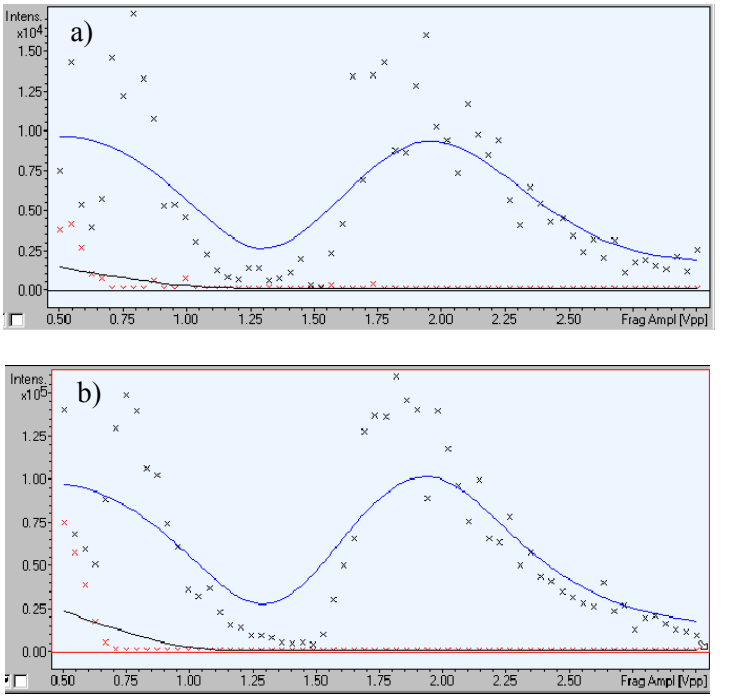


Figure 16. Two figures represent the optimization curves when optimizing FragAmp value for FOSF-Phe: a) for procedure A (program chose the value of 0.5) and b) for procedure B (program chose the value of 1.95)

This phenomenon was investigated further by comparing obtained peak areas with three methods 1) the FragAmp value chosen by the software with procedure B, 2) the FragAmp value chosen by the software with procedure A, and 3) the FragAmp value corresponding to the minimum on the optimization curve. For other MS^1 parameters, values obtained with procedure B were used. If the maximum for the procedures A and B were the same, the other maximum was estimated from the optimization graph and used for comparison. Results for comparison between peak areas obtained with FragAmp value 1) and 2) are presented in the Table 8.

Table 8. Ratios of signals obtained with FragAmp value 1) and 2).

	Deemm n=4	**Fmoc-Cl** n=4	**DNS** n=4	**TAHS** n=4	**FOSF** n=2
Arg	125%	129%	150%	113%	92%
Asp	189%	180%	[a]	114%	112%
Gly	127%	71%	176%	109%	131%
β-Ala	74%	146%	64%	94%	115%
Pro	115%	140%	79%	100%	121%
Trp	112%	133%	133%	56%	98%
Phe	61%	80%	90%	139%	94%

[a] – the signal of Asp was not obtained for DNS analysis.

For Fmoc-Cl, the FragAmp values chosen by the software were different for procedures A and B for all cases except Pro (without two maximums) and Gly (with two maximums). Results show that the FragAmp value chosen by the software provides better or similar results as the value at the other maximum. Only for Gly, the other FragAmp value (lower one), which was not chosen on either case by the software, can provide 9–22% better signal (this can also be seen on the Figure 14, where default MS^2 provides the best signal). This illustrates how important it is to visually check the work of the automatic optimization since better signal can be achieved for some cases. It can also be concluded from the work that the lower value of FragAmp is better for all amino acids derivatives, except for the two last eluting ones, Trp and Phe.

In the case of TAHS, both FragAmp values provide very similar results, except for Trp. For Trp, the FragAmp value (lower value), which was chosen by the software with the procedure A, can provide twice the signal. This is a significant difference. Like in the case with Fmoc-Cl, lower FragAmp values are preferred.

For DNS, there is a clear difference between using the FragAmp value either from the optimization procedure A or B. For Pro, the value obtained by the procedure A can provide much better signal, especially at low concentrations. For β-Ala, both procedures chose the same FragAmp value, 2.02, but much better results were obtained by the value, which was estimated from the optimization curve (which was 0.7). This again emphasizes the error that software can make when doing optimization. In the case of DNS, lower FragAmp are in the most cases a better choice (except Arg).

In the case of Deemm, for most cases, the sofware has made the better choice, meaning that better signal is observed. However, software chose the same FragAmp value for both optimizations in the case of β-Ala and Phe, but trying the other maximum (which is lower by value), it turns out that this can actually provide almost twice the signal. Therefore, it is important not to only

look at the numbers that software provides, but also look at the curves what software generates while going through an optimization procedure.

In the case of FOSF, there was no significant signal difference when using both maximums. Lower value might provide slightly better results but not significantly. This is interesting how two very different FragAmp values (one around 0.5 and another around 2) can give very similar signal. Moreover, the middle value (around 1.25) also gives very similar results. It could be concluded that when using FOSF as a reagent, not much effort has to be put in the optimization of MS^2. Moreover, there is not much need for further testing if the optimization is still working if the method is used over a longer period of time since reagent is resilient to changes in parameters.

One of the conclusions that could be drawn from the results is that the reagents with better ionization have better results by default values. It could be that they might be less sensitive to the MS parameters because they are by nature more sensitive analytes for ESI/MS analysis.

As for the FragAmp value that situates between the two maximums (method 3), when signals obtained with method 1) and 2) are similar, similar signal is obtained with method 3 also. If values are different, method 3 provides lower signal than the signal obtained with the better method.

Moreover, in conclusion, results show that there are some problems with choosing the right Fragmentation Amplitude by the Chemstation software. Software seems to prefer larger FragAmp values, when actually the lower value is preferred in the real chromatographic analysis. This is a very important thing to keep in mind when doing a very sensitive MS^2 analysis. If only 15% of the signal remains when choosing a wrong FragAmp value by the software, this means a significant change in method LoD and LoQ values. Moreover, if the time is a limiting factor, it could be concluded that MS^2 parameters could be optimized for couple of analytes, choosing those that are chromatographically further apart. Another possibility is to use reagent, which is not strongly affected by the optimization parameters, such as FOSF. This makes a good case if the developed method is planned for use over a long period of time.

4.6. Influence of boric acid on ionization

Influence of boric acid is one of the main topics in this work since it is present in almost all amino acid derivatization mixtures in borate buffer in order to provide pH from 9–11. Preliminary experiments showed constant problems with various derivatization reagents when using borate buffers in LC/ESI/MS and therefore, the influence of boric acid was taken into investigation. In order to avoid excessive amount of involatile cations (e.g. sodium) boric acid was used in experiments instead of sodium borate. However, not much investigation has been made about this nonvolatile compound.

4.6.1. Influence on standard amino acid derivatives (Deemm and Fmoc-Cl) [Paper IV]

Deemm and Fmoc-Cl amino acid derivatives were obtained as pure compounds i.e. no borate was present in the solutions. Other compounds (diphenylamine (DPA), 2-nitroaniline (2-NA), tetraethylammonium perchlorate (TEA), dimethyl glutarate (DMG) and diphenyl phthalate (DPhP)) were chosen according to the ESI efficiency scale [60] of organic compounds in order to include compounds with higher and lower logRIE values than Deemm and Fmoc-Cl derivatives of amino acids (preliminary results of logIE determinations showed values below 2.3 for Fmoc-Cl derivatives and 3.5 for Deemm derivatives). Structures of all the analytes are presented in Figure 17. Table 9 summarizes the information about the analytes. Experiment was repeated for 5 times over the period of one month.

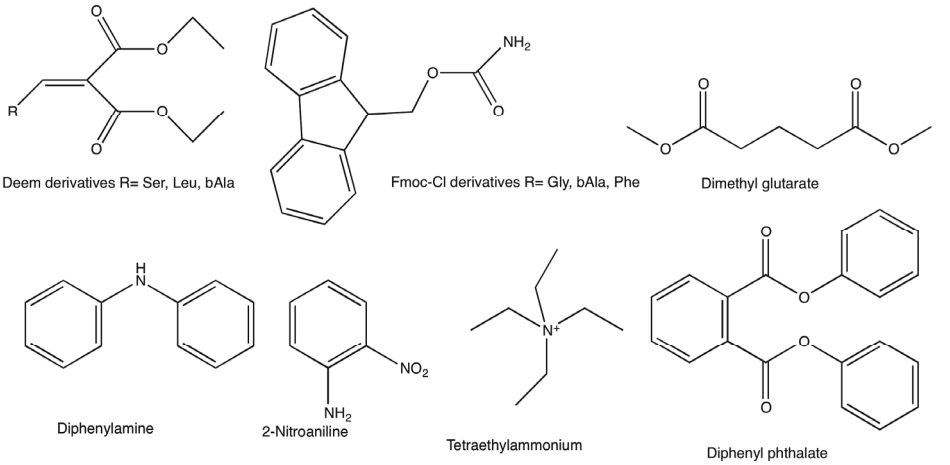


Figure 17. Structures of the analytes

Concentrations of the compounds were chosen so that they would result in similar peak areas. Moreover, if possible, it was preferred to have signal in UV, which would help to confirm the stability of the samples. For compounds for which UV signal was detected, no problems with sample stability were observed.

The peak area for MS signal was obtained from extracted ion chromatogram (EIC). Comparison between the peak areas of analytes with and without added boric acid solution was evaluated by means of Equation 9 (*%ME*), i.e. ionization suppression or enhancement due to boric acid will be revealed.

Table 9. Ionization efficiencies (logIE), retention times, used *m/z* in positive and negative mode and the concentrations in the injected sample of analytes when analysis is carried out with 0.1% formic acid.

Analyte	logIE	t_R (min)	+ m/z	– m/z[a]	c (mg/g)
tetraethylammonium perchlorate (TEA)	3.95	3.9	131		0.022
dimethyl glutarate (DMG)	2.88	14.6	161		0.396
2-nitroaniline (2-NA)	2.44	18.0	139		0.219
diphenylamine (DPA)	4.18	25.7	170		0.026
diphenyl phthalate (DPhP)	4.10	28.2	319	317	0.121
Fmoc-Gly		18.2	298	296	0.059
Fmoc-β-Ala		18.7	312	310	0.084
Fmoc-Phe		26.8	388	386	0.067
Deemm-Ser		15.2/10.8[b]	276	274	0.009
Deemm-β-Ala		18.1/18.8[b]	260	258	0.002
Deemm-Leu		27.2/43.2[b]	302	300	0.031

[a] – TEA, DMG, 2-NA and DPA were not observed in negative ion mode ESI.
[b] – retention times when analysis is carried out with 1mM ammonium acetate in 0.1% formic acid

In order to evaluate the influence of boric acid, an experiment without boric acid was carried out, and ultrapure water was added to the ESI source in order to keep concentration of analytes identical. For the boric acid experiments, 0.2 M boric acid in eluent (1 mM ammonium acetate in 0.1% formic acid) was infused. Identical results were obtained if the boric acid was dissolved in ultrapure water or 0.1% formic acid. At lower boric acid concentrations, no effect on signal intensity was observed, moreover proving that the effect on ionization is due to the boric acid, not the solvent, in which boric acid is dissolved.

