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Characterization of amino acid derivatization reagents for LC-MS analysis

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Institute of Chemistry, Faculty of Science and Technology. University of Tartu, Estonia.

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Supervisors: Riin Rebane, PhD, Institute of Chemistry, University of Tartu, Estonia

Associate Professor Koit Herodes, Institute of Chemistry, University of Tartu, Estonia

Opponent: Prof. Jonas Bergquist, Dr Med Sci, PhD, Uppsala University, Sweden

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Author's contribution

Paper I: Participated in carrying out the experiments and writing the manuscript.

Paper II: Participated in carrying out the experiments.

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Paper V: Main person responsible for planning and performing the experimental work and writing the manuscript.

ABBREVIATIONS

AA	amino acid
AA-DEEMM	pure standard of derivative of respective amino acid
AA-FMOC	pure standard of derivative of respective amino acid
APCI	atmospheric pressure chemical ionization
API	atmospheric pressure ionization
DBEMM	dibenzyl ethoxymethylenemalonate
DEEMM	diethyl ethoxymethylenemalonate
DNS	5-(dimethyamino)naphthalene-1-sulfonyl chloride, dansyl chloride
ESI	electrospray ionization
FL	fluorescence
FMOC-AA	procedural standard of derivative of respective amino acid
FMOC-Cl	9-fluorenylmethoxycarbonyl chloride, 9-fluorenylmethyl chloroformate
FOSF	2,5-dioxopyrrolidin-1-yl N tri(pyrrolidino)-phosphoranylideneamino carbamate
IE	ionization efficiency
ILIS	isotopically labelled internal standard
LC	liquid chromatography
ME	matrix effect
MS	mass spectrometry
MS/MS	tandem mass spectrometry
m/z	mass-to-charge ratio
TAHS	*p-N,N,N*-trimethylammonioanilyl *N'*-hydroxysuccinimidyl carbamate iodide
UV	ultraviolet

1. INTRODUCTION

Each analytical method provides a sensitive and selective analysis for analytes with specific properties. If these properties are not met, analytical derivatization can be used in order to extend the applicability or increase the sensitivity of the method. This means that the analyte is turned into its derivative which is suitable for analysis or more easily analyzed with the respective method.

Currently, liquid chromatography atmospheric pressure ionization mass spectrometry (LC-API-MS) is often the method of choice for analyzing low concentrations in complex matrices due to its high sensitivity and selectivity. Derivatization can improve all steps of LC-API-MS analysis, starting from the sample preparation, increasing chromatographic retention, enhancing ionization efficiency and improving structural elucidation with MS. Until now, LC-API-MS analysis has exploited mainly derivatization reagents which have been developed for ultraviolet (UV) or fluorescence (FL) detection. At the same time, derivatization reagents aiming at increased ionization efficiency of the analytes have been also developed specifically for API-MS analysis.

In addition to sensitivity, matrix effect (ME) is an important aspect influencing both trueness and precision of LC-API-MS analysis. This makes ME an essential parameter of quantitative method validation. Therefore, characterization of the derivatization reagents is important for achieving analysis which would be less affected by ME. The comparison of derivatization reagents is necessary for choosing a suitable derivatization reagent for specific applications. In addition, this information would be valuable for designing novel derivatization reagents which would provide both sensitive and ME free LC-MS analysis.

The present work aimed at characterization of amino acid derivatization reagents from the ME point of view. Amino acids were chosen for model analytes as their analysis with reversed phase (RP) chromatography is generally carried out using derivatization. Altogether six derivatization reagents, from which two have been synthesized in our work group in University of Tartu, were applied to the analysis of seven amino acids in standard solutions and matrices. Three approaches were used for evaluating ME: post-column infusion, post-derivatization spiking and sample dilution experiments. In addition to the derivative's structure, the influence of instrument setup was evaluated for comparing ME in case of ESI and APCI, and with one reagent for ESI positive and negative ion modes.

The characterization of six derivatization reagents on the one system (same samples and instrument) provided information, which can be useful for choosing a suitable derivatization reagent for future applications and for designing new derivatization reagents for LC-MS analysis with high sensitivity and accuracy.

2. REVIEW OF LITERATURE

2.1. LC-API-MS analysis

When searching for higher sensitivity and selectivity, liquid chromatography mass spectrometry (LC-MS) has become one of the most widely used analytical methods applied to the analysis of low concentrations in a wide variety of matrices.[1,2] The sample components are first separated chromatographically and then by their mass-to-charge ratio (*m/z)*.[3] The use of LC-MS method has become more popular with the use of atmospheric pressure ionization (API) sources, which connect these two instruments by generating gas phase ions from the LC effluent at atmospheric pressure and introducing them to the vacuum region of MS.[4,5] The wide applicability of API comes from its ability to ionize a wide variety of compounds and from the softness of the ionization technique, which preserves the structural integrity of the analyte and results in dominating quasimolecular ion in the mass spectra.[3]

The most widely used electrospray ionization (ESI) source is suitable for moderately non-polar to ionic analytes [6] and allows ionization of thermally labile, nonvolatile compounds and multiply charged large molecules (e.g. proteins).[7] The ionization takes place in the liquid phase as the effluent flows through a metal capillary which has electric potential of several kV relative to the MS inlet. Due to the electric field, the effluent forms a Taylor cone at the tip of the capillary, resulting in a fine mist of charged droplets. The solvent evaporation (aided by the gas flow and heating) causes the droplets to shrink to the nanometer range until the surface tension is balanced by the Coulombic repulsion forces (the Rayleigh limit).[3] From that point forward, there are three main mechanisms describing the formation of gas phase ions in ESI: the ion evaporation model (IEM) for low molecular weight analytes [7], the charge residue model (CRM) for large globular molecules [8] and the chain ejection model (CEM) for disordered macromolecules [9].

Another widely used ionization technique is atmospheric pressure chemical ionization (APCI).[10] Differently from ESI, the ionization takes place after the pneumatic nebulization of the effluent into the gas phase. In APCI, the corona needle provides electrical charge for ionization – high voltage applied to the needle creates a corona discharge, which produces high energy electrons, which are able to ionize carrier gas or solvent molecules.[11] Ionization can occur according to at least two mechanisms: (1) the charge exchange and (2) the proton transfer or hydrogen atom abstraction, resulting in both molecular ions $M^{\cdot +}$ and quasimolecular ions $[M+H]^+$.[12] The higher the corona needle voltage, the higher is the ion current.[13]

Depending on the ionization mode either positively or negatively charged ions can be generated with ESI and APCI. In practice, due to different ionization mechanism and depending on the application (analyte) either ESI [14,15] or APCI [16] or both could allow the most sensitive analysis.

2.2. Ionization efficiency

The sensitivity of the analysis depends on the ionization efficiency (IE) of the analyte. IE describes the efficiency of generating gas-phase ions from molecules or ions in the solution.[17] IE depends on the analyte and analysis conditions (e.g. ion source and mode, solvent) and it can vary to a great extent.

The two main requirement for high IE in ESI are chargeability and presence of non-polar moiety in the molecule structure. Firstly, the ionization in ESI can occur via protonation and deprotonation or adduct formation (e.g. NH_4^+, Na^+, K^+, Cl^-) depending on the ionization mode. Therefore, the analyte has to be ionic or readily ionizable in the solution or be able to form adducts in the gas phase.[18–21] Secondly, as the surface activity of the compound determines its ability to move to the surface of the charged droplet and enter the gas phase more efficiently, a non-polar region in analyte's structure is beneficial in both positive and negative ion modes.[22] Moreover, non-polar region increases the retention in RP-LC and results in elution at higher organic content in the eluent. This reduces effluent's surface tension and lowers boiling point, which in turn increase the IE.[23,24]

Due to the different ionization mechanism in APCI, low solvation energy is important for high IE in both ion modes, while gas phase basicity is important for positive and gas phase acidity for negative ion mode.[13] In addition, while adduct formation can be abundant in case of positive ion mode ESI, adducts are usually not observed with APCI.[6,16] For example, it has been hypothesized that the sodium ion is not easily volatilized and therefore adducts don't form in the gas phase or the weak interaction of sodium adduct (Na-adduct) is broken in high temperature APCI source.[6]

In order to better understand the ionization processes and predict the IE of specific compounds, log*IE* is used for quantifying IE and experimental log*IE* scales have been previously compiled for ESI [17,25–27] and APCI [28]. The most important parameters affecting the log*IE* through protonation in ESI are the basicity of the analyte in solution (pK_a of the conjugated acid or pK_b) [25] and molecular volume [26]. This means that compounds with higher basicity and more voluminous molecules ionize better in positive ion mode ESI. The log*IE* scale for APCI indicates some similarities between ESI and APCI ionization mechanisms and shows that the compounds with high log*IE* in ESI have high log*IE* in APCI regardless of the volatility or molecular volume. Though, compounds ionizing in both sources generally have higher IE in ESI.[28] ESI and APCI are complementary as they are suitable for analytes with different polarity: ESI is more suitable for ionic or polar analytes (acidic or basic properties in solution), while APCI is more suitable for low- to medium-polarity analytes.[6]

One of the experimental factors affecting the log*IE* is the solvent composition. In ESI, the conductivity of the effluent is important and both methanol and acetonitrile are suitable from this aspect, however, methanol is often preferred due to providing better peak shape for basic compounds in LC and higher

sensitivity.[29,30] In APCI, as ionization takes place in the gas phase, analytes with higher proton affinity than the solvent have higher log*IE*. Methanol is considered more suitable than acetonitrile due to the relatively lower proton affinity.[29,31] As evaporated solvent molecules act as reagent gas, higher sensitivity is obtained at higher effluent flow rates.[14]

When sensitive LC-MS analysis of compounds with low log*IE* is necessary, derivatization can be applied.[23] The desired properties of the derivative depend on the ionization source and it's polarity. While hydrophobic moiety of the derivative leads to higher log*IE* in both ESI ion modes, derivatives with permanent charge, e.g. tertiary ammonium ion [32,33] or strong basic center [Paper I], are preferred for positive ion mode. Meanwhile, chromatographic separation of charged compounds could be challenging due to weak retention in RP-LC.[34] In the negative ion mode, acidic functional groups enhance log*IE*.[19] For positive ion APCI analysis, derivatization has been used for decreasing the polarity of analyte or introducing an atom with high proton affinity, e.g. fatty acids are turned into neutral esters which still contain oxygen with high proton affinity.[21] Adding functional groups with high electron-affinity (e.g. nitro, trifluoromethyl) to neutral steroids has resulted in more sensitive negative ion APCI-MS analysis.[35]

In addition to higher log*IE*, derivatization offers other advantages for MS detection. First, MS signal of the derivative with higher molecular weight is further from low molecular weight matrix compounds and noise is decreased.[21] Secondly, the ability of the analyte to produce intense and characteristic product ions during fragmentation in MS/MS is a prerequisite for selective and sensitive analysis. Fragmentation of the derivatives can offer additional information about the structure of the analyte and aid the identification of small molecules.[36]

Derivatization reagents applied to the amino acid LC-API-MS analysis are further discussed in section 2.5.