When analysis was carried out without boric acid, pure MilliQ water was added to the ESI-source. Water was used as a reference and not simple injection since then the comparison is more accurate. Meaning, when nothing is added through the tee-piece, the signal is a little bit higher than when adding water. Compared to the eluent flow rate (0.8 mL/min) the infusion rate of either boric acid or water was low (8.3 μL/min). pH of the original eluents (1 mM ammonium acetate in 0.1% formic acid and 0.1% formic acid) and pH of the eluent after addition of boric acid or water were measured. Identical pH values were recorded for the eluent before and after addition of boric acid or water. Consequently, the observed effects cannot be explained by eluent pH change and must be attributed to the presence of boric acid in the ESI source.

The influence of boric acid on the mass spectra was also observed. The effects were similar for all compounds. In positive ion ESI mode, boric acid has no influence (Figure 18a). However, in negative ESI mode (Figure 18b), addition of boric acid gives rise to multitude of new peaks. These can be related to various anionic complexes of boric acid [85]. It is interesting to note, that despite of the presence of these ions, the signal of an analyte is enhanced, like in the case of Fmoc-Cl derivatives (Figure 19). Additional peaks due to the presence of boric acid complicate the interpretation of mass spectra.

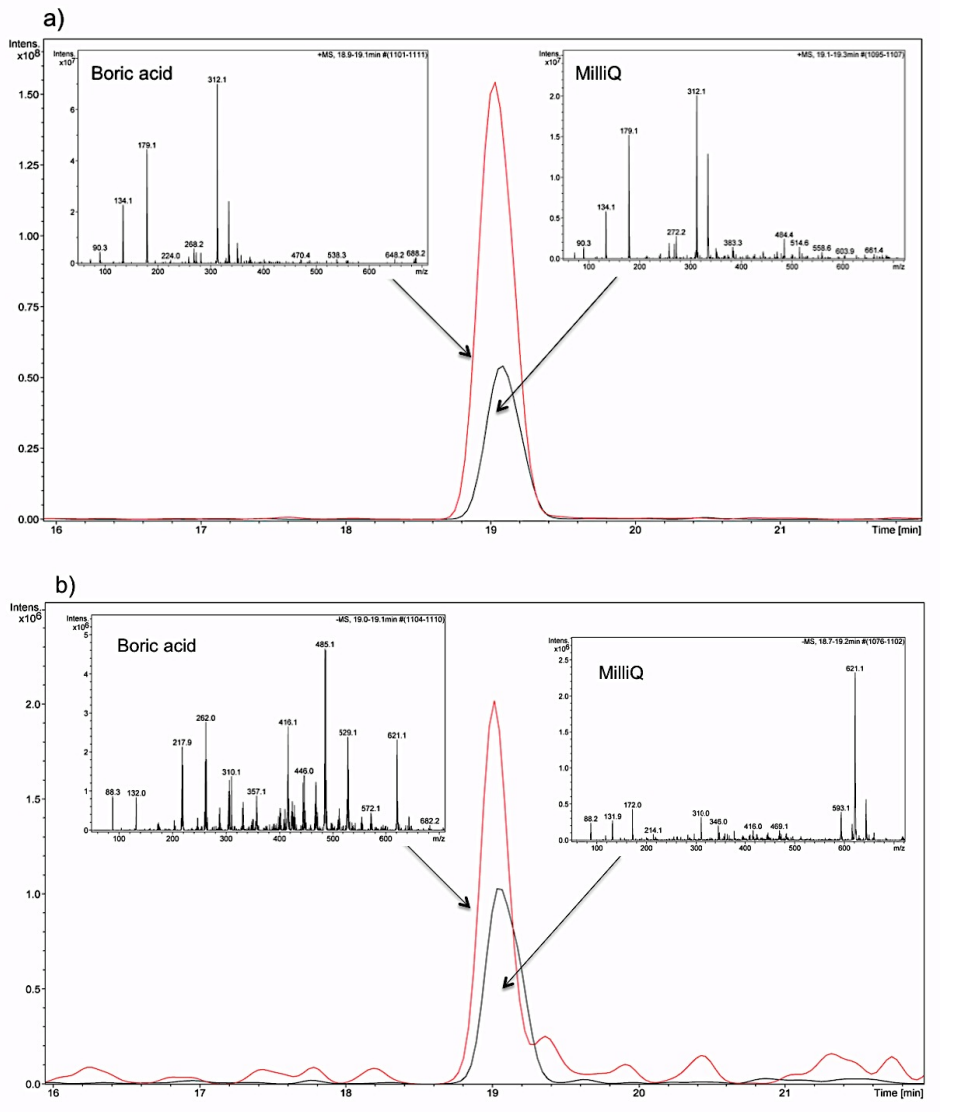


Figure 18. Comparison of chromatograms (EIC) of Fmoc-β-Alanine with boric acid and with ultrapure (MilliQ) water: a) positive ion mode (*m/z* 312); b) negative ion mode (*m/z* 310). Insets are the respective mass spectra.

Postive ion mode

All the tested analytes ionized in positive ion mode ESI and calculated matrix effect values *%ME* presented in Figure 19 show that the effect of boric acid varies strongly. Firstly, validated methods were compared, meaning that 0.1% formic acid is used as an eluent for the mixture of compounds and Fmoc-Cl derivatives and 1mM ammonium acetate in 0.1% formic acid for Deemm derivatives. For most compounds, the signal was enhanced if the boric acid was present. Enhancement can be up to 267% as in the case of Fmoc-Phe. For Fmoc-Cl derivatives, the enhancement was most substantial. On the opposite, for Deemm derivatives, the signal was strongly suppressed in presence of boric acid when buffer containing ammonium acetate was used – this is an originally validated method for analysis of Deemm derivatives [Paper II, Paper III]. Strongest suppression of the signal was observed for Deemm-β-Ala (6%). In case of the rest of the compounds, for all, except 2-NA, the signal was enhanced if boric acid was present.

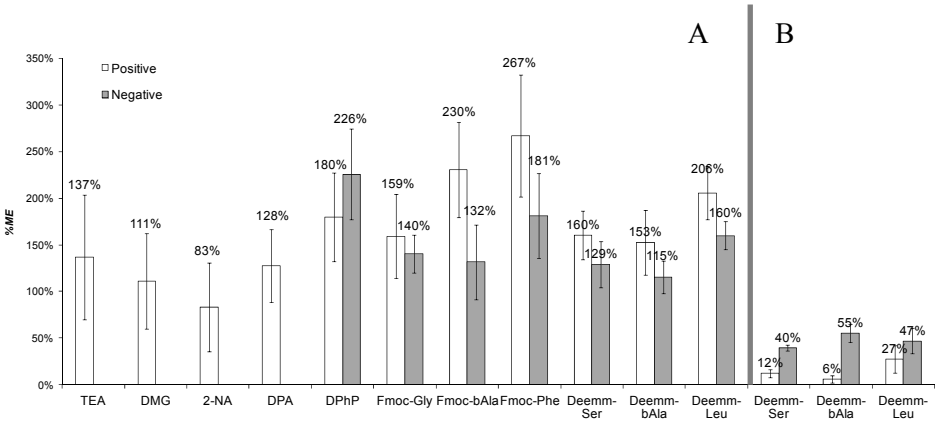


Figure 19. Calculated matrix effect values *%ME* for analytes in positive and negative ion mode. A: with 0.1% formic acid as an eluent component and B: with 1 mM ammonium acetate in 0.1% formic acid as an eluent component (n = 5).

Since suppression was observed only in the case of Deemm derivatives, for which the analytical method differs from analysis of other compounds, eluent of the method was changed to 0.1% formic acid instead of eluent containing ammonium acetate. Results showed that when 0.1% formic is used, the signal is also enhanced for Deemm derivatives, up to 206% in the case of Deemm-Leu. Therefore, it can be concluded that the ionization enhancement is related to the use of boric acid and formic acid but if ammonium acetate is present, suppression occurs. This is confirmed by preliminary results of boric acid influence on Fmoc-Cl derivatives with ammonium acetate present.

Two other tendencies should be mentioned. Signal enhancement is stronger when analytes have longer retention times, meaning that the percentage of organic component in the solvent is higher (Fmoc-Phe, Deemm-Leu and diphenyl phthalate), and compounds with higher logIE values are more likely to observe signal enhancement in positive ion mode if borate buffer is present. But considering the limited volume of experimental data, these results are inconclusive.

Negative ion mode

Negative ion mode was used for amino acid derivatives and DPhP (Figure 19). The remaining compounds did not ionize in negative mode ESI. It is distinct that for Deemm derivatives strong signal suppression (40–55%) was observed when ammonium acetate is present. However, this suppression was not as strong as in the positive ion mode. With only formic acid present in the eluent, similarly to positive ion mode, enhancement was observed (116–160%). It is not as strong as in the positive ion mode, but still over 100%. For Fmoc-Cl derivatives there is also signal enhancement like in the positive mode. The enhancement is not as high but still significant being up to 181% in the case of Fmoc-Phe. For DPhP, the signal is also strongly enhanced being more than twice the signal of the case when no boric acid is present.

Mechanism of action of boric acid

Error bars in Figure 19 represent standard deviations of five measurements carried out at approximately one-week intervals. In most cases, the reproducibility of the matrix effect is rather large. This observation has an important consequence. If borate is present in injected sample solutions, large deviations are to be expected.

Poor reproducibility could be due to the fact that borate is not volatile and precipitates on the internal surfaces of the ESI source. As analytes were injected in random order and time intervals between different injections were variable, then the amount of precipitated borate was different each time, which could lead to variability of results. Still, in general, all the results for one compound were on the same side of the 100%, i.e. suppression or enhancement.

Results demonstrate that boric acid strongly influences the ESI ionization. Surface activity of matrix compounds is most often regarded as the cause of suppressing matrix effect [105]. As boric acid caused both, enhancement and suppression, then other mechanisms must be sought.

Borates are known to form complexes [85,86]. One may assume that borate ion forms complexes with analyte molecules and those complexes facilitate evaporation of analytes into gas phase. This could be the case with all the experiments where ammonium acetate was not present (Figure 19). If ammonium acetate simply prevents formation of the complex then there should be no

matrix effect (*%ME* = 100%). But this is not the case since severe ionization suppression is observed when ammonium acetate is present (Figure 19). Consequently, borate complexation with analyte can hardly be the reason for the observed effects.

As other mechanisms of action, one should consider Lewis acidity [85] and electrochemical properties of boric acid. Influence of borate on solvent structure may also play a role.

The mechanism of action of borate remains unclear, but it certainly deserves further investigation.

Practical considerations

For compounds for which boric acid has substantial enhancement effect, it could be used to enhance sensitivity of ESI/MS determinations and if reproducibility is adequate, quantitative analysis is possible.

As for amino acid derivatization, where borate buffers are widely used, these should be given another look at and made sure that they do not suppress the signal or cause poor reproducibility, especially when buffers containing ammonium acetate are used. To solve this problem, buffers other than borate buffer could be considered for amino acid derivatization. Another solution would be diverting effluent from the initial part of chromatographic run into waste.

4.6.2. Influence on the amino acid analysis

There are not many standard amino acid derivatives commercially available but the experiments with standards showed an idea of using a boric acid as a signal modifier when an analysis of derivatized standards is carried out. Therefore, the experiment for using boric acid as a modifier was set up the way 2-(2-Methoxyethoxy)ethanol has been used for negative ion mode chromatography to enhance signal for screening ibuprofen [77]. A tee-piece was used after the chromatographic column to add boric acid into the mobile phase. The concentration of boric acid 0.2 M was chosen from the experiments done previously for other compounds. This provided the maximum signal enhancement and also boric acid did not cause visually detectable precipitate on the ESI source. Boric acid was added at the flow rate of 0.5 mL/h, which is small compared to the mobile phase flow rate in order to insure that all the effects are due to the boric acid. However, the boric acid solution was still prepared in the buffer of the mobile phase (0.1% formic acid for DNS and Fmoc-Cl, 1 mM ammonium acetate in 0.1% formic acid for Deemm and TAHS and ammonium bicarbonate buffer pH 7 for FOSF).

For assessing the influence, the peak areas obtained from the LC/ESI/MS^2 analysis of standards were taken as 100% and the influence of the mobile phase (same as component A of the corresponding chromatographic run) or boric acid infusion was assessed as a comparison to the default analysis. Following experi-

ments are carried out under MS^2 conditions for the seven amino acid mixture since previous experiments showed signal enhancement properties of boric acid.

Influence of boric acid on Deemm ionization

Previous experiments from Paper IV had shown that the boric acid suppressed signal of Deemm derivatives when using MS^1 method. Same was observed for the validated MS^2 method. Improvement is only seen when ammonium acetate would be removed from the eluent. Results for changing the buffer component for Deemm derivatives is discussed in Section 4.9.1. Moreover, in further section (Section 4.8), it is investigated how to remove the suppression effect of the boric acid for Deemm derivatives.

Influence of boric acid on Fmoc-Cl ionization

From the previous experiments, it was seen that most dramatic signal enhancement effects were for Fmoc-Cl. Influence of boric acid was tested at two concentration levels (Figure 20). Compared to the results in Paper IV, the effects remained the same when carrying out the analysis described previously. Results showed that when just 0.1% formic acid was added to the source, a slight improvement of the signal intensity is observed. However, the addition of boric acid increased the signal even further. Signal increase is as high as 2.5 times compared to the regular LC/ESI/MS^2 analysis for higher concentration samples. Slight decrease in signal was observed only for Asp, where around 30% of the original signal remains. Signal improvement was not as significant for less concentrated sample, except for Gly and Phe. Due to these experiments, the possibilities for using boric acid as a signal modifier is further discussed in the next section (Section 4.7) of this work.

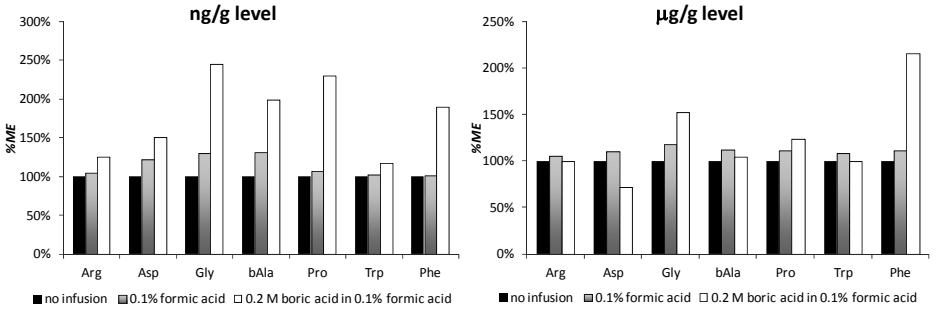


Figure 20. Influence of boric acid on Fmoc-Cl ionization.

Influence of boric acid on DNS ionization

DNS amino acid derivatives were not included in the previous studies and therefore, the effect of boric acid was unknown. Derivatization procedure of DNS differs from other reagents since boric acid is not included in the derivatization mixture. Therefore, results provide an insight to the use of boric acid as a signal modifier.

Results show that at lower concentrations for Arg, there is a slight suppression of the signal, around 30% of the signal remains when boric acid is added to the effluent (Figure 21). However, for all other cases there is a signal enhancement and the signal is enhanced up to 2.5 times. These results suggest that boric acid could be used to obtain better sensitivity in amino acid analysis when using a DNS derivatization reagent (these possibilities are further discussed in the Section 4.7).

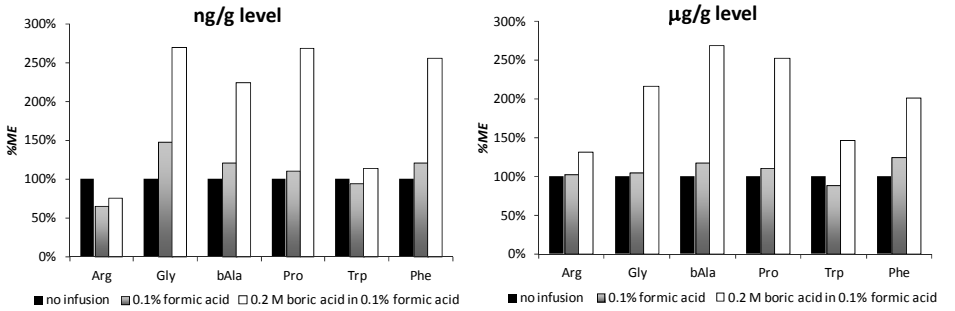


Figure 21. Influence of boric acid on DNS ionization.

Influence of boric acid on TAHS ionization

TAHS is a derivatization reagent, which is specially designed for very sensitive amino acid analysis and also contains boric acid in its derivatization mixture. Results show that the signal of TAHS amino acid derivatives is significantly suppressed similarly to the case of Deemm (Figure 22). Depending on amino acid, 47% to 0% signal is left of the original when the boric acid is added. However, like in the case of Deemm, it could be related to the use of ammonium acetate buffer. There seems to be no significant difference if the concentration is close to LoD value or 50 times larger. Possibilities for eluent change are discussed in Section 4.9.1.

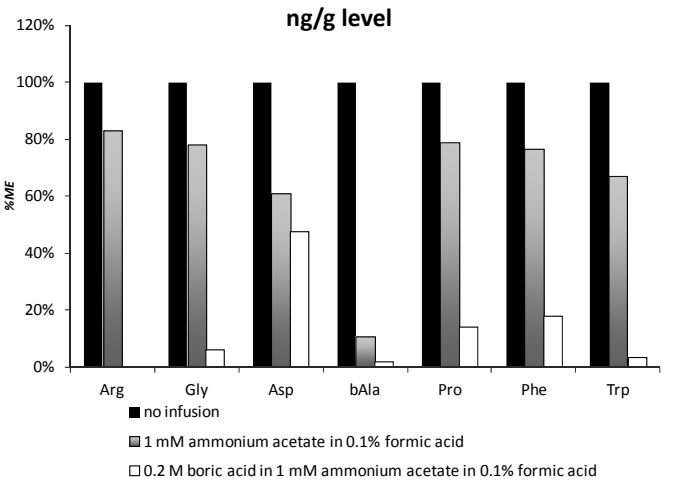


Figure 22. Influence of boric acid TAHS ionization

Influence of boric acid on FOSF ionization

Since previously the influence of boric acid on FOSF amino acid derivatives was unknown, it was also tested. One concentration level was tested for preliminary tests. When only buffer was added with the tee-piece, the signal was enhanced in some cases up to 60%. However, when boric acid was added, the signal dropped dramatically. Only 5–38% of the original signal remained after the addition. Meaning that boric acid cannot be used as a signal enhancer but the removal of boric acid influence should be under interest.

Conclusions

It can be concluded that boric acid has a suprising effect on the amino acid analysis, either strong signal suppression or signal enhacement. For FOSF, TAHS and Deemm there is a strong signal suppression and results suggest that if the ammonium acetate is removed from the eluent buffer, boric acid property to enhance signal could be applied (further discussed in Section 4.9.1). Due to the use of boric acid in derivatization procedure for TAHS and Deemm, it is of interest to investigate how to remove borate from the analysis either by not allowing it to reach the ESI-source or replace borate buffer by some other pH 9 buffers for the derivatization procedure (Section 4.8.).

From the enhancement point of view, for DNS and Fmoc-Cl, boric acid could be used as a signal enhancer and the application of boric acid should be further investigated.

4.7. Boric acid as signal-enhancing modifier for DNS and Fmoc-Cl

Investigation of the influence of boric acid on ionization showed that for some reagents boric acid has strong signal enhancing effect. Therefore, opportunities to use this phenomenon as a signal-enhancing modifier for amino acid analysis were searched for. Since previous experiments showed that there was a strong suppression for Deemm, TAHS and FOSF, these derivatization reagents were not further investigated at this point. However, in order to get more information about the mechanism of signal enhancement and the possibilities for applications, more thorough experimentation was carried out for Fmoc-Cl and DNS.

4.7.1. Post-column addition

In literature [54,77], post-column modifiers have been used for LC/MS analysis and at first it was looked at if boric acid could be used that way.

Experiments described in Section 4.6 were carried out with adding boric acid with a tee-piece. The same technique could be used to add boric acid during amino acid analysis.

Using boric acid as a modifier for DNS

Experiments with amino acids confirmed the findings with the standards and signal enhancement was onbserved for all amino acid derivatives with DNS derivatization. Therefore, other practical aspects were tested for the possible use in quantitative analysis.

One important thing to consider when using boric acid as a post-column modifier is the signal stability. By adding a modifier, which has that significant influence on the ionization, the accumulation of that compound (in this case boric acid) could have a strong influence on stability of the signal. However, results showed that once the boric acid is in the ESI source, equilibrium is reached and the signal remains stable. An observation was made that when the addition of the boric acid is stopped for some time, the influence is retained. In order to get rid of the boric acid, the surface of the ESI source was cleaned thoroughly with 1:1 water:2-propanol mixture in order to establish the original state without the boric acid. However, even after that cleaning, it takes some time before the system returns to condition before addition of boric acid (3–4 chromatographic runs, around 120 min of eluent flow).

Once the conditions for stable signal were obtained, another application was considered. Since addition of the enhancement component to the chromatographic eluent is more convenient, 0.1% formic acid was prepared with 4mM boric acid. Results are discussed below.

Using boric acid as a modifier for Fmoc-Cl

More thorough investigation was needed for Fmoc-Cl since at the higher concentration, the enhancement of the signal was significant and can be as high as 260%, but this phenomenom was not as intense at lower concentrations and was not very reproducible either. This was further investigated and the analysis was returned to the MS^1 instead of MS^2 and the negative ion mode was also taken into comparison.

The mass spectrums of Fmoc-Cl derivatives in MS^1 were compared when boric acid was added and when it wasn't. Results showed that when boric acid is not present, the main ion present is the Na-adduct, which is also used for the MS^2 analysis (except Arg). However, when boric acid is added to the effluent, the most abundant ion becomes the protonated ion $[M+H]^+$. This explains the case when at the lower concentrations the signal with MS^2 seems to be suppressed by the boric acid since the Na-adduct signal disappears.

In order to get better overview of the effect from the boric acid, the influence of boric acid was researched at different *m/z* for compounds with molecular mass M: $[M+H]^+$, $[M+Na]^+$, $[M–H]^–$ and also for one comparison the peak areas of extracted ion chromatograms of $[M+H]^+$ and $[M+Na]^+$ were summed. Results showed that in negative MS^1 mode, the signal is enhanced by boric acid, up to 1.5 times like in the case of standard Fmoc-Cl derivatives (Section 4.6.1). For positive ion mode, when $[M+H]^+$ is looked at, the signal is enhanced up to 10 times and for Na-adduct, signal is suppressed and signals as low as one third of the original are observed (Figure 23). When for observing the effect of boric acid in total, the $[M+H]^+$ and $[M+Na]^+$ intensities are added together, the signal is enhanced up to 3 times. Except for Arg, for which the Na-adduct is not observed and the signal is not affected by the addition of boric acid. One possible explanation for this is that when Arg is derivatized with Fmoc-Cl, instead of reacting with the alpha-amino group, it reacts with the side-chain amino. This explanation is supported by the different MS^2 fragmentation pattern as discussed in Section 4.3.8.