2.3. Matrix effect

Signal in LC-MS can be seriously affected by the sample matrix, i.e. the components of sample other than the analyte. In general, any influence of matrix components on the analytical result caused by the matrix components is called matrix effect (ME).[37] In the context of LC-API-MS analysis, ME is the change in analyte's IE caused by the co-eluting sample components.[38] Two types of ME can affect the analysis, first, the difference of analyte signal in pure solution versus spiked matrix extract (absolute ME), and second, the change of signal in different sample lots (relative ME).[39] The interference from the sample matrix can influence the quantitative analysis (i.e. precision, trueness, limit of detection (LoD) and limit of quantitation (LoQ)), hence, the importance of evaluating ME during method development and validation has been addressed.[38,40]

Investigation of ME began with the study by Tang and Kebarle showing that the presence of other electrolytes can reduce sensitivity of the analysis.[41] Since then, several models have been suggested for describing this phenomenon. For example, analyte's signal could be altered due to competition for the limited amount of excess charge on the surface of ESI droplet or the change in the effluent's viscosity so that the analyte's ionization is hindered. In addition, unknown adducts can form so that the MS would not recognize the ion as the analyte or non-volatile compounds can prevent the analyte from leaving the droplet.[42]

As ME depends on the analyte and matrix or both, the ME should be determined for each case. Additionally, ME depends on the ion source, its design and polarity.[43,44] King et al. compared the differences of ME in case of ESI and APCI. It was concluded that while both solvent and gas phase processes can be the cause of ME, the former is dominant in causing ME.[45] Therefore, ESI is more susceptible to ME in some cases, e.g. estradiol in human serum and tissue [46], for patulin in apple [47], various drug candidates in bioanalysis [48]. Moreover, negative ion mode has been shown to be less affected by ME compared to positive ion mode.[49] ME can also affect analysis when derivatization is applied [50] and even novel derivatives designed specifically aiming at signal enhancement for LC-MS analysis suffer from ME.[51] As ME affects quantitative analysis, suitable methods for evaluating its scope, eliminating it or taking it into account are needed.

2.3.1. Evaluation of ME

Evaluation of the presence of ME is an important step in LC-MS method validation.[40,52] Two of the most often used methods are post-column infusion and post-extraction spiking method. Both of these require analyte free blank matrix and a standard substance of the analyte.

The post-column infusion experiment provides qualitative information about ME. In order to estimate the influence of sample matrix on the signal of the analyte, the effluent of the chromatographic analysis of the blank sample is mixed (using a T-piece) with the continuous flow of the analyte solution before the ion source. The signal of the analyte is monitored and alterations in the signal, other than the influence due to changing eluent composition during gradient elution [53], indicate either signal suppression or enhancement caused by the components in the sample [54].

Quantitative evaluation of ME is sometimes required and this can be done with the post-extraction spiking method. ME is evaluated by comparing the signals of equal concentration of analyte spiked into blank sample extract (A_{matrix}) and into the solvent (A_{standard}). The estimate of the ME can be found using Eq. 1, where ME values higher than 100% indicate signal enhancement and values below 100% indicate signal suppression.[55]

$$\%\boldsymbol{ME} = \frac{\boldsymbol{A_{matrix}}}{\boldsymbol{A_{standard}}} \cdot \mathbf{100}\% \qquad \textbf{Equation 1}$$

However, in some cases the requirement to have a blank sample and a standard material of the analyte is difficult or even impossible to fulfil. For example, analyte free matrix is not available for analysis of endogenous substances in biological matrices, where the presence of analytes is an intrinsic property of the sample, e.g. amino acids in human plasma.[1] Since ME evaluation is also needed in cases when blank samples are not available, modifications of the mentioned methods have been used. In case of derivatization, there is a possibility to use an underivatized sample as a blank sample (does not contain the derivative of the analyte) for post-extraction addition and spike it with derivatized analyte standard solution [56,57]. However, as the sample is altered during the derivatization, i.e. sample components can also be derivatized, their retention time is changed, meaning that the components co-eluting with the analyte can also change.

Moreover, reference materials cannot be obtained for many analytes, e.g. in case of derivatization, where reference material could be considered an analyte already in derivatized form. Pure standards of derivatives are commercially available only for few analytes and derivatization reagents. For certain cases, pure derivatives could be either synthesized in-house or purified from the derivatization mixture. Uutela et al.[53] used solid-phase extraction (SPE) for purifying propyl chloroformate (PrCl) derivatized β-Ala, which was used for post-column infusion experiment for evaluating possible ME for other PrCl-derivatives. Either way it is laborious to obtain pure derivatives and also require knowledge and instrumentation often not available for the laboratory. In addition, comparison of slopes of standard addition graph and solvent calibration graphs have been used for evaluating ME for derivatives of dansyl chloride (DNS).[58]

2.3.2. Reducing or taking ME into account

Once the presence and extent of ME has been determined, there are several methods proposed for reducing or taking it into account. First of all, in order to reduce ME, changes in sample preparation and optimization of instrumental parameters could be considered during method development. For example, analyte can be extracted from sample using SPE [59], but this can also concentrate sample components and does not always eliminate the ME [60]. Incorporating derivatization can increase the retention of analytes resulting in better separation from matrix components and decreased number of compounds co-eluting with the analyte.[30,56,61] Another option is diluting the samples, since it has been shown that there is a linear correlation between ME and logarithm of sample dilution factor. It was also demonstrated, that 25–40 fold dilutions can reduce strong signal suppression and allow acceptable accuracy for many analytes.[62] E_n scores have been previously used for differentiating between the analytes affected and not affected by ME. When ME is decreased, but not eliminated with dilution, extrapolative dilution approach can be used for

taking the ME into account by estimating the analyte concentration at infinite dilution, where ME has been eliminated.[63] In addition, selection of suitable ion source and ion mode, suitable effluent flow rate (nano-LC or flow splitting after LC) can decrease ME.[64,65]

If the ME is not eliminated, then one of the most effective methods for taking the ME into account is matrix matched calibration.[66] This approach also needs analyte free matrix and presumes that ME is consistent from sample to sample, which does not always hold.[48] If ME is not present or is eliminated, solvent based calibration solutions could be used instead of matrix matched standards.[67,68]

Another option for taking ME into account is standard addition method, which is resource intensive and there may not be enough sample available.[69] Internal standards can also be used and as ME depends on the retention time of the analyte, isotopically labelled internal standard (ILIS) are suitable for this purpose. This assumes that ILIS is affected by ME to the same extent as the analyte and the ratio of the two signals remains constant. Nevertheless, differences in ME of the analyte and ILIS can occur.[70,71]

2.4. Amino acid analysis

Amino acids (AAs) can be found in various fields of chemistry. They have an important role as the building blocks for proteins and 20 of them are encoded in the DNA. In addition, they are the precursors for other vital compounds, e.g. hormones, nucleic acids and neurotransmitters.[72] Due to their importance in the human physiology, nine of the encoded AAs are considered essential (His, Ile, Leu, Lys, Met, Phe, Thr, Trp, Val), as they cannot be synthesized by human body and need to be obtained with food.[73] This also means that AA analysis is carried out in a wide variety of applications and matrices: pharmaceutical industry [74], diagnosis of medical disorders [75], in food and beverages to assess AA's influence on their taste, aroma and color [76] or to determine their adulteration [77], geographical or botanical origin [78]. AA content can be used to differentiate between varieties of foods such as coffees [79] or teas [80]. In addition, depending on the application, analysis at very low concentrations is often aimed at, e.g. selenoamino acids in liver cells [81].

Most AAs are polar compounds and have poor retention in RP-LC.[82] In many cases, derivatization has been used for AA analysis to enhance chromatographic separation and detection.[76] Due to their acidic/basic properties, most AAs can be ionized for MS detection in both positive and negative ion modes [75,77]. However, effort has to be made in order to analyze free AAs with ESI-MS [74,75] and APCI-MS [83]. Moreover, derivatization can improve the retention of AAs enabling their chromatographic separation from each other and from matrix components and less ME is expected.