In conclusion, boric acid does have a signal enhancing effect on Fmoc-Cl derivatives in positive ion MS^1 mode for $[M+H]^+$ and suppressing effect for $[M+Na]^+$. However, possibilities for more sensitive MS^2 are limited with the use of boric acid since in addition to the suppression of the $[M+Na]^+$ signal, the intensity of the $[M+H]^+$ is lower than the signal of the $[M+Na]^+$ with the unmodified method.

In Section 4.9.1 two types of eluents are tested for Fmoc-Cl analysis and the discussion of $[M+H]^+$, $[M+Na]^+$ and $[M–H]^–$ continues.

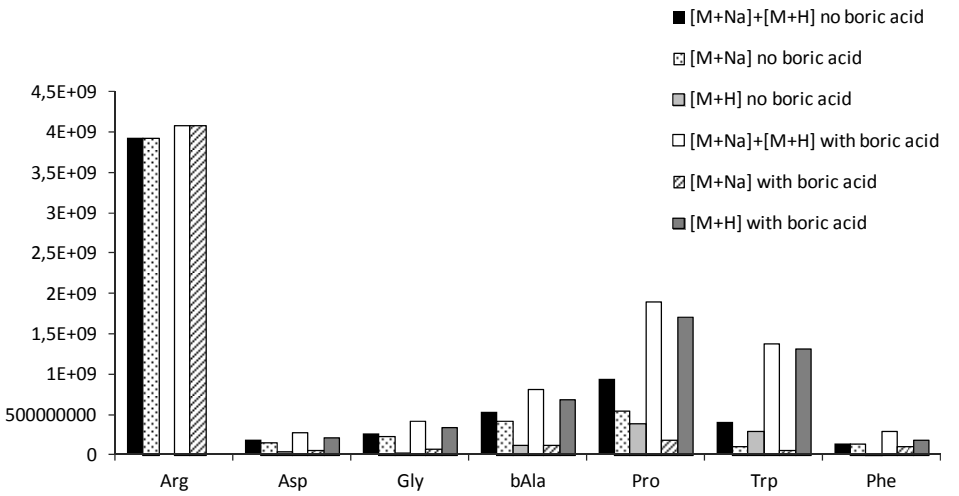


Figure 23. Signal intensities for $[M+H]^+$, $[M+Na]^+$ and their sum for Fmoc-Cl derivatives with and without boric acid (at the concentration level of μg/g).

4.7.2. Addition to the chromatographic eluent

From the previous results, it is observed that if it would be possible to add boric acid to the chromatographic eluent, it might be possible to obtain better detection limits. Compared to the post-column addition, addition of boric acid to the chromatographic eluent is more convenient for routine analysis. Specially when the syringe used for post-column addition has a small volume and needs to be changed often. Moreover, addition to the eluent provides better reproducibility and control. The only downside is that if boric acid is added only to the aqueous phase, the concentration of boric acid is not constant with the gradient elution. It was tested if boric acid could be added to the organic component, but boric acid has poor solubility in organic solvents and therefore boric acid is added only to the aqueous component.

Since for the original method boric acid provided signal enhancing for Fmoc-Cl and DNS derivatives, it was tested if it would be possible to use boric acid in the aqueous eluent component instead of adding it after the column.

Results showed that concentration of 4 mM boric acid in 0.1% formic acid does not change the retention times of the compounds and could be used as an eluent component without any major changes to the already developed methods. However, the ESI source must be in close observation since this concentration of boric acid can have visible effects on the source. Despite that the repeatability of the signal is very good after the boric acid has reached an equilibrium state in the source. Meaning that while some of the boric acid is deposited, at the same time, some of it is also washing away and stable signal remains.

Boric acid in eluent for DNS analysis

It can be concluded that when boric acid is added to the eluent, better results are obtained for DNS. Two types of comparisons were made, first, multiple injections of one sample were made first with regular eluent and then the eluent was changed to the one containing boric acid and results show significant improvement in signals. Difference is more significant in the beginning of the chromatogram where the content of aqueous eluent component is larger. For Pro-derivative the signal enhancement is the largest.

In order to gain information if this effect is transferrable to quantitative analysis, LoQ values were compared with eluent containing boric acid and not containing it. Results showed that with boric acid signal is very stable and also offers 2 to 4 times better LoQ values (Table 10). Effects are biggest for Gly- and β-Ala-derivatives, for which the LoQ values are improved 4 times. Therefore, for DNS analysis, using boric acid in the eluent can significantly improve the sensitivity of the analysis. The mechanisms of action of boric acid certainly deserves further investigation.

Table 10. LoQ (n = 6) values for the studied amino acid derivatives and expressed in fmol on column. a) LoQ values for developed DNS analysis without boric acid; and b) 4 mM boric acid in the chromatographic eluent.

	DNS without boric acid	**DNS with boric acid**
Arg	893	350
Gly	1171	286
β-Ala	1020	251
Pro	3336	976
Trp	237	109
Phe	198	103

Boric acid in eluent for Fmoc-Cl analysis

As it was observed in previous paragraph, results in the case of Fmoc-Cl are more complicated. Experiments showed that when boric acid is in the system, quasimolecular ions $[M+H]^+$ are more abundant. Therefore, in order to gain information about the preferred MS^2 analysis, fragmentation parameters were optimized for $[M+H]^+$ of the amino acid derivatives when boric acid is present in the eluent. Calibration graphs were linear, but the LoD and LoQ values were different than for Na-adducts. After the optimization, results showed that only for Arg- and β-Ala-derivatives, LoD and LoQ are slightly elevated but on the other hand, for Trp- and Phe-derivatives no adequate signal is observed (Table 11). It could be due to the fact that at higher organic content the influence of boric acid is not evident.

Table 11. LoQ (n = 6) values for the studied amino acid derivatives and expressed in fmol on column. a) LoQ values for developed Fmoc-Cl analysis [Paper VI] and b) for developed Fmoc-Cl analysis with 4 mM boric acid in the chromatographic eluent.

	Fmoc-Cl without boric acid	**Fmoc-Cl with boric acid**
Arg	259	186
Asp	943	1052
Gly	3615	9511
β-Ala	1687	623
Pro	174	376
Trp	164	–
Phe	193	–

In conclusion, it is difficult to use the signal enhancing effect of boric acid for Fmoc-Cl. However, if protonated forms ($[M+H]^+$) are preferred, boric acid could be used. If more sensitive analysis is looked for, Na-adducts should be used.

4.8. Approaches for removing suppression caused by boric acid

4.8.1. Replacing borate buffer with other suitable buffers in derivatization mixture [Paper V]

Since boric acid seems to have suppressing effects for Deemm and TAHS, it is sensible to look for substitutions for boric acid containing borate buffer used in derivatization procedures. For the possibilities of changing the buffer component for derivatization mixtures, several approaches were used. For Deemm and Fmoc-Cl, the very first screening experiments were carried out with different amino acids (Glu, Asn, Ser, β-Ala, Pro, Me-Cys, Met, Val, Phe and Lys) also at higher concentrations and using MS^1.

Three buffer systems were tested with respect to their suitability in amino acid derivatization with Fmoc-Cl and Deemm for detection with LC/ESI/MS. Buffers chosen were borate, carbonate and HFIP buffers at pH 9 needed for amino acid derivatization reactions.

Even though borate buffer is the most commonly used for derivatization, it has serious influence on the ESI ionization. For Deemm, in positive ion mode, borate buffer has strong signal enhancement with 0.1% formic acid as an eluent and in negative ion mode – ionization suppression occurs. HFIP buffer has most stable properties for Deemm derivatization. This makes it a good candidate for derivatization procedures since it will provide more reproducible results than borate or carbonate buffers.

In case of Fmoc-Cl derivatization, HFIP buffer appeared to cause low yield of derivatization products. Therefore only carbonate and borate buffers were compared by means of their effect on ESI ionization.

It was demonstrated that borate deposited on internal surfaces of ESI source can cause ionization enhancement or suppression. As the amount of borate deposited depends on many factors, including the sample injected earlier then ill-reproducible results are to be expected when borate buffer is used for derivatization.

The results of the study can be used as guidance for choosing the buffer for derivatization reaction in case of relatively clean samples. The applicability of the results to samples with complex matrices is yet to be more thoroughly investigated.

Moreover, the scope of derivatization buffer suitability with ESI/MS is broader than these two reagents and can be expanded to other derivatization reagents that use borate buffer and can be detected by LC/ESI/MS such as (OPA) [43,107], (APDS) [26] and (BCEOC) [44].

More elaborate discussions are available in Paper V.

Application to real samples

Applicability of the results was studied on two types of matrices: honey and tea. Preliminary results showed that different buffers act differently in the case of samples and standard solutions. And also the matrix, tea or honey, has an impact on the results. For example, Fmoc-Cl derivatization of amino acid standard solutions in HFIP buffer does not proceed and the same holds for tea matrix. But amino acids in honey matrix were effectively derivatized by Fmoc-Cl in HFIP buffer. The same situation was present for all amino acids in positive and negative ion mode and preliminary results are presented on the example of Fmoc-β-Ala on Figure 24. On the Figure 24, it must be noted that the concentration of Fmoc-β-Ala differs in the standard, tea and honey samples. Meaning that the responses are not comparable between different matrices, only in the scope of one matrix.

In case of Deemm derivatization of honey samples ionization is better in waste mode than in regular mode. In case of tea samples, waste mode has little advantage. Borate buffer caused ionization suppression in case of both tested matrices. Regarding ionization efficiency, carbonate buffer appears to be better suited for honey samples and HFIP for tea samples.

For more conclusive summary of the results, more experiments should be carried out. However, it could be concluded from the results, that the choice of buffer is crucial when complex samples are derivatized. Results also emphasize the need for the use of matrix-matched calibration.

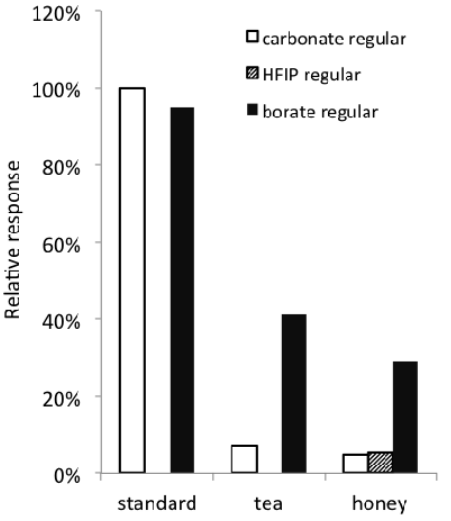


Figure 24. Derivatization of Fmoc-β-Ala with different buffers in standard, tea and honey samples.

4.8.2. Comparison of derivatization buffers for Fmoc-Cl, Deemm, FOSF and TAHS

Once it was concluded, that the choice of buffer used in derivatization reaction could provide significant input to the method development strategies for amino acid analysis with derivatization, more experiments were carried out with the fully developed MS^2 methods and for four derivatization reagents (Fmoc-Cl, Deemm, FOSF and TAHS) which have borate buffer in the derivatization mixture. Three types of buffers were compared: borate buffer, carbonate buffer and HFIP all with the pH of 9.

Derivatization buffer comparison for Fmoc-Cl

As concluded earlier, HFIP is not a suitable buffer for Fmoc-Cl (Figure 25). However, when comparing borate and carbonate buffers, results show that borate offers a little bit better results. For higher concentration level, the difference is not as significant, but for the lower concentration sample, borate provides up to twice the signal as carbonate buffer. Therefore, the borate buffer is more suitable for Fmoc-Cl applications and the replacement is not needed. It is in accordance with the results where boric acid provides signal enhancement for Fmoc-Cl derivatives [Paper V].

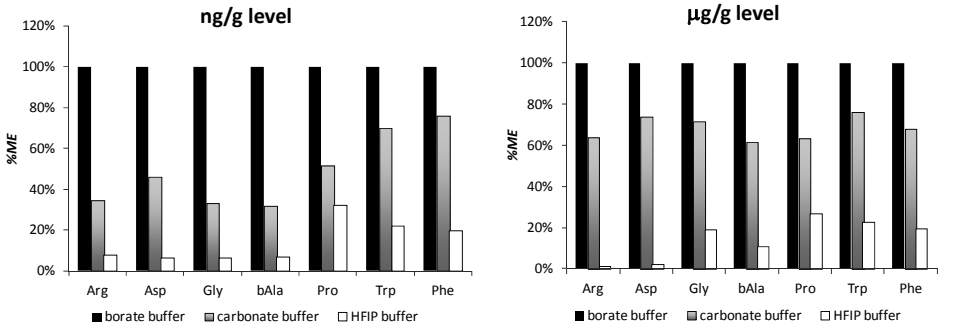


Figure 25. Comparison of dependence of Fmoc-Cl derivatives' signal intensities on derivatization buffer (signals relative borate buffer).

Derivatization buffer comparison for Deemm

Results show that there is a signal difference for different amino acids with various buffers (Figure 26). The differences are more significant in the beginning of the chromatogram where carbonate buffer offers almost two times higher signal than HFIP or borate buffers with 1 mM ammonium acetate in 0.1% formic acid as an eluent. The replacement of borate buffer with carbonate buffer should be considered. Moreover, the continuous analysis of Deemm derivatives showed that the analysis is sensitive towards the borate in the chromatographic and mass-spectrometric system. It may be the source of poor signal stability and repeatability of LoD and LoQ values.

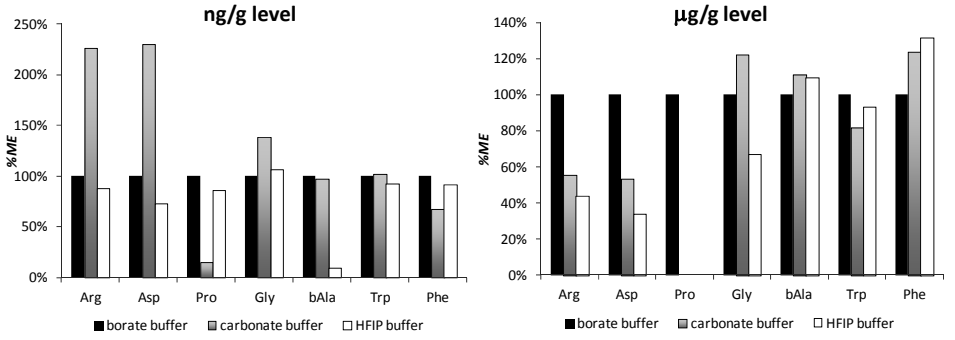


Figure 26. Comparison of dependence of Deemm derivatives' signal intensities on derivatization buffer (signals relative borate buffer).

Derivatization buffer comparison for FOSF and TAHS

At two different concentration levels of amino acids, HFIP and carbonate buffers were used instead of borate buffer and neither was suitable for the deri-

vatization reaction (Figure 27). Results showed significantly lower responses or even no response at all. It is an interesting case since all the buffers offer the same pH values. This would mean that the borate buffer also acts as some kind of a catalyst as well, not only the pH provider. However, this was not further investigated.

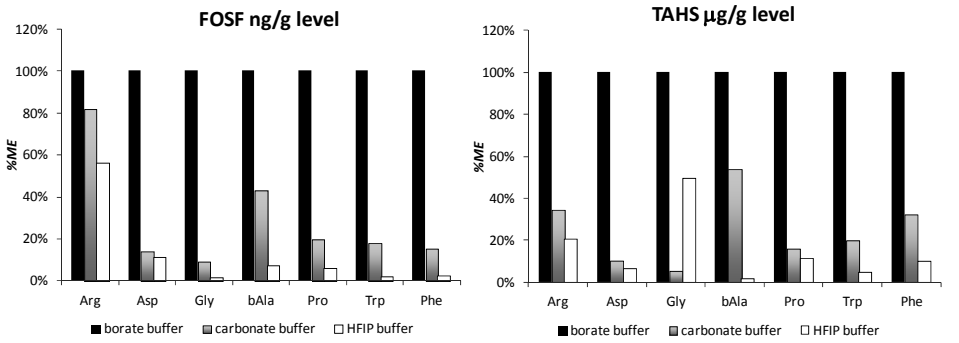


Figure 27. Comparison of dependence of FOSF and TAHS derivatives' signal intensities on derivatization buffer (signals relative borate buffer).

4.8.3. Diverting boric acid away from the ESI source

For many cases, the replacement of a borate buffer is not possible since there is not a suitable alternative available. Therefore, other ways to remove the suppression by a borate buffers must be sought. One of the possible solutions is to divert into waste the buffer components that elute early on the chromatogram and could possibly precipitate on the ESI source and influence the ionization of the amino acid derivatives. Similar technique has been used in analysis of drug metabolites to divert salts and other unretained analytes away from the MS [81].

The LC/ESI/MS instrument used in these experiments allows to use a switching valve, which can divert salts and other unretained analytes away from the ESI source in the beginning of the chromatographic run. For the following experiments, this technique was tested and called the waste run, which means that for the first 5 minutes of the chromatographic run, the effluent is diverted into waste. Results are compared to the results when all the effluent passes the ESI source.

Since the signal enhancement effect by the boric acid for Fmoc-Cl has been confirmed by the previous experiments and DNS reaction mixture does not contain boric acid, the following experiments were only carried out for the derivatization reagents, which could possibly benefit from the waste run – Deemm, FOSF and TAHS. For the next discussion, the use of the switching valve is referred to as the waste run.

Experiments were repeated multiple times at two concentration levels.

Deemm amino acid derivatives

For Deemm derivatives, boric acid has a strong suppression and it could be predicted that there would be a strong signal enhancement if waste run is employed, especially for the amino acids that elute in the beginning of the chromatogram.

Results, however, show no indication of the strong signal enhancement for either of the concentration levels (Table 12). Results are similar for both concentration levels and for amino acids that elute in the beginning of the chromatogram or in the end. On the contrary, results suggest that the signal is higher if all the effluent reaches the ESI source. It could be due to the fact that for 5 minutes the ESI source has no effluent running and stays dry for that period of time. Reequilibration of ESI source with liquid can, in principle, result in signal suppression. Another pump could be used to provide neat eluent to the ESI source during initial 5 minutes, but has not been tested.

Table 12. The ratios of signals obtained with waste and regular run for Deemm derivatives at two concentration levels.

	µg/g level	**ng/g level**
Arg	89%	114%
Gly	116%	72%
Asp	223%	–
β-Ala	119%	127%
Pro	108%	84%
Trp	96%	84%
Phe	86%	96%

FOSF amino acid derivatives

For FOSF, two concentration levels were also tested and the results suggest that the use of a waste run can provide signal enhancement meaning that during the first 5 minutes, borate, which suppresses the signal, is carried away from the ESI source, providing a better ionization environment for amino acid derivatives. Results show that the use of a waste run is more beneficial at low concentrations (Table 13). Depending on the amino acid, the signal can be up to 148% of the original when using the waste mode. Signal enhancement is similar for amino acid derivatives regardless of their retention time. This could also be due to the fact that the chromatographic run for FOSF analysis is quite short compared to the other amino acid derivatization reagents.

In conclusion, when using FOSF as an amino acid derivatization reagent and using borate buffer, it would be suggested to use waste run in order to avoid the strong signal suppression by boric acid.

Table 13. The ratios of signals obtained with waste and regular run for FOSF derivatives at two concentration levels.

	µg/g level	ng/g level
Asp	104%	129%
β-Ala	100%	137%
Gly	128%	148%
Arg	106%	124%
Pro	111%	108%
Trp	104%	140%
Phe	116%	148%

TAHS amino acid derivatives

Since for TAHS there is also suppression by the boric acid, signal enhancement could be predicted with the waste run. However, results show that at both concentration levels, only a little enhancement is observed for early eluting amino acids and for most amino acids, there is a insignificant signal difference between the regular run and the waste run (Table 14). This can be related to the structure of TAHS having permanent charge, and implies that influence of borate on other derivatives can be related to removal or addition of hydrogen ion. However, it is interesting to note that the benefit is bigger when the ESI source is freshly cleaned, up to 180% from the original signal. Once the source has more injections of the samples, the difference is not as significant, around 110% from the original signal. This brings about the issue of the cleanliness of the LC/ESI/MS system and also the fact that when very sensitive analysis of amino acids is carried out, aspects that usually can be disregarded become under interest.

It can be concluded that for TAHS, it is not very likely to obtain significantly better LoD and LoQ values with the waste run.

Table 14. The ratios of signals obtained with waste and regular run for TAHS derivatives at two concentration levels.

	µg/g level	ng/g level
Arg	124%	127%
Gly	122%	135%
Asp	115%	127%
β-Ala	123%	114%
Pro	123%	111%
Phe	118%	85%
Trp	124%	93%

4.8.4. Removing ammonium acetate from the eluent

Experiments with the boric acid brought about an interesting finding that boric acid has a suppressing effect only if the ammonium acetate is used in the eluent. This means that removing ammonium acetate from the eluent could improve the signal. Therefore, a simple experiment was carried out with the amino acid standards of Deemm and Fmoc-Cl, to test how much effect would there be to the overall signal. For TAHS the analysis of mixture of seven amino acid derivatives is used for evaluation and for FOSF, that type of experiments were not considered since for FOSF, eluent is different.

Results for Deemm and Fmoc-Cl are presented in the next section along with discussions on the eluent compatibility.

4.9. Other techniques for signal enhancement

4.9.1. Eluent compatibility

Results in Section 4.6 with boric acid influence on the ionization with ammonium acetate present and absent in the eluent composition, brought about the idea of how does the choice of an eluent buffer components can have an effect on the ionization. Ammonium acetate is sometimes added to eluent buffers to improve the ionization in the ESI source. This is also the case for Deemm and TAHS derivatives.

Since the change in the buffer composition can have a significant effect on the retention times, the following experiments were carried out for Fmoc-Cl and Deemm standard amino acid derivatives. Experiments were carried out in the MS^1 mode in order to get information about the positive and negative ion mode. Moreover, this gives more information about the Na-adduct formation. In addition to eluent compatibility experiments, it was looked into the influence of boric acid on the ionization of the analytes when different eluents were used.