2.5. AA derivatization for LC-API-MS analysis

Several aspects need to be kept in mind for successful derivatization. Depending on the analyte's functional group or groups, different reaction types can be used for derivatization.[23] Therefore, derivatization reagents should have a reactive group corresponding to specific functional group of the analyte and the derivatization reaction should be as selective as possible. In addition, fast one-pot derivatization reaction in mild conditions (e.g. pH, temperature), resulting in stable water soluble derivatives is preferred.[51]

In case of LC-MS, derivatization often aims at enhancing analyte's log*IE*. Other possible intentions include (1) improving chromatography (selectivity, separation and peak shape), (2) facilitating enantiomer separation without chiral stationary phase, (3) improving stability or extraction efficiency of analyte, (4) removing endogenous interferences (separation from structurally similar compounds) and (5) improving structural elucidation.[23] Depending on the specific aim of the derivatization, it can be carried out at different steps during the analysis and categorized as pre-, on- or post-column derivatization. Since in many cases the chromatographic separation is also one of the main aims, pre-column derivatization is mainly used.[23] This means that derivatization focused on API-MS should also meet the criteria set by the previous steps of the analysis: sample preparation and chromatographic analysis.[84,85]

Derivatization reagents for amines and AAs have been discussed in several reviews for LC-ESI-MS [23,36,84,86] and LC-APCI-MS analysis [61] and some examples are brought in Figure 1. Various derivatization reagents originally meant for UV or FL detection have been applied to LC-ESI-MS analysis of AAs in positive ion mode. The derivatives of 9-fluorenyl-methoxycarbonyl chloride, 9-fluorenylmethyl chloroformate (FMOC-Cl) [87], 5-(dimethyamino)naphthalene-1-sulfonyl chloride (dansyl chloride, DNS) [88] and diethyl ethoxymethylenemalonate (DEEMM) [89] have also been analyzed in negative ion mode. Although derivatives of FMOC-Cl [90] and DNS [91] have been analyzed with LC-APCI-MS, their application for LC-APCI-MS analysis of AAs has not been previously published.

In addition, several novel derivatization reagents aiming at sensitive LC-ESI-MS analysis have been developed for amines and AAs. For example, *p-N,N,N*-trimethylammonioanilyl *N´*-hydroxysuccinimidyl carbamate iodide (TAHS [33], (5-N-Succinimidoxy-5-oxopentyl)triphenylphosphonium bromide (SPTPP) [92] and 2,5-dioxopyrrolidin-1-yl N-tri(pyrrolidino)-phosphoranylideneamino carbamate (FOSF) [Paper I] provide LoD at subfemtomole to attomole levels. The design of the new reagents differs and some of them have been developed based on the existing ones. For example, FMOC-Cl, a common blocking group in peptide synthesis, has been the starting point for development of several new derivatization reagents, e.g. 1,2-benzo-3,4-dihydrocarbazole-9-ethyl chloroformate (BCEOC-Cl) [93] and 2-(11H-benzo[a]-carbazol-11-yl) ethyl chloroformate (BCEC-Cl) [83] for LC analysis aiming fluorescence detection, but also applied for ESI- and APCI-MS analyses, respectively. The development of novel

permanently charged TAHS lead to the development of 3-aminopyridyl-*N*-hydroxysuccinimidyl carbamate (APDS) without a permanent charge, in order to achieve stronger retention in RP-LC.[34]

Three novel derivatization reagents have been designed in our work group at University of Tartu. Firstly, FOSF was developed based on the hydrophilic phosphazenes, which have the highest log*IE*s in ESI IE scale [26] due to a practically permanent positive charge (high basicity) and large molecular volume. [Paper I] Secondly, two derivatization reagents, dibenzyl ethoxymethylenemalonate (DBEMM) and benzyl ethyl ethoxymethylenemalonate (EBEMM), were developed based on the structural features of DEEMM, which provided LoD values comparable to TAHS. Their design was also based on the ESI log*IE* scale, but differently from FOSF, these structures were designed without a permanent charge in order to improve chromatographic separation. Moreover, it was important that the structures of DBEMM and EBEMM were able to chelate the charge carrier (H^+ or Na^+) and have a large hydrophobic moiety to facilitate ESI ionization.[94]

Since log*IE* values of derivatization reagents can help towards the design of reagents, log*IE* values of selected derivatization reagents and respective derivatives have been measured. In case of APCI with acetonitrile as eluent, log*IE* values of derivatization reagents decrease in the order of DNS (3.28), EBEMM (2.92), DBEMM (2.43) and DEEMM (1.18). In case of ESI with acetonitrile, DBEMM (3.56) and EBEMM (3.17) are superior to DEEMM (2.04). The DEEMM-derivatives of Phe and Gly have higher log*IE* (3.79 and 3.36) compared to respective FMOC-derivatives (3.56 and 3.23) in ESI with methanol.[85,94,95] The results demonstrate that derivatization reagents with higher log*IE* can be designed based on the ionization efficiency scales [17,26,28].

Five AA derivatization reagents (FOSF, TAHS, DNS, FMOC-Cl and DEEMM) have also been compared for LC-MS analysis using standard solutions and it was demonstrated that the reagent meant for UV detection (DEEMM) can provide LoQ comparable with novel derivatization reagents carrying a permanent positive charge (TAHS and FOSF). As a result of absence of permanent charge the former also provide better chromatographic separation and wider linear ranges.[Paper I]

However, when choosing the most suitable derivatization reagent, the IE in the sense of sensitivity is not the only parameter that needs to be considered. For practical applications, especially with complex matrices, the question of ME rises. Matrix influence has been evaluated for LC-MS analysis of AAs using derivatization, for example, with PrCl [53], DNS [58], 2H,2H,3H,3H-perfluoroundecan-1-al [57] and 10-ethyl-acridone-3-sulfonyl chloride (EASC)[56]. Although ME has been evaluated for specific LC-MS applications, a comparison of different derivatization reagents from ME point of view has not been conducted on the same instrument and samples. The information about ME for analyses using various derivatization reagents would be valuable for choosing the best possible reagent during method development depending on whether the sensitivity or absence of ME is critical from the analysis point of

view. Moreover, this information would support characterizing classical and novel derivatization reagents and also provide an input for developing new derivatization reagents, which would provide the highest possible ionization efficiency at the same time being immune to ME.

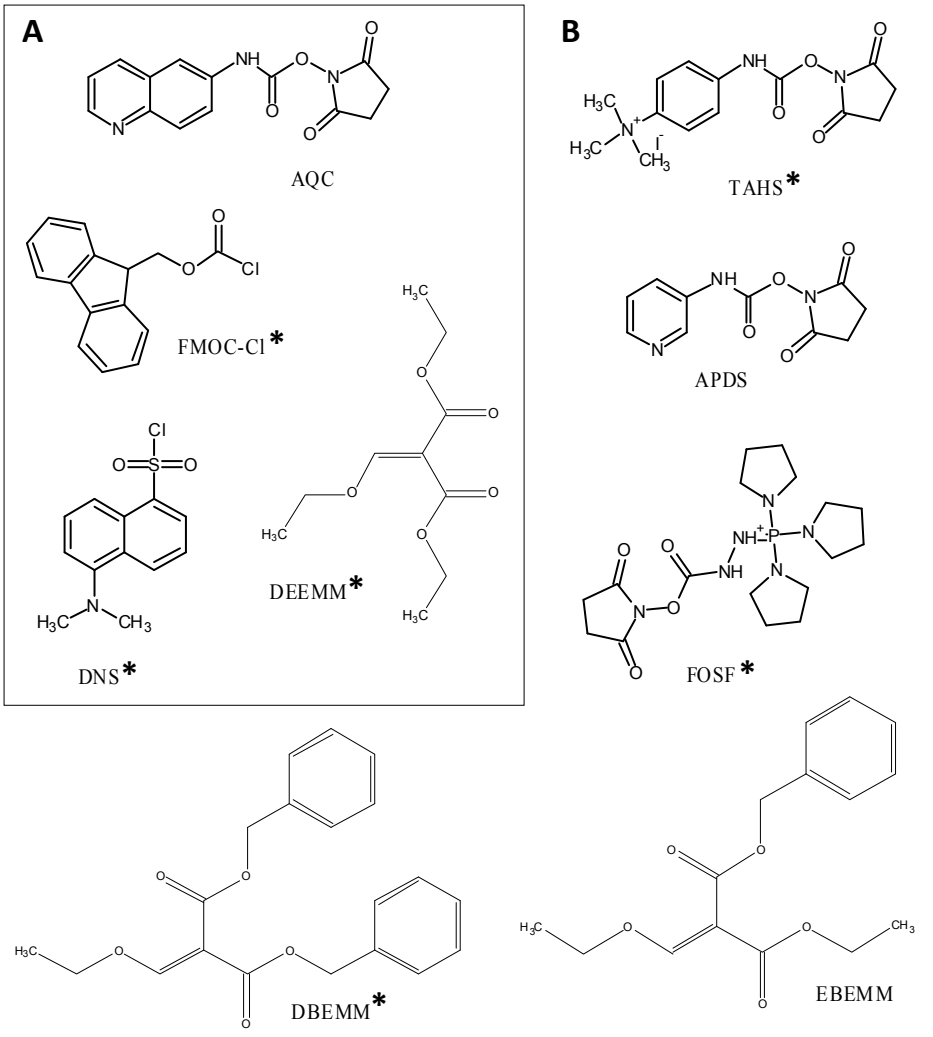

Figure 1. Derivatization reagents applied for LC-MS analysis, which have been originally designed for (A) UV or FL and for (B) API-MS detection. The ones used in the present work have been marked with asterisk (*).
Derivatization reagents: 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate (AQC) [96], DNS* [97], FMOC-Cl* [98], DEEMM* [78], TAHS* [33], APDS [34], FOSF* [Paper I], DBEMM* [94], EBEMM [94].

3. EXPERIMENTAL

3.1. Chemicals and materials

Amino acids: The kit of α-amino acids (Sigma), β-Ala (Fluka). Pure derivatives of AAs β-Ala- and Gly-FMOC (Fluka), Phe-FMOC (Aldrich), Gly-, Phe- and β-Ala-DEEMM (synthesized in-house according to Ref. [99]).