Formic acid with or without ammonium acetate as an eluent – Fmoc-Cl

For Fmoc-Cl, three amino acids standards were used: Fmoc-Met, -β-Ala and -Phe. Difference between the performance of the two eluents is evident from Figure 28. Results clearly show that when ammonium acetate is present in the eluent, the signal of Fmoc-Cl derivatives decreases significantly. The situation is independent of the mode used, results are the same for the $[M–H]^-$, $[M+H]^+$, and $[M+Na]^+$. Effect is most significant with the negative ions, when the signal is completely lost at the ng/g concentration level if ammonium acetate is used. For $[M+H]^+$, the signal with ammonium acetate is around 4 times lower than without the ammonium acetate. Moreover, in the light of previous experiments, the formation of Na-adducts was observed and since the same tendency (better signal without ammonium acetate) is applicable, it could be confirmed that the

difference between the two eluents is not related to the different ratio of $[M+H]^+$, and $[M+Na]^+$.

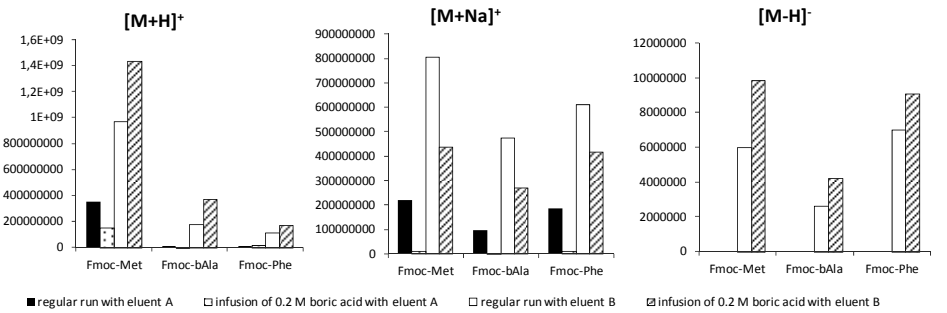


Figure 28. Signals with different eluent combinations for Fmoc-derivatives (eluent A: 1 mM ammonium acetate in 0.1% formic acid and B: 0.1% formic acid).

With different experiments, effects from the boric acid vary. Results of boric acid are in accordance with the previously acquired results, showing that when ammonium acetate is missing from the eluent, boric acid enhances the signal of Fmoc-Cl derivatives in the negative and positive mode (Figure 28). However, with boric acid, the signal of $[M+Na]^+$ is suppressed and the same tendency is observed with the buffer containing ammonium acetate. Similarly to the previous experiments, for $[M+H]^+$, the mixture of ammonium acetate and boric acid results in signal suppression.

Formic acid with or without ammonium acetate as an eluent – Deemm

At first, for Deemm, three amino acids standards were used for experiments: Deemm-Ser, -β-Ala and -Leu and MS^1 analysis was carried out (Figure 29). In the negative ion mode, at concentration levels of ng/g, very weak signals were registered for Deemm-β-Ala and Deemm-Leu. However, for Deemm-Ser, results are similar to the Fmoc-Cl results where formic acid alone in eluent enables better ionization than together with ammonium acetate.

In positive ion mode, for Deemm, when the signal of the protonated derivative is observed, signals in both eluents are similar. When boric acid is added to the eluent, in both cases, the loss of intensity is significant.

The situation for the Na-adduct is different. When ammonium acetate is present in the eluent, the signal is somewhat better. Addition of boric acid lowers the response in both cases. Consequently, boric acid is not a suitable signal modifier for Deemm, as was demonstrated earlier.

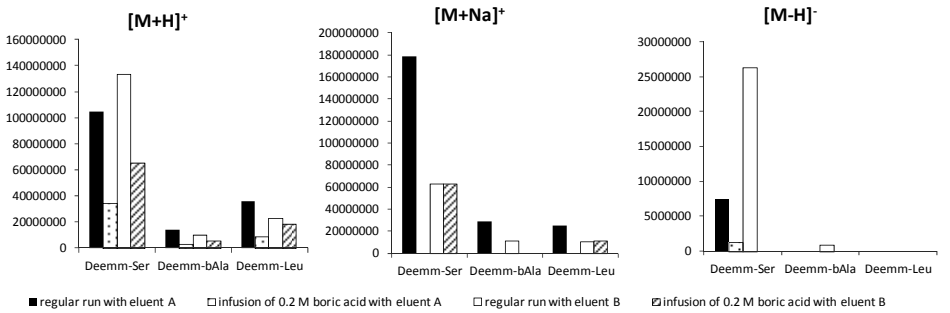


Figure 29. Signals with different eluent combinations for Deemm derivatives (eluent A: 1 mM ammonium acetate in 0.1% formic acid and B: 0.1% formic acid).

In conclusion, for Deemm analysis, ammonium acetate in the eluent provides better results and should be a choice of an eluent component for analysis of amino acids with Deemm derivatization. Therefore, change from ammonium acetate-containing eluent to the ammonium acetate-free eluent could benefit to the lowering of the LoD and LoQ values and the change of eluents was applied to the analysis of mixture of 7 amino acids.

The same experiment was applied to the developed and optimized MS^2 method. Experiment was carried out at two concentration levels (Figure 30). When for Deemm analysis, formic acid with and without ammonium acetate are compared, the results show that when just formic acid is used as an eluent, the signal is enhanced, especially for Asp, Gly and β-Ala. However, when boric acid is added to the mixture, the signal is suppressed again. The conclusion that formic acid (without ammonium acetate) provides better ionization than the eluent with ammonium acetate is not in accordance with the results obtained with MS^1.

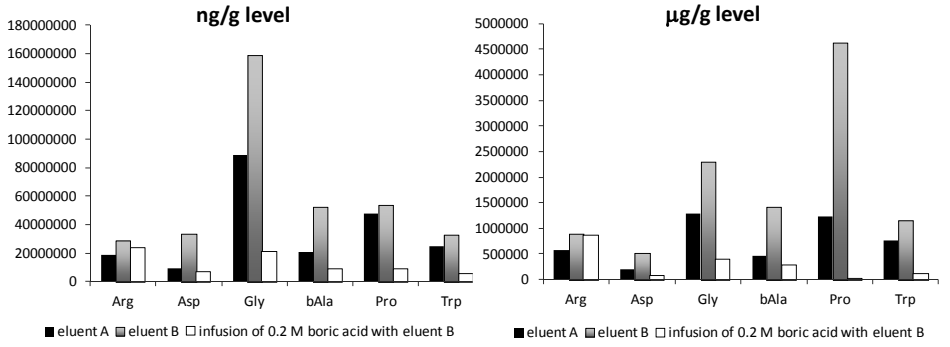


Figure 30. Comparison of signals for analysis of Deemm derivatives with different eluent components (eluent A: 1 mM ammonium acetate in 0.1% formic acid and B: 0.1% formic acid).

In order to get practical information about which eluent would be more suitable, LoD and LoQ values were measured with both eluents (Table 15). Results confirmed that for some amino acids signal is enhanced in presence of ammonium acetate and for others higher signals are obtained in absence of ammonium acetate.

Table 15. LoQ (n = 6) values for the studied amino acid Deemm-derivatives and expressed in fmol on column: a) with 1 mM ammonium acetate in 0.1% formic acid (eluent A) as an eluent and b) with 0.1% formic acid as an eluent (eluent B).

	Eluent A	**Eluent B**
Arg	112	227
Asp	188	70
Gly	758	216
β-Ala	147	568
Pro	–	–
Trp	108	69
Phe	67	99

In conclusion, from the eluent suitability point of few, it is acceptable to use either of the eluents.

Formic acid with or without ammonium acetate as an eluent – TAHS

In the light of previous experiments, for TAHS, MS^1 and MS^2 measurements were conducted (Figure 31). For two concentration levels, both eluents provided very similar results, ammonium acetate providing slightly better ionization. Differently from the case of Fmoc-Cl and Deemm, the *m/z* base peak (most intense peak in mass spectrum) was the same and independent of the eluent. Moreover, boric acid in combination with 0.1% formic acid as an eluent does not cause signal enhancement.

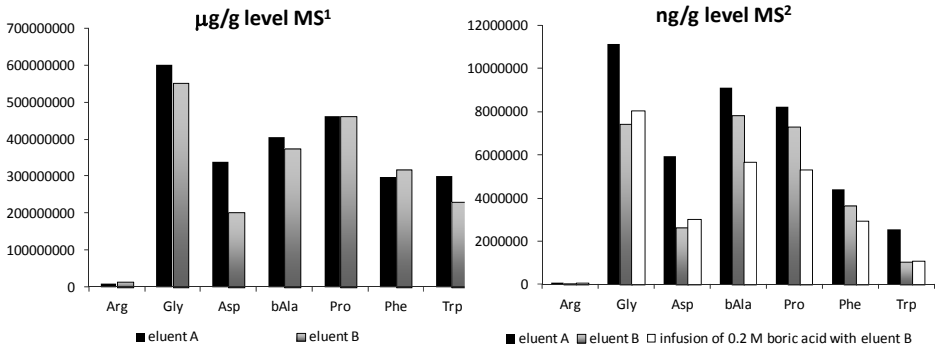


Figure 31. Comparison of signals for analysis of TAHS derivatives with different eluent components (eluent A: 1 mM ammonium acetate in 0.1% formic acid and B: 0.1% formic acid).

In conclusion, the use of the chromatographic eluent containing ammonium acetate is justified.

Formic acid with or without ammonium acetate as an eluent – FOSF

For FOSF, it was not possible to try other eluents due to poor chromatographic separation. Only eluent with pH 7 was suitable.

4.9.2. Replacing acetonitrile with methanol

In reversed phase liquid chromatography acetonitrile (MeCN) has higher eluting power than methanol (MeOH). Therefore, when MeCN is changed for MeOH, the main influence is that in reversed phase chromatography the retention gets better, consequently, longer retention times are observed. This brings about that when the analytes arrive in the ESI source, the percentage of an organic eluent is higher and ionization better. Consequently, it could be expected that with MeOH the ionization is improved. It has been previously published that at equal conditions, MeOH provides better ionization than MeCN [54]. On the other hand, for some applications and analytes, MeCN can provide better chromatographic separation.

Retention times of all the reagents (except TAHS) were significantly longer when MeOH was used. Due to that, the methods were slightly modified in order to do MS^2 analysis. The same optimization parameters were used as for MeCN with the assumption that the optimization parameters are not very strongly influenced by the organic component. Assumption is based on the fact that in most cases optimization parameters without the eluent flow (meaning, insignificant if MeOH or MeCN is used) provided very similar results to optimization parameters with the eluent flow.

Replacement for Deemm derivatives

For Deemm, it was observed that the increase in retention times led to significantly better ionization with MeOH. Increase was as high as nine times. In order to get practical comparison to the developed method with MeCN, new LoQ values were measured (Table 16). However, this showed that despite the better ionization, MeOH provided significantly higher LoQ values for Arg, Gly and Phe. This could be related to the poorer repeatability when MeOH is used. For rest of the amino acids LoQ values were similar or slightly better. In conclusion, it is justifiable to use MeCN as an organic component.

Table 16. LoQ (n = 6) values for the studied amino acid Deemm derivatives and expressed in fmol on column with MeCN and MeOH as an organic component.

	MeCN	MeOH
Arg	84	549
Asp	154	86
Gly	384	837
β-Ala	227	226
Pro	–	–
Trp	53	44
Phe	26	129

Replacement for Fmoc-Cl derivatives

Like in the case of Deemm, for Fmoc-Cl, MeOH provided enhanced ionization. However, the effect was not pronounced, being highest when twice the signal was observed. Again, LoD and LoQ values were measured and calculated, and results showed that for Fmoc-Cl, MeOH indeed could be a better choice of an organic component (Table 17). At this point is hard to predict how much difference this swap would do to the chromatographic separation of all the 24 amino acid derivatives since it should be more difficult to obtain good separation with MeOH than with MeCN.

Table 17. LoQ (n = 6) values for the studied amino acid Fmoc-Cl derivatives and expressed in fmol on column with MeCN and MeOH as an organic component.

	MeCN	MeOH
Arg	259	106
Asp	943	134
Gly	3615	593
β-Ala	1687	1243
Pro	174	185
Trp	164	52
Phe	193	79

Replacement for DNS derivatives

The case of DNS was different from Fmoc-Cl and Deemm, since experiments showed that MeOH is not suitable for DNS analysis. Results clearly showed that signal is lost when MeOH is used instead of MeCN. Loss of the signal compared to MeCN is dependent on the content of organic component. In the beginning of the chromatogram, less signal is lost (about 20%). However, for

analytes eluting at higher organic content, such as Trp and Phe, 80% of the original signal is lost. Shift in retention times was also observed.

Replacement for TAHS derivatives

Since TAHS is carrying a permanent charge, change in retention times was not observed, meaning that not much effect was detected from the ionization point of view that is related to the change in organic component precentage. Signals with MeOH and MeCN were comparable and no further testing was conducted.

Replacement for FOSF derivatives

For FOSF, shift in retention times was observed. Longer retention times brought about a situation where the identification of the derivatives with methanol got more complicated since the separation got significantly worse. After several tries, it was concluded, that even though in the beginning of the chromatogram, some signal enhancement (up to 1.5 times) was observed, MeOH is not suitable for FOSF analysis.

4.9.3. Use of post-column flow splitting device

It has been proposed in literature that the post-column splitting can be used to provide high flow rate for the LC separation and low flow rate for the ESI/MS detection [76,78]. This could possibly be a technique to enhance the signal of amino acid derivatives since the chromatographic methods used are most commonly with high flow rates in order to achieve the best possible chromatographic separation for amino acid derivatives.

For getting the best overview of the possible effects of the flow splitting, amino acid solutions with two concentrations were used. One was around the LoQ values and the higher one 50 times higher. Three different splitting ratios were used: around 65%, 75% and 85% was splitted away from the flow. Lower splitting was briefly tested but did not provide stable signal and was then decided not to further analyze.

Post-column flow splitting for Deemm derivatives

The effect of the splitting is more significant on the analytes that elute in the end of the chromatogram. The effect is different for the high and low concentration samples. The signal enhancing is more significantly present for the low concentration sample and for three amino acid derivatives that elute the latest – β-Ala, Trp and Phe. In all cases, 64% and 72% splitters offer 14–40% better signal than for without the splitter. However, the splitter of 86% provides significantly lower peak areas than the analysis without the splitter. The signal enhancements are all within the range of reproducibility. It is important to note

that for the amino acids that elute in the beginning, signal is lost when using a splitter.

Post-column flow splitting for Fmoc-Cl derivatives

For Fmoc-Cl derivatives also two concentration levels were evaluated and the results showed that at higher concentration, there is no benefit from using the splitter, on the contrary, signal is lost. However, at low concentration, there is a small benefit from using a 65% splitter. Benefit is for amino acids that elute in the end – β-Ala, Pro, Trp and Phe. The signal improvement is in the range of 10–30%. For other amino acids, signal either remains the same when using a splitter or some signal is lost.

Post-column splitting for DNS derivatives

For DNS derivatives, there seems to be no benefit when using a splitter. In few cases, a small improvement can be present when using a 69% splitter, but it is in the range of reproducibility and therefore not significant.

Post-column flow splitting for TAHS derivatives

The use of a splitter can offer a small benefit for the higher concentration samples (however, never for the highest 86% splitting). For the low concentration sample, the only benefit is for the last eluting analyte, Trp (13–30% with a 67% splitter). Experiments show a small enhancement for the Phe also, but this could result from the poor reproducibility of the signal also. For the higher concentration, the benefit to the ionization is present to some extent for all amino acids except β-Ala. Enhancement is never present for the highest 86% splitter. All amino acids benefit from the 67% splitter (8% and 42% depending on amino acid) and some amino acids (mostly Phe and Trp) also benefit from the 74% splitter (30–53%). Again, the highest benefit is to the amino acid derivatives that elute in the end of the chromatogram.

Post-column flow splitting for FOSF derivatives

Signal enhancement was not observed in any cases.

Conclusions

Results show that the use of a splitter offers no signal enhancement for FOSF and no significant signal enhancement for DNS derivatization reagents. This is not uncommon; it has been observed previously that not for all compounds, splitting will result in signal enhancement [76]. However, when using TAHS, Fmoc-Cl or Deemm derivatives, the splitter in the range of 60–80% could provide a small signal enhancement, which could be enough for some applications. Moreover, the signal enhancement is mostly present for amino acid derivatives,

which elute in the end of the chromatogram, meaning with higher MeCN content. It can be concluded that splitting can be beneficial when the method is developed so that amino acid derivatives elute with quite high organic solvent percentage. Or if the derivatization reagents would be designed in a way that allow chromatographic separation with high content of organic in mobile phase.

In conclusion, it can be said that using the standard ESI source there is no significant enhancement from the flow splitting for the analysis of amino acid standards. However, this technique should be reconsidered when real samples are used since there is a possibility of reduced matrix effects when using a flow splitter [76].

4.10. Amino acid analysis in real samples

4.10.1. Practical considerations

The choice of amino acid derivatization reagents is wide and the comparison of five reagents for the LC/ESI/MS^2 analysis brings out the differences between the commercially available reagents and the novel reagents. From practical point of view, it is better to use reagents that have been in use longer and are also suitable for LC/ESI/MS^2 analysis. If very sensitive analysis is targeted, the use of novel reagents could be considered. However, since little method optimization info is available, much care must be taken in order to optimize the derivatization procedure and chromatographic separation.

A wide range of aspects (derivatization procedure, chromatographic separation etc.) that need to be under consideration when choosing a suitable derivatization reagent for analysis are discussed in Section 4.3. A choice of a reagent depends on the application and for example whether a sensitive analysis is targeted (LoD/LoQ) or a wide range of concentrations is expected from the samples (linearity).

Moreover, out of the five derivatization reagents discussed, pricewise, Deemm is significantly cheaper than other reagents. DNS and Fmoc-Cl are both commercially available but price difference with Deemm is tens of times. As for TAHS and FOSF, in addition to the necessary synthesis effort, materials for their synthesis are expensive. Therefore, from the availability point of view, Deemm is one of the best choices for amino acid derivatization.

4.10.2. Analysis of amino acids in honey samples [Paper II]

Full method for amino acid analysis in honey was developed and validated. The method consists of sample preparation (including SPE), derivatization and chromagraphic analysis. All steps were extensively studied and optimized for analytical performance. This is the first report of application of SPE to honey amino acid analysis. Careful examination revealed that secondary retention

between aromatic ring containing amino acids and SPE sorbent exists if organic modifier is not used in eluent. This observation has not been reported in previous works on honey amino acid analysis.

The derivatization procedure of the new and promising derivatization reagent Deemm was under close testing. It appeared that derivatized samples must be analysed not earlier than 24 hours and not later than 48 hours after derivatization. Derivatized samples are stable if refrigerated.

Total chromatographic separation of all amino acids was achieved by extensive testing of different columns and gradient programs.

Major advantage of the developed method is that it is suitable for UV and MS detection. MS detection was used for peak confirmation. Therefore, all steps in optimized amino acid analysis method are MS compatible. Regarding the proposed procedure as generic method for amino acid analysis in different matrices MS and especially MS/MS detection would allow lower detections limits compared to UV detection.

4.10.3. Analysis of selenoamino acids in serum and onion [Paper III]

A method to analyze SeMet and Se-MeSeCys with LC/ESI/MS/MS was developed. The method offers the best detection and quantitation limits published for precolumn derivatization methods with HPLC-FL or LC/ESI/MS. Diethyl ethoxymethylenemalonate (Deemm) was used for precolumn derivatization of samples. Separation of SeMet and Se-MeSeCys from each other and from other naturally occurring amino acids was accomplished in 15.3 min. SeMet oxidation was investigated. It was demonstrated that introduction of 2-mercaptoethanol into the standard and sample solutions prevented SeMet oxidation. For both compounds, the detection limits were 0.1 pmol, which are comparable to LC-ICP-MS analysis and therefore, the developed method offers an alternative to LC-ICP-MS providing similar sensitivity and additionally allowing identification.

The method was demonstrated to be applicable for the analysis of SeMet and Se-MeSeCys in onion. Moreover, in addition to results published in Paper III, the method has been even further developed and researched and applied to serum and onion samples.

5. SUMMARY

The aim of this work was to systematically approach the topic of method development for derivatization LC/ESI/MS on the example of amino acid analysis. Various aspects related to the sample preparation, derivatization, chromatographic analysis and mass spectrometric detection were under interest.

The first phase of the work was to find a sample preparation procedure compatible with derivatization LC/ESI/MS and it was determined that solid phase extraction is most suitable. Moreover, it should be quaranteed that solvents used are suitable for further derivatization and LC/ESI/MS analysis.

The largest part of the work is dedicated to the derivatization reagents: commercially available Deemm, Fmoc-Cl, DNS; previously published and specially designed for sensitive LC/ESI/MS analysis TAHS; and in-house designed and synthesized FOSF. FOSF is a novel phosphazene based derivatization reagent that was designed and synthesized in this work keeping in mind the ionization efficiency scale. Comparison of developed methods showed a difference in properties and performances of these reagents. It was found out that novel reagents TAHS and FOSF provide sensitive analysis. However, their chromatographic separation is not as good as for commercially available reagents, which is important when analyzing complex matrices. It was concluded that Deemm is an optimal choice for derivatization reagent since it provides sensitive analysis and better chromatographic separation.

Generally it is not regared as a problem if nonvolatile compounds are present in an injected sample when using LC/ESI/MS. In this work, derivatization procedures of the derivatization reagents were evaluated considering LC/ESI/MS compatibility, including the presence of nonvolatile compounds. It was concluded that the choice of a derivatization buffer can have significant effect on the stability and intensity of the signal. Main influences were related to the use of borate buffer. It was concluded that for reagents Deemm, TAHS and FOSF, boric acid can cause signal suppression in the ESI source and the removal is advised either through the change of the derivatization buffer (as in the case of Deemm) or simply modifying the LC/ESI/MS method so that the initial part of the chromatographic run is diverted away from the ESI source. On the contrary, for Fmoc-Cl, boric acid caused ambiguity in analysis and for DNS, it was found that the use of a boric acid as a signal modifier could significantly lower the LoQ of an analysis.

Since the goal of a derivatization LC/ESI/MS is to provide sensitive analysis, other aspects were evaluated. For all reagents, most suitable eluent components were determined. It was concluded that the presence of ammonium acetate in the chromatographic eluent is favorable for some reagents (Deemm, TAHS) and unfavorable to others (Fmoc-Cl). The same can be concluded for the use of either methanol or acetonitrile as an organic component. Method modifications such as the post-column flow splitter did not provide significant difference to the signal enhancement.