Derivatization reagents: DEEMM and DNS (Fluka), FMOC-Cl (Aldrich), TAHS (synthesized in-house with modifications from Ref. [33]) [Paper III], FOSF (synthesized in-house) [Paper III] and DBEMM (synthesized in-house) [94].

Chemicals for derivatizations: acetic acid (Lach-Ner); acetone (Lach-Ner); sodium hydroxide (Chemapol); boric acid, ammonium hydroxide (Reakhim); 1,1,1,3,3,3-Hexafluoro-2-propanol (HFIP) (Fluka).

Other chemicals: hydrochloric acid (HCl), orthophosphoric acid, sodium dihydrogenphosphate (Reakhim), 2-mercaptoethanol (Sigma).

LC eluents: HPLC grade methanol and acetonitrile (Sigma-Aldrich), formic acid and ammonium acetate (Fluka).
Aqueous solutions were prepared with ultrapure water purified by Millipore Milli-Q Advantage A10 (Millipore).
All reagents were of analytical grade if not otherwise stated.

Materials: strong cation exchange solid phase extraction (SPE) cartridges (Alltech, 500 mg styrenedivinylbenzene polymer), wide-pore paper filter (388 FILTRAK), syringe filters regenerated cellulose (RC; 0.45 μm, Econofilter, Agilent) and cellulose-acetate (GD/X; 0.45 μm, Whatman).

3.2. Preparation of standard solutions

In this work, "pure standard" stands for the derivative of AA, i.e. without any excess derivatization reagent, buffer salts or other components used for derivatization reaction. In an abbreviation of a specific pure standard amino acid is marked first, e.g. Gly-FMOC. "Procedural standard" stands for AA standard solution which is derivatized, i.e. contains excess of derivatization reagent, reaction by byproducts and buffer components. In an abbreviation of a specific procedural standard derivatization reagent is marked first, e.g. DEEMM-Phe.

Pure derivatives of AAs:
A calibration graph was constructed using pure standards for evaluating the derivatization yield of procedural standards. Stock solutions of each pure standard were prepared in methanol at concentration of 50 μg g^{-1}, expressed as the concentration of AA (not the derivative). Working solutions (0.5–5 μg g^{-1}) were prepared in 0.1 M HCl with 30% methanol.

For post-column infusion experiments, stock solutions of each pure standard of Gly-, Phe- and β-Ala-FMOC and β-Ala-DEEMM were prepared in acetonitrile with 0.1% aqueous formic acid solution (80:20) at two concentration levels: 40 μg g^{-1} and 1 mg g^{-1}.

Procedural standards:
All procedural standards were prepared by derivatization of AA standard solutions.

Mixture of 7 amino acids: Individual AA stock solutions (4–16 mg g^{-1}) were prepared in 0.1 M HCl with 30% methanol. A stock solution of 7 AAs (Arg, Asp, Gly, β-Ala, Pro, Trp, Phe) was prepared so that individual AA concentration was in the range of 5–12 μg g^{-1}. Working standard solutions with appropriate concentrations were prepared prior to derivatization with 0.1 M HCl with 30% methanol.

Mixture of 22 amino acids: Individual AA stock solutions (1.6–6.5 mg g^{-1}) were prepared in 0.1 M HCl with 30% methanol. A stock solution of 22 AAs, with AA concentrations of 20–28 μg g^{-1} was prepared. Working standard solutions were prepared prior to derivatization by dilution with ultrapure water (Milli-Q) in the AA concentration range 8–7000 ng g^{-1}.
All stock solutions were stored at –20 ºC.

3.3. Preparation of samples

Samples: Green tea was the main sample used for most of the experiments: post-column infusion and sample dilution experiments with ESI and APCI (see sections 4.2–4.5). Honey samples were involved for sample dilution experiments with ESI (see section 4.3) and, together with 11 herbal extracts, for estimating the influence of ionization polarity in case of ESI (see section 4.5).

Herbs: Green tea (Ahmad Tea, London, Green Tea); herbs purchased from local stores: pine growth (*Pini gemma*), yarrow (*Achillea millefolium*), heather (*Calluna vulgaris*), linden1 (*Tilia cordata*), chamomile1 (*Chamomilla recutita*), cowslip (*Primula veris*)); herbs obtained directly from consumers (self-grown): linden2 (*Tilia cordata*), chamomile2 (*Chamomilla recutita*), St. John's wort (*Hyperici perforatum*) and peppermint (*Mentha × piperita*).

Honeys: Heather (*Calluna Vulgaris*) honey from Estonia, honey1 (Fauchon Paris: floral honey. Provence), honey2 (Fauchon Paris: Acacia honey. Rhône Alpes), honey3 and honey4 were Estonian polyfloral honeys (purchased locally).

Sample preparation methods:
Preparation of herbal extracts: Sample preparation from Ref. [80] was used for all of the herbal extracts (including green tea). 10 mL of Milli-Q was added to 0.25 g of herb and heated at 80 ºC for 25 min. The mixture was cooled to room temperature and filtered through wide pore paper filter. The volume of the

extract was taken to 10 mL with Milli-Q water. In addition, as a modification, green tea extract with 2-times higher amount of sample (0.5 g) was also prepared.

Solid phase extraction (SPE) procedure: Sample preparation form Ref. [78] was carried out for all honeys and one green tea extract. Honey (1 g) or green tea extract (1 mL) was diluted with 25 mL of phosphate buffer (0.03 M, pH 2.12), which was filtered through a wide-pore paper filter. Strong cation exchange SPE cartridge was used for extracting AAs from the buffered sample. First, the SPE cartridge was conditioned with 10 mL of 0.1 M HCl (4 mL min^{-1}). The buffered sample was applied to the cartridge (1.5 mL min^{-1}). AAs were eluted from the cartridge with 15 mL ammonium hydroxide (2.5 M, 10% of acetonitrile) and the eluate was evaporated to dryness under nitrogen flow. The evaporation residue was dissolved in 1 mL (0.25 mL and 0.5 mL for concentrated samples) ultrapure water. The extracts were stored at 4 ºC and diluted prior to derivatization with Milli-Q water (section 3.5.2).

Derivatization procedures:

DEEMM: 250 µL of sample was mixed with 375 µL of DEEMM solution (1:50 in methanol) and 875 µL of borate buffer (0.75 M, pH 9). Derivatized sample was kept in the dark at room temperature. The analysis was carried out during 24–48 h after the derivatization. Derivatized samples were filtered using GD/X syringe filter.

DNS: 100 µL of sample was mixed with 20 µL of NaOH (2 M) and 500 µL of DNS solution in acetone (5 mg mL^{-1} for green tea and 10 mg mL^{-1} for honey). After 45 min in the dark (at 4 ºC) the reaction was ended with 10 µL of concentrated ammonium hydroxide. Samples were filtered using RC syringe filter.

FMOC-Cl: 300 µL of sample was mixed with 300 µL of borate buffer (0.75 M, pH 9) and 300 µL of FMOC-Cl solution (1 mg mL^{-1} in acetonitrile). The derivatization reaction was ended after 30 min by adding 300 µL of histidine solution (4 mg mL^{-1}). Samples were filtered using RC syringe filter.

TAHS and FOSF: 10 µL of sample was mixed with 30 µL of borate buffer (0.2 M, pH 8.8) and 20 µL of TAHS or FOSF solution in acetonitrile (20 mg mL^{-1}). The derivatization reaction was ended after 30 min by adding 200 µL of acetic acid solution (0.2% in Milli-Q water). Samples were filtered using RC syringe filter.

DBEMM: 50 µL of sample was mixed with 300 µL of DBEMM solution (1:150 in isopropanol) and 200 µL of HFIP buffer (0.56 M, pH 9.0). Samples can be filtered using either RC or GD/X syringe filters.

3.4. LC-API-MS analysis

Two different instrument setups were used in this work, one with ion trap (XCT) mass analyzer and the other with triple quadrupole (QqQ) mass analyzer. Following, the operational parameters of these systems are presented.

XCT: LC-API-MS^n system Agilent Series 1100 LC/MSD Trap XCT was used. Detection was carried out using a photodiode array detector (PDA) with 6 mm path length flow cell and electrospray (ESI) or atmospheric pressure chemical ionization (APCI) interface mass spectrometer (MS). ESI parameters: nebulizer gas (nitrogen) pressure 50 psi (345 kPa), drying gas (nitrogen) flow rate 12 L min^{-1} and drying gas temperature 350 ºC. Optimized APCI parameters for each reagent are presented in Table 1.

The chromatographic methods, retention times (t_R) and the *m/z* values of the 7 AA derivatives are in presented Table 2, Table 3 and Table 4, respectively.

QqQ: LC-ESI-MS/MS system Agilent 1290 UHPLC with Agilent 6495 Triple Quad LC/MS with Jet Stream ion source was used. Ion source parameters were the following: nebulizer gas (N_2) pressure 30 psi (207 kPa), drying gas (N_2) flow rate 15 L min^{-1} and temperature 250 ºC, sheath gas (N_2) flow rate 12 L min^{-1} and temperature 350˚C.

Analysis of 22 AA derivatives was carried out using analytical column Agilent Zorbax RRHD SB-C18 (2.1 × 50 mm, 1.8 μm) with inline filter 0.3 μm (Agilent). Eluent components were (A) 0.1% formic acid in water and (B) acetonitrile (with 4% Milli-Q water). The following gradient of (B) was used at flow rate 0.4 ml min^{-1}: 0–1 min 10%, 1–1.5 min 10–15%, 1.5–4.5 min 15%, 4.5–6.5 min 15–35%, 6.5–11 min 35%, 11–12 min 35–100%. Injection volume was 2 μL. The MS parameters for each analyte are presented in Table 5.