Experiments conducted in addition to the previously mentioned, such as the measurements of ionization efficiency in terms of logIE scale and a thorough MS optimization investigation, provided useful additional information for even more complete and systematic method development. Practical applications showed the high performance of the derivatization LC/ESI/MS for analysis of amino acid in honey and selenoamino acid in bloodserum and food (onion).

In conclusion, in this work, it is shown that for a sensitive derivatization LC/ESI/MS analysis, new aspects of method development are needed and therefore combines together most important aspects and proposes an advanced method development strategy for derivatization LC/ESI/MS.

6. SUMMARY IN ESTONIAN

Parendatud meetodiarenduse strateegia derivatiseerimise LC/ESI/MS jaoks

Aminohapped on ühendid, mis esinevad väga erinevates maatriksites. Aminohapete sisalduse määramine võib olla seotud nii bioloogiliste protsesside uurimisega kui ka näiteks toiduainete päritolu kindlaks tegemisega. Seetõttu toimub aminohapete analüüs tihti keerulistes segudes ning määratavad sisaldused on madalad.

Kui eesmärgiks on madalad määramispiirid, on üks levinumaid valikuid analüüsiks vedelikkromatograafia-elektropihustus-ionisatsioon-massispektromeetria (LC/ESI/MS). LC/ESI/MS võimaldab lisaks madalatele määramispiiridele ka ühendite identifitseerimist. Selleks et parandada kromatograafilist lahutust ning alandada määramispiire, aminohapped derivatiseeritakse. Klassikaliselt kasutatavad derivatiseerivad reagendid on algselt loodud ultraviolett- ja fluorestsentdetektorite jaoks, nagu näiteks 9-fluorenüülmetüül kloroformiaat (Fmoc-Cl), dietüül etoksümetüleenmalonaat (Deemm) ja dansüülkloriid (DNS). Uueks suunaks derivatiseerimisel on aga spetsiaalselt MS detektorite jaoks disainitud väga tundlikku analüüsi võimaldavad derivatiseerivad reagendid nagu *p*-*N,N,N*-trimetüülammooniumanilüül-*N'*-hüdroksüsuktsiinimidüül karbamaat jodiid (TAHS).

Käesoleva töö eesmärgiks oli tervikliku meetodiarenduse strateegia väljatöötamine derivatiseerimise LC/ESI/MS jaoks, et kontrollida kõikide metoodika etappide (proovi ettevalmistus, derivatiseerimine, kromatograafia, massianalüüs) sobivust aminohapete analüüsiks LC/ESI/MSiga.

Töö esimese etapi käigus tehti kindlaks, et kõige paremini sobivaks proovi-ettevalmistuse meetodiks on antud analüüsi korral tahkefaasiekstraktsioon, mis võimaldab solventide õigel valimisel edasi derivatiseerimist ja ka LC/MS analüüsi.

Töö käigus töötati välja metoodikad viie derivatiseeriva reagendi kasutamiseks: Fmoc-Cl, DNS, Deemm, TAHS ning laboris sünteesitud uudse fosfaseenil põhineva reagendi (FOSF) jaoks. FOSF on käesolevas töös disainitud ja sünteesitud esmakordselt silmas pidades ionisatsiooniefektiivsuste skaalal asuvate paremini ioniseeruvate ainete struktuuri. Väljatöötatud metoodikate võrdlemine näitas, et reagendid on erinevate omadustega. Parimat tundlikkust pakuvad uudsed reagendid TAHS ja FOSF, kuid võrreldes klassikaliste reagentidega on neil tunduvalt kehvem kromatograafiline lahutus, mis on oluline eelkõige keeruliste maatriksite analüüsi korral. Kokkuvõtvalt on Deemm parim derivatiseeriv reagent pakkudes kompromissi hea tundlikkuse ja kromatograafilise lahutuse vahel.

Üldjuhul ei peeta LC/ESI/MS analüüsi korral probleemiks mittelenduvate ühendite esinemist süstitavas proovis. Käesolevas töös uuriti esmakorselt derivatiseerimisprotseduuride sobivust LC/ESI/MS analüüsiga. Tulemused näitasid,

et derivatiseeriva segu puhvri valik mõjutab oluliselt signaali stabiilsust ja intensiivsust. Enamik mõjust oli seotud boraatpuhvriga, mida kasutatakse derivatiseerimisel keskkonna pH kontrollimiseks. Tulemused näitasid, et Deemm, TAHS ja FOSF korral boraatpuhvris sisalduv boorhape põhjustab signaali mahasurumist ning seega tuleb see kas välja vahetada (Deemm korral) või suunata kromatograafilise jooksu alguses ESI allikast eemale. Fmoc-Cl korral põhjustab boorhape spektrite identifitseerimisraskusi ning DNS korral saab boorhapet kasutada signaali tugevdajana määramispiiride alandamiseks.

Kuna LC/ESI/MS eesmärgiks on tundlik analüüs, siis tehti töö käigus kindlaks reagentidele kõige paremini sobivad kromatograafilised eluendid. Leiti, et ammooniumatsetaadi sisaldumine eluendis sobib vaid osadele reagentidele (Deemm, TAHS) ja teiste puhul tuleks neist hoiduda (Fmoc-Cl). Samuti näitasid tulemused, et oluline on ka orgaanilise komponendi valik (kas metanool või atsetonitriil). Metoodika modifikatsioonid nagu kolonnijärgne eluendijagamine tundlikkust oluliselt ei parandanud.

Lisaks eelmainitule teostati ka ionisatsiooni efektiivuste mõõtmisi logIE skaala täiendamiseks ning uuriti põhjalikumalt MS optimeerimist, mis andsid olulist lisainformatsiooni, et meetodiarendus oleks veelgi täielikum ja süstemaatilisem. Praktilised rakendused näitasid derivatiseerimise LC/ESI/MS analüüsi head sobivust tundlikuks aminohapete sisalduse määramiseks meeproovidest ning selenoaminohapete määramiseks vereseerumist ja sibulast.

Töö tulemusena näidati, et derivatiseerimise LC/ESI/MS meetodiarenduseks on vaja kaasata uusi aspekte, et võimaldada veelgi madalamaid määramispiire. Antud töö koondab kokku olulisemad aspektid ning pakub seega välja parendatud meetodiarenduse strateegia derivatiseerimise LC/ESI/MS jaoks.

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PUBLICATIONS

CURRICULUM VITAE

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Education

2008–…	University of Tartu, PhD student
Fall 2007	Hong Kong Baptist University, chemistry, exchange student
2006–2008	University of Tartu, Master of Science in Natural Sciences (analytical and physical chemistry), *cum laude*
2003–2006	University of Tartu, chemistry, Bachelor's degree, *cum laude*
2003–2004	University of Maryland, USA. Bachelor's studies

Professional employment

July 2008–…	University of Tartu, Institute of Chemistry, chemist
Summer 2006	Competence Center of Food and Fermentation Technologies; Researcher

Professional self-improvement

Introduction for Sample Preparation (for chromatography). Lectors: Harold M. McNair, Sandy Fuchs. 2007, Tartu, Estonia.
Waters LC/MS training course "Mass Spectrometry", 2008, Helsinki, Finland
Measurement Science in Chemistry Summer School 2008, Celje, Slovenia
Estonian Accreditation Centre training for assessors, 8.–11. June 2010, Pühajärve, Estonia
Effective College Teaching Workshop, 17.–18. October 2011, Tallinn, Estonia
Red Cross First Aid European Certificate training, 5.–6. December 2011, Tartu, Estonia.
Conflict Management, 12.–13. March 2012, Tartu, Estonia.
Mediation, 4. May 2012, Tartu, Estonia.
Juhtimiskool, 15.–17. June 2012, Tartu, Estonia.

Scientific Publications

1. Riin Rebane, Koit Herodes. Evaluation of botanical origin of Estonian uni- and polyfloral honeys by amino acid content. *Journal of Agriculture and Food Chemistry,* **2008**, *56(22)*, 10716–10720
2. Riin Rebane, Ivo Leito, Sergei Yurchenko, Koit Herodes. A review of analytical techniques for determination of Sudan I–IV dyes in food matrixes. *Journal of Chromatography A*, **2010**, *1217(17)*, 2747–2757.
3. Riin Rebane, Koit Herodes. A sensitive method for free amino acids analysis by liquid chromatography with ultraviolet and mass spectrometric detection using precolumn derivatization with diethyl ethoxymethylenemalonate: Application to the honey analysis. *Analytica Chimica Acta*. **2010**, *672*, 79–84.
4. Kivirand, K., Rebane, R., Rinken, T. A simple biosensor for biogenic diamines, comprising amine oxidase-containing threads and oxygen sensor. *Sensor Letters*, **2011**, *9(5)*, 1794–1800.
5. Riin Rebane, Ivo Leito, Koit Herodes. Analysis of selenomethylselenocysteine and selenomethionine by LC-ESI-MS/MS with diethyl ethoxymethylenemalonate derivatization. *Analyst*, **2011**, *136(24)*, 5241–5246.
6. Riin Rebane, Koit Herodes. Influence of boric acid on ESI ionization efficiency. *European Journal of Mass Spectrometry,* **2012**, *18(1)*, 71–75.
7. Rebane, R.; Herodes, K. Comparison of three buffer solutions for amino acid derivatization and following analysis by liquid chromatography electrospray mass spectrometry. *Journal of Chromatography A*, **2012**, *1245*, 134–142.

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Haridus

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Sügis 2007	Hong Kong Baptist University, keemia, vahetusüliõpilane
2006–2008	Tartu Ülikool, loodusteaduse magister (analüütiline ja füüsikaline keemia) *cum laude*
2003–2006	Tartu Ülikool, keemia eriala, bakalaureusekraad *cum laude*
2003–2004	University of Maryland, Ameerika Ühendriigid, bakalaureuseõpingud

Teenistuskäik

Juuli 2008–…	Tartu Ülikooli Loodus- ja tehnoloogiateaduskond, Keemia Instituut, keemik
Juuni 2006	Toidu- ja Fermentatsioonitehnoloogia Arenduskeskus, teadur

Erialane enesetäiendus

Proovide ettevalmistamine kromatograafiliseks analüüsiks. Lektorid: Harold M. McNair, Sandy Fuchs. 2007, Tartu, Eesti.

Fundamentals of LC-MS. Firma Waters seminar, 2008, Helsingi, Soome.

HPLC column selection and method development. 2008, Tartu, Eesti. Lektorid: Liisa Kanner ja Esa Lehtorinne.

Measurements Science in Chemistry Suvekool 2008. Celje, Sloveenia.

Eesti Akrediteerimiskeskuse Assessorite Koolituskursus, 2010, Pühajärve, Eesti.

Effective College Teaching Workshop, 2011, Tallinn, Eesti.

„Konflikti juhtimine" Lektor: Tõnu Lehtsaar. 2012, Tartu, Eesti.

„Lepitamine" Lektor: Tõnu Lehtsaar. 2012, Tartu, Eesti.

„Juhtimiskool 2012" Noored Liidrid. 2012, Tartu, Eesti.

Teaduspublikatsioonid

1. Riin Rebane, Koit Herodes. Evaluation of botanical origin of Estonian uni- and polyfloral honeys by amino acid content. *Journal of Agriculture and Food Chemistry,* **2008**, *56(22)*, 10716–10720
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