Table 1. Optimized values of APCI parameters. Final values different from the default values are marked in bold.

Parameter	**DBEMM**	**DNS**	**DEEMM**	**FMOC-Cl**
Corona needle voltage (V)	4000	4000	**3500**	4000
Nebulizer gas pressure (psi)	60	**50**	**50**	60
Drying gas flow (L min^{-1})	**5**	**5**	**5**	**5**
Drying gas temp (ºC)	**325**	**325**	350	350
Vaporizer gas temp (ºC)	**350**	**350**	**350**	400

Table 2. Chromatographic methods for LC-ESI-MS analysis of 7 AAs on XCT instrument.

		DEEMM	TAHS	FMOC-Cl	DNS	FOSF
Papers I, II, III	**Column**	Synergi Hydro-RP 80A (4.60 mm × 250 mm, 4 µm) (Phenomenex), guard cartridge (4.0 mm × 2.0 mm, polar endcapped C18) (Phenomenex)		Eclipse XDB-C18 (4.6 mm × 250 mm, 5 µm) (Zorbax), guard column (4.6 mm × 12.5 mm, 5 µm) (Agilent)		Eclipse XDB-C18 4.6 mm × 150 mm, 5 µm, guard column (4.6 mm × 12.5 mm, 5 µm (Agilent)
	Eluent composition	(A) acetate buffer (B) acetonitrile		(A) 0.1% formic acid (B) acetonitrile		(A) 1 mM ammonium bicarbonate (pH=7) (B) acetonitrile
	Flow rate	0.9 ml min^{-1}	0.8 ml min^{-1}	0.8 ml min^{-1}	0.8 ml min^{-1}	0.8 ml min^{-1}
	Gradient (% B)	0–12 min 20–25%, 12–20 min 25%, 20–50 min 25–60%	0–30 min 5–35%	0–45 min 30–100%	0–45 min 10–100%	0–20 min 20–40%
	Injection volume	5 µL	5 µL	10 µL	5 µL	5 µL
Paper V	**Column**	**DEEMM**	**DBEMM**	**FMOC-Cl**	**DNS**	**FOSF**
	Column; flow rate	Zorbax Eclipse XDB-C18 (4.6 × 150 mm, 5 µm, Agilent) with guard column (4.6 mm × 12.5 mm, 5 µm, Agilent); 0.8 ml min^{-1}				
	Gradient (% B)	0–18 min, 25–70%	0–18 min, 45–70% B	0–20 min, 30–100% B	0–20 min, 20–90% B	0–18 min, 15–40% B
	Injection volume	5 µL				

Table 3. Retention times (t_R (min)) of derivatives of 7 AAs

	Agilent 1100 system XCT [Papers I and III]					Agilent 1290 system QqQ [Paper V]				
	DEEMM	**DNS**	**FMOC-Cl**	**FOSF**	**TAHS**	**DEEMM**	**DNS**	**FMOC-Cl**	**FOSF**	**DBEMM**
Arg	14.2	15.2	13.0	14.0	14.8	2.6	5.6	5.3	4.2	2.9
Asp	23	-[a]	19.8	9.0	15.6	4.1	7.9	7.9	5.8	7.1
Gly	27.5	22.0	22.1	13.5	15.2	5.2	9.4	9.5	7.8	9.3
β-Ala	31.4	23.1	22.5	12.8	17.3	5.7	10.1	9.8	8.1	9.9
Pro	35.0	27.4	25.8	15.8	19.1	7.2	12.3	11.2	10.2	10.3
Trp	45.6	28.7	28.7	19.7	31.6	10.8	13.2	12.8	14.6	14.0
Phe	47.0	30.4	30.2	20.3	29.9	12.9	14.0	14.5	14.6	15.9

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

Table 4. Precursor and product ions (*m/z*) of 7 AA derivatives for LC-ESI-MS^2 and LC-APCI-MS^2 analysis with XCT.

	ESI						APCI			
	DEEMM	**DNS**	**DBEMM**	**FMOC-Cl**	**FOSF**	**TAHS**	**DEEMM**	**DNS**	**DBEMM**	**FMOC-Cl**
Arg	345→321	408→252	469→253	397→336	472→298	351→177	345→253 [a]*	408→170 [g]*	469→253 [e]	397→179
Asp	326→280	–	428→384	378→263	431→298	310→177	258→240 [b]	367→252 [h]*	428→384 [c]	356→179
Gly	268→222	309→252	392→284	320→263	373→298	252→177	200→156 [c]	309→170 [g]	370→326 [c]	298→179
β-Ala	282→236	323→252	406→254	334→263	387→298	266→177	214→170 [c]	323→252 [h]	384→276 [f]	312→179
Pro	308→262	349→252	410→302	360→263	413→298	292→177	240→196 [c]*	349→303 [i]	410→302 [f]	338→179
Trp	397→351	438→252	499→481	449→263	502→298	381→177	375→329 [d]	438→261 [j]	499→481 [b]	427→179
Phe	358→312	399→252	460→352	410→263	463→298	342→177	336→290 [d]	399→170 [g]	460→352 [f]	388→179

*The signal was not stable for quantitative analysis.
The leaving fragments are:
[a] two molecules of EtOH (M = 92); [b] H_2O (M = 18); [c] CO_2 (M = 44); [d] EtOH (M = 46); [e] two benzyl alcohols (M = 216); [f] benzyl alcohol (M = 108); [g] AA and SO_2; [h] DNS and H_2O; [i] H_2O and CO (M = 46); [j] fragmentation is unclear and needs further investigation.
For DNS-derivatives both *m/z* 252 [18] and *m/z* 170 [33] have been used in literature.

Table 5. Analysis parameters (retention times (t_R), *m/z* of precursor and product ions, collision energy (CE)) for alternating polarity mode LC-ESI-MS/MS analysis with QqQ.

AA	t_R	Positive ion mode		Negative ion mode	
		m/z	**CE (V)**	***m/z***	**CE (V)**
His	1.33	326 → 280	10	324→234**	10
Arg	2.18	345 → 299	10	343→299	10
Asn	2.37	303 → 257	10	301→257	10
Gln	2.69	317 → 271	10	315→271	10
Ser	2.79	276 → 230	10	274→184**	10
Asp	3.13	326 → 280*	10	302→258	10
Gly	4.10	246 → 200	10	244→154**	10
Thr	4.25	290 → 244	10	288→244	5
β-Ala	5.30	260 → 214	10	258→168**	5
GABA	6.11	296 → 250*	10	272→182**	5
α-Ala	6.33	260 → 214	10	258→168**	10
Pro	6.40	286 → 240	10	284→194**	10
Glu	6.43	318 → 272	10	316→272	5
Tyr	6.86	352 → 306	20	350→306	10
Met	7.59	320 → 274	10	318→274	10
Val	7.78	288 → 242	10	286→242	5
Trp	8.40	397 → 351	20	373→283**	10
Orn	8.88	473 → 427	10	471→427	15
Phe	8.98	336 → 290	10	334→290	5
Ile	9.08	302 → 256	10	300→256	10
Leu	9.34	302 → 256	10	300→256	10
Lys	9.95	509 → 463*	10	485→441	15

CE – collision energy

* In positive ion mode: the precursor ion corresponds to $[M+Na]^+$, unmarked corresponds to $[M+H]^+$.** In negative ion mode: the product ion corresponds to $[M-H-90]^-$, unmarked corresponds to $[M-H-44]^-$.

3.5. Matrix influence evaluation methods

3.5.1. Post-column infusion of pure derivatives [Papers III and V]

Qualitative evaluation of ME was carried out using the post-column infusion experiment setup shown on Figure 2. The pure standard of the derivative was infused while the derivatized sample was chromatographically analyzed. The two solutions were mixed using a T-piece and introduced to the API source. The solutions of pure standard of the derivative (DEEMM or FMOC-Cl) at two concentration levels (low: 40 μg g^{-1}; high: 1 mg g^{-1}) were infused using a syringe pump (0.0033 mL min^{-1}). Derivatized sample was analyzed chromatographically as blank sample does not exist. The chromatographic conditions described in section 3.4 were used for the chromatographic analysis. The MS/MS signal of the infused analyte was recorded.

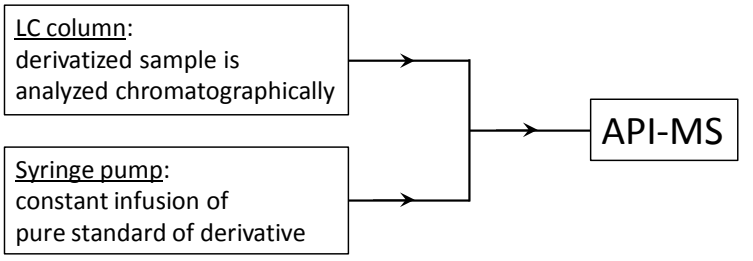

Figure 2. Setup of the post-column infusion experiments for LC-API-MS analysis involving derivatization.

3.5.2. Post-derivatization spiking [Paper II]

Three types of samples were prepared with DEEMM, DNS, FMOC-Cl and TAHS. First, a standard solution was derivatized and analyzed giving signal A_{standard}. Secondly, post-extraction spiking was carried out, meaning that blank matrix was spiked after sample preparation, but before derivatization and analysis resulted in signal A^{*}_{matrix}. Ratio of these signals yields *ME*%* (Eq. 2), which reflects both derivatization reaction yield and ionization ME, but not sample preparation recovery.

$$\boldsymbol{ME\%^{*} = \frac{A^{*}_{matrix}}{A_{standard}} \cdot 100\%} \qquad \textbf{Equation 2}$$

Thirdly, a post-derivatization spiking was carried out by mixing separately derivatized standard solution and sample matrix giving signal A_{matrix}, which is unaffected by the derivatization reaction yield. The resultant ME estimation

dME% (Eq. 3) takes into account the ionization ME without the influence of derivatization yield.

$$\boldsymbol{dME\%} = \frac{\boldsymbol{A_{matrix}}}{\boldsymbol{A_{standard}}} \cdot \mathbf{100\%} \qquad \textbf{Equation 3}$$

The relative yield of derivatization reaction can be calculated from the two ME values (Eq. 4).

$$\boldsymbol{Y_{rel}} = \frac{\boldsymbol{ME\%^{*}}}{\boldsymbol{dME\%}} \cdot \mathbf{100\%} \qquad \textbf{Equation 4}$$

3.5.3. Sample dilution approach for evaluating the ME [Papers III,IV,V]

In order to assess the influence of sample dilution on the ME, analyte concentration was evaluated in the sample extract (C_1) and its dilutions ($C_2 \ldots C_n$), resulting in respective back-calculated concentrations $C_{1(bc)} \ldots C_{n(bc)}$ (Eq. 5).

$$\boldsymbol{C_{n(bc)}} = \frac{\boldsymbol{C_n}}{\boldsymbol{d_n}} \qquad \textbf{Equation 5}$$

where n represents each dilution and d_n is the dilution factor calculated according to Eq. 6.

$$\boldsymbol{d} = \frac{\boldsymbol{m_{sample}}}{\boldsymbol{m_{total}}} \qquad \textbf{Equation 6}$$

Where m_{sample} is the mass of sample taken for dilution and m_{total} is the mass of extract after dilution.

In order to characterize the derivatization reagents in the present work, the sample dilution influence on the back-calculated concentration was assessed using relative pooled standard deviation (RSD) of these concentrations (Eq. 7).

$$\boldsymbol{RSD} = \frac{\sqrt{\frac{(\boldsymbol{C_{1(bc)}} - \boldsymbol{C_{mean}})^2 + \ldots + (\boldsymbol{C_{n(bc)}} - \boldsymbol{C_{mean}})^2}{\boldsymbol{n}}}}{\boldsymbol{C_{mean}}} \qquad \textbf{Equation 7}$$

This RSD describes the sample dilution influence on the quantitative analysis and a criterion was needed for making a decision about the presence of ME. SANTE validation guideline for pesticide analysis considers acceptable ME between 80–120% [100], which corresponds to RSD 16%. As there is no blank matrix available, the concentrations from undiluted and diluted samples were estimated and the RSD of concentrations (obtained from different dilution

factors) was chosen for this criterion. In addition, due to additional error from sample dilution and derivatization reaction reproducibility, the RSD above 20% was considered as indication of ME in the present work.

The decision about matrix influence on the analysis was made for each analyte and the results of these experiments were divided into three main cases as shown on Figure 3. In the first case, RSD was below 20% meaning that the sample matrix did not have any effect on the analysis. When RSD was above 20%, sample matrix influenced the analysis and two possible cases emerged. For cases where sample dilution eliminated matrix influence, a plateau of concentrations was reached (with RSD of at least 2 concentrations below 20%). The last case was when sample dilution decreased matrix influence, but a plateau of concentrations was not reached and extrapolative dilution approach [63] was used to evaluate the analyte's concentration. This was based on extrapolating the graph of analyte concentration dependence on dilution factor to infinitely small dilution factor, which corresponds to the matrix-free solution. In order to do that, a linear range on the graph (with at least three points) was needed.[63]

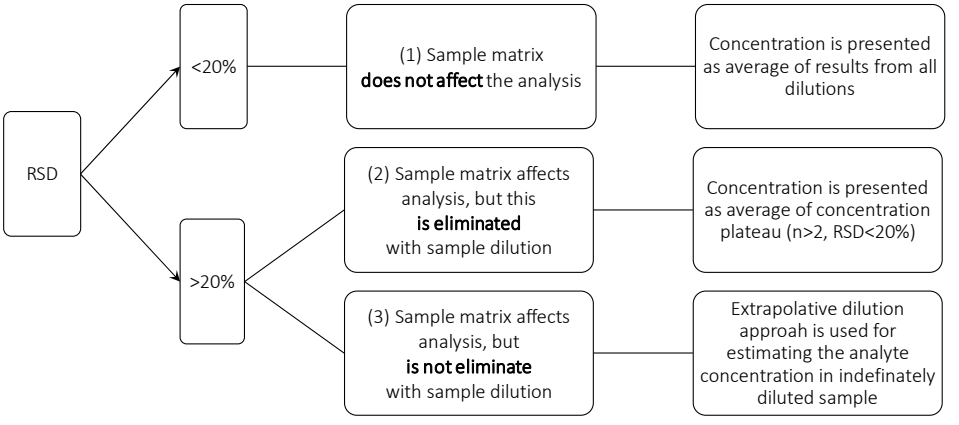

Figure 3. The analysis of results from sample dilution experiments.

4. RESULTS AND DISCUSSION

Matrix effect (ME), as a part of trueness, is one of the most important parameters for LC-MS method validation. Additionally, it can be used as a criterion for selecting derivatization reagents applied to LC-MS analysis. Since ME is not always evaluated for derivatization reagents and also new derivatization reagents have been developed in our work group, there are several reagents that need to be characterized and compared from ME point of view. Moreover, this information would be beneficial when designing new derivatization reagents aiming at sensitive and ME free LC-API-MS analysis.

Present work covers characterization of six AA derivatization reagents using standard solutions [Paper I] and samples [Papers II, III, IV, V] including different experimental setups. Three of these derivatization reagents have been originally developed for UV or FL detection, but have also been applied to LC-API-MS analysis: DEEMM, DNS and FMOC-Cl. Remaining three were novel derivatization reagents, which have been designed specifically for sensitive LC-ESI-MS analysis. Firstly, a derivatization reagent published in literature TAHS [33], and secondly, DBEMM [4] and FOSF [Paper I], which have been designed and synthesized in-house. A set of seven AAs was chosen as model analytes to represent the wide variety of AA properties: Arg, Asp, Gly, β-Ala, Pro, Trp and Phe. DEEMM was also applied for analysis of 22 AAs [Paper IV]. Applications covered 11 herbal extracts and five honeys.

The main results of this research are discussed starting from the method optimization, leading to the analysis of samples, evaluation of ME and finishing with aspects to keep in mind when designing novel derivatization reagents.

4.1. Method development

Firstly, optimization of derivatization procedure is needed for ensuring the trueness of the analysis and, secondly, LC-API-MS analysis needs to be optimized in order to achieve the highest sensitivity. Prior to analysis of samples and evaluation of ME, additional optimization of derivatization reactions in samples, chromatographic separation, ionization source and mass spectrometric parameters was carried out.

4.1.1. Optimization of derivatization procedures

The yield of DEEMM and FMOC-Cl procedural standards [Paper III]

For quantitative analysis using derivatization, there are in principle two types of standards which can be used: pure and procedural standards.[100] For AA analysis, the “pure standard” indicates the AA derivative without any additional compounds from the derivatization reaction, i.e. derivatization reagent itself, buffer salts or other components or products of the derivatization reaction. These can be either purchased or synthesized in-house. In the present work, the “procedural standard” stands for the standard solution of the derivative, which is

prepared by derivatization of the analyte standard solution. Hence, procedural standards can contain excess of the derivatization reagent, by-products of the derivatization reaction and other components necessary for the reaction, e.g. buffer components. Preparing standard solutions according to the same derivatization procedure as samples assures that the derivatization reaction yield is as similar as possible in standards and samples.

In this work, pure standards of Gly-, β-Ala- and Phe-FMOC were obtained commercially and pure standards for respective DEEMM-derivatives were synthesized in-house. These were used for verifying the derivatization yield of DEEMM- and FMOC-Cl procedural standards in order to confirm the reliability of the quantitative analysis. The calibration graphs based on the UV signals of pure standards were used to calculate the AA concentrations detected in procedural standards. In this case, the UV detection has an advantage over API-MS, as it is not influenced by the ME.

The average derivatization yield in standard solutions for three derivatives (n=6 for each derivative) was 109–117% for DEEMM-derivatives and 104–116% for FMOC-derivatives. Derivatization yield higher than 100% could be explained by possible impurities in pure standards synthesized in-house or the degradation of pure derivatives. In general, the recovery was close to 100% with RSD below 11% for all cases, meaning that the derivatization yield is not a problem for procedural standards and concentration of the standard solutions can be used for quantification.

In the present work, procedural standards were used for quantitative analysis with all the derivatization reagents. This is the general approach and especially relevant for MS detection, due to possible influences caused by other components in the derivatization mixture. Moreover, preparing the standard solutions as similarly as possible to the samples may compensate for some of the ME.[100]

Optimization of derivatization reactions in samples [Papers I, II, III]

The present work was focused on estimating the ME, but both recovery and ME contribute to the overall process efficiency of analysis. In order to avoid analyte loss during sample preparation, two sample types with high recovery sample preparation methods were chosen. First sample was honey, for which the sample preparation has high AA recovery and has been previously validated in our work group.[78,101] Second type of samples were green tea and herbal extracts, which were prepared using a simple extraction procedure without additional purification steps (section 3.3).

The complexity of derivatization procedure is also of high importance for characterizing the derivatization reagents. As the analyte concentration and sample matrices vary to a great extent, it was important to optimize the derivatization procedure for samples. Contrary to standard solutions, samples contain other components beside analytes. If these can also react with the derivatization reagent, it might result in deficiency of the derivatization reagent or longer reaction time.

The optimal derivatization procedures for samples are summarized in experimental section 3.3. It was concluded that derivatization of samples with FMOC-Cl needed longer reaction time (30 min) compared to standard solutions. Sample derivatization with TAHS needed both higher concentration and longer reaction time (30 min) and derivatization with FOSF was carried out similar to TAHS. This shows that these derivatization procedures are not robust across different matrices and need to be optimized. The derivatization with DNS was suitable for selenoamino acid analysis in onion and was minimally modified for green tea and honey as lower DNS concentration was suitable for green tea.

The original derivatization procedure with DEEMM was suitable for AA analysis in honey and selenoamino acid analysis in onion. In case of DEEMM, the derivatization reagent is added in large excess and reaction takes place immediately, only Pro needs reaction time of 24 h. For reducing the necessary sample size for derivatization from 1 mL as in Ref. [78] to 250 µL, the amount of all reagent solutions were proportionately reduced 4 times, without any effect to quantitative performance.

DBEMM derivatization has been optimized for onion and honey. It reacts within minutes with most AAs, only Pro needed 2 h reaction time. The fast reaction and satisfactory repeatability for Pro has made it possible to use automated derivatization.[94]

In general, derivatization procedures of DNS, DEEMM and DBEMM could be suitable for a wide range of applications. Moreover, as ending the reaction is not essential for DEEMM and DBEMM, these reactions are carried out in one step.

4.1.2. Optimization of chromatographic analysis

Chromatographic separation of the analyte peak from other matrix components can influence the analysis by causing ME. In order to minimize the possibility for such influences, it is important to achieve sufficient chromatographic separation. In the present work, the separation for seven AAs was aimed at, mostly so that the analytes would elute at a retention time far enough from the hold-up time in order to reduce the possible ME coming from early eluting compounds on RP, e.g. buffer components.

Eluent composition and chromatographic separation was thoroughly optimized for LC-ESI-MS analysis with DEEMM, DNS, FMOC-Cl, TAHS and FOSF [Papers I and III]. Some additional optimization was needed due to using shorter chromatographic columns and evaluating suitability of LC eluent composition with LC-APCI-MS [Paper V].

In addition to chromatographic separation, mobile phase composition influences the IE and optimal conditions can vary for different ionization sources. In this work, APCI was used for the first time for the analysis of AA derivatives of these reagents and a shorter column compared to previous experiments was used. Therefore, the optimization of mobile phase composition was needed and different combinations of two organic components (methanol and acetonitrile) and two aqueous phases (0.1% formic acid and ammonium acetate buffer (pH = 3.2)) were

tested. Acetonitrile was used for all derivatization reagents since it provided good chromatographic separation and the highest signal intensities (Figure 4).

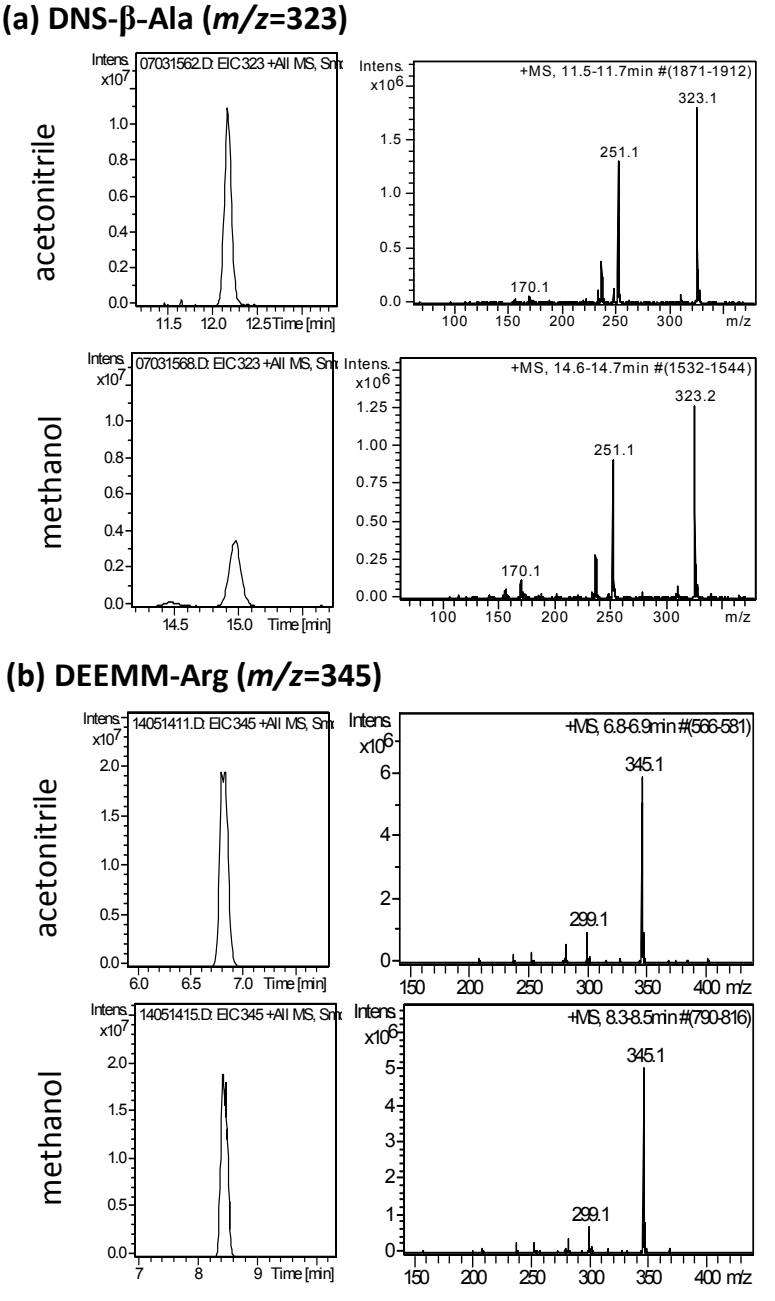

Figure 4. Comparison of organic solvent effect on signal intensity. Extracted ion chromatograms and APCI-MS spectra in case of acetonitrile and methanol as eluent components with ammonium acetate buffer for (a) DNS-β-Ala and (b) DEEMM-Arg.

Combinations of the two aqueous phase components (0.1% formic acid and ammonium acetate) with acetonitrile resulted in equal signals, but formic acid resulted in lower signals only for DBEMM. Consequently, the combination of acetonitrile and aqueous ammonium acetate buffer was used for further analyses with all the derivatization reagents. Detailed descriptions of the chromatographic methods are presented in Experimental section 3.4 in Table 2, the retention times of seven AAs are presented in Table 3 and for 22 AAs with DEEMM in Table 5.

4.1.3. LC-ESI-MS methods [Papers I–V]

LC-ESI-MS experiments in this work were carried out on two different mass spectrometers, ion trap (XCT) [Papers I, II, III and V] and triple quadrupole (QqQ) types [Paper IV], which both used different LC systems. The *m/z* values of precursor and product ions used with XCT are in Table 4 in Experimental section 3.4.

ESI-MS analysis in positive ion mode using XCT ion trap mass spectrometer was optimized and validated for analysis of seven AA standard solutions for DEEMM, FMOC-Cl, DNS, TAHS and FOSF [Paper I] and DBEMM [94]. DNS, TAHS and FOSF ionize through protonation and neutral AA is lost during fragmentation. Both protonated derivatives and Na-adducts were used as precursor ions for FMOC-Cl, DEEMM and DBEMM derivatives' detection.

The QqQ method development for DEEMM derivatization consisted of optimizing the ESI-MS parameters for 22 AAs in positive and negative ion mode. In positive ion mode, both protonated and Na-adducts of DEEMM-derivatives were fragmented in order to find the optimal transition. The product ions were formed by loss of a neutral fragment of 46 amu from the derivatization reagent part (CH_3CH_2OH, M = 46 g mol^{-1}). In some cases, in MS spectra Na-adduct yielded higher signal than protonated form, but fragments of protonated derivative resulted in higher MS/MS signal than fragments of the Na-adduct. As a result, Na-adducts were suitable precursor ions for Asp, GABA and Lys, while protonated derivatives were used for other AAs (Table 5, in section 3.4). In general, protonated precursor ions proved to be better suited for MS/MS analysis. This was attributed to the eluent composition, as the ammonium acetate additive contributes to the stability of Na-adduct signal [89], but formic acid was used in this work.

In negative ion mode, LC-ESI-MS analysis of DEEMM-derivatives was carried out for the first time. Analysis with QqQ instrument used precursor ions which had formed via loss of proton ($[M-H]^-$) and two kinds of product ions were produced during fragmentation. Firstly, carbon dioxide (CO_2, M = 44 g mol^{-1}) was lost from the AA part of the derivative resulting in product ion $[M-H-44]^-$. This is characteristic to AA derivatives with other reagents, also.[87] Secondly, $[M-H]^-$ fragmented to give product ion $[M-H-90]^-$ corresponding to loss of both CO_2 and ethanol (M=46 g mol^{-1}). Product ion which

had lost only ethanol, like in positive ion mode, was not observed. The selection of precursor and product ions was carried out simultaneously with optimization of collision energies (Table 5 in section 3.4).

4.1.4. LC-APCI-MS methods [Paper V]

AA analysis with these derivatization reagents was carried out with LC-APCI-MS for the first time. Due to the different ionization mechanisms compared to ESI, differences in the mass spectra can occur and the ionization and fragmentation needs to be determined. APCI-MS parameters were optimized for DEEMM, DBEMM, DNS, FMOC-Cl and FOSF derivatives of seven AAs.

First, a novel derivatization reagent designed for LC-ESI-MS, FOSF, was tested, but a stable signal was not obtained with APCI. One reason could be the permanent positive charge on the derivatives, as similar problems have been seen before for permanently charged pesticides.[6] Therefore, FOSF was not considered a suitable derivatization reagent for APCI applications and could not be used for further experiments.

DEEMM, DBEMM, FMOC-Cl and DNS proved to be suitable for APCI and *m/z* of the precursor and product ion of their derivatives are presented in Table 4 in section 3.4. Protonated derivatives were observed for all of the analytes, e.g. DEEMM-β-Ala in Figure 5, and low signals of Na-adducts were observed for some DEEMM-derivatives (less than 8% of the signal of protonated form). The presence of Na-adducts shows that mixed mode ionization can occur in APCI and a part of the analyte molecules are ionized similarly as in ESI [28].

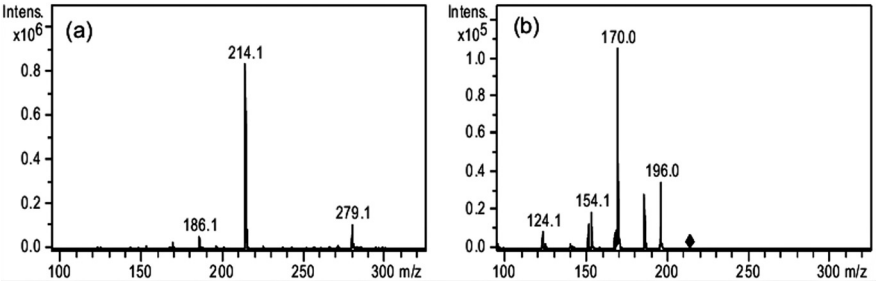

Figure 5. Example of (a) ionization (MS) and (b) fragmentation (MS^2) spectra of DEEMM- β -Ala in case of APCI (MS^2 precursor ion *m/z* = 214). Note that Na-adduct is absent (*m/z* = 282) in MS spectrum (a).

The protonated derivatives were used as precursor ions for all DBEMM-, DNS- and FMOC-derivatives. For DEEMM, the precursor ions were either protonated forms or the protonated derivatives, which had already undergone fragmentation by loss of ethanol fragment $[M+H-46]^+$. The fragmentation of DEEMM-derivatives in the ion source did not depend on the ion source parameters (e.g. vaporizer temperature) and was considered characteristic to APCI ionization.

For all FMOC-derivatives, product ion with *m/z* 179 was used.[32] DNS derivatives ionized through protonation, but fragmentation differed depending on the analyte. All fragmentations are described in Table 4 in section 3.4. The signals of DNS-Asp and DEEMM-Pro [18], DEEMM-Arg and DNS-Arg were not obtained for quantitative analysis with APCI.

Optimal ionization source and mass spectrometer parameters are necessary for sensitive analysis. Optimizing the parameters using constant infusion with effluent flow and the software of the instrument was used for pure derivatives of FMOC-Cl and DEEMM. For other reagents and analytes, the following APCI parameters were optimized by focusing on higher LC-MS signal: corona needle voltage, nebulizer gas pressure, drying gas flow, drying gas temperature and vaporizer gas temperature (Table 1 in section 3.4). Results showed that APCI is robust to changing the parameters and from these parameters, only drying gas influenced the results as lower value resulted in 2-times higher signals. The lower optimal drying gas flow could be connected with the additional vaporizer gas temperature compared to ESI source.

The optimized parameters were used for evaluating the repeatability and lower limit of quantitation (LLOQ, expressed as the lowest concentration of linear calibration graph). The RSD of repeatability was under 15% for all detected analytes (n = 3, at concentration 50 pmol on column). From the LLOQ point of view (Table 6), ESI and APCI were comparable in case of DEEMM and DNS, LLOQ-s were more than 10 times lower with ESI for FMOC-Cl and DBEMM. An example of APCI spectra at LLOQ is presented on Figure 6. As a result, LC-APCI-MS methods suitable for quantitative analysis with four derivatization reagents were obtained: DEEMM, DBEMM, DNS and FMOC-Cl. These methods were used for further analysis in order to determine the matrix influence.

Table 6. The values of lower limit of quantitation (LLOQ) in pmol on column.

Amino acid	**DEEMM**		**FMOC-Cl**		**DNS**		**DBEMM**	
	APCI	**ESI**	**APCI**	**ESI**	**APCI**	**ESI**	**APCI**	**ESI**
Arg	- [b]	0.7	9.4	1.1	- [b]	1.3	5.9	0.4
Asp	25	29	43	4.7	- [a]	- [a]	5.2	1.7
Gly	0.8	2.4	73	3.5	0.2	0.5	3.9	0.1
β-Ala	0.9	17	38	0.6	8.2	0.3	2.0	0.1
Pro	- [a]	- [a]	15	0.5	0.7	0.7	5.4	0.2
Trp	1.6	1.6	2.4	0.3	1.6	2.7	2.7	0.3
Phe	0.9	4.1	28	2.0	0.4	0.8	7.7	1.0

[a] – stable signal was not obtained for both ESI and APCI
[b] – stable signal was not obtained with APCI

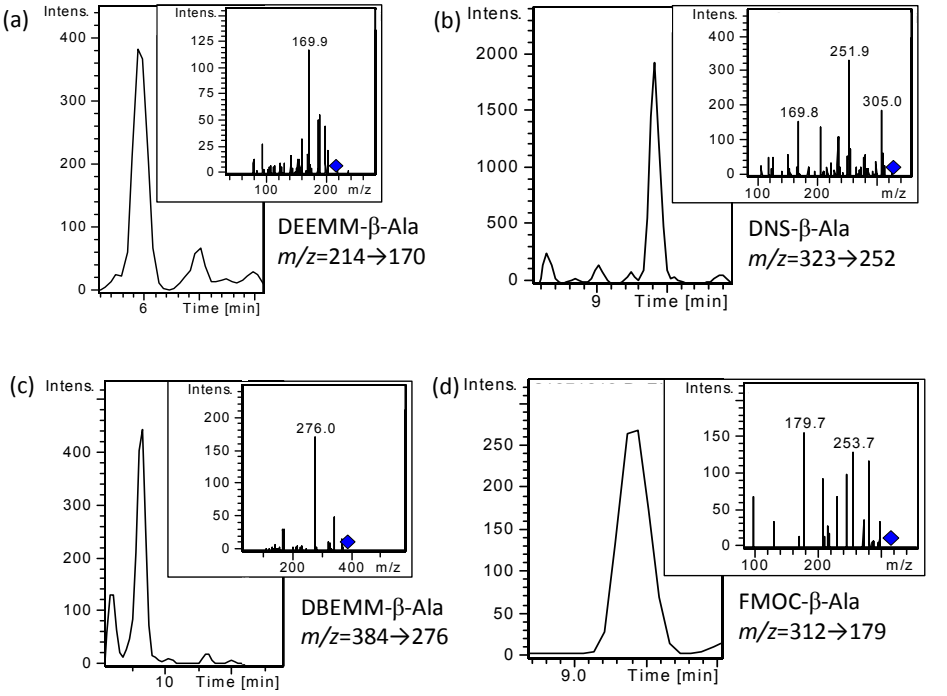

Figure 6. Examples of extracted ion chromatograms and APCI-MS^2 spectra of β-Ala derivatives at LLOQ for (a) DEEMM, (b) DNS, (c) DBEMM and (d) FMOC-Cl.

4.2. Evaluating ME – post-column infusion

The characterization of derivatization reagents from ME point of view using several approaches was in the focus of this work. Firstly, qualitative evaluation of ME was carried out using post-column infusion experiments with green tea extract using ESI and APCI (described in section 3.5.1). Since this approach needs pure standard of the analyte, only DEEMM and FMOC-Cl were used as the pure derivatives of FMOC-Cl are commercially available and were synthesized in-house for DEEMM.

This experiment also requires blank matrix, which is not available for AA analysis in green tea and a modification from the original post-column infusion experiment was used. Three types of derivatized samples were chromatographically analyzed, while the pure derivative was infused and its MS/MS signal was monitored. These samples were (1) derivatized deionized water as a matrix and analyte free sample, (2) derivatized AA standard solution and (3) derivatized green tea extract. Different samples were required in order to distinguish between the origins of possible ME sources.

The difference from original post-column infusion is that the infused analyte is also present in the chromatographically analyzed sample (samples 2 and 3). Therefore, the analyte's signal from the chromatographically analyzed sample

adds to the signal of the infused analyte. This results in a peak on the extracted ion chromatogram at the retention time of the analyte rendering it impossible to evaluate the presence of ME in that point, which would be most valuable. Regardless, the general influence of ME on the analysis could still be characterized, especially if it is caused by the derivatization mixture components. The results of post-column infusion experiments are discussed separately for ESI and APCI in the following chapters.

4.2.1. Post-column infusion experiments with ESI-MS [Paper III]

The post-column infusion experiments with ESI-MS showed that signal suppression was present for both FMOC- and DEEMM-derivatives. However, the nature and cause of ME were different. In case of FMOC-Cl, several signal suppression areas in the extracted ion chromatograms of infused FMOC-derivatives were observed when samples (1)–(3) were chromatographically analyzed (Figure 7).

Comparison of the result of all three infused FMOC-derivatives allowed to draw the following conclusions. Firstly, a suppression area was observed at the FMOC-histidine retention time meaning that FMOC-histidine was the cause of strong signal suppression in all the derivatized samples. Histidine is added to the reaction mixture (up to 1 mg mL^{-1} in the derivatized sample) in order to end the derivatization reaction and consequently it is present in all derivatized samples. Signal suppression was the strongest in analyte free sample (1) (Figure 7b*), as there are no analytes which could react with FMOC-Cl. Consequently, more FMOC-histidine is formed due to the higher concentration of unreacted FMOC-Cl compared to samples containing AAs (as in case of samples (2) and (3)).

Secondly, additional suppression areas emerged when derivatized AA standard solution (2) and tea samples (3) were chromatographically analyzed (Figure 7b,c). The retention times of these suppression areas matched with the retention times of FMOC-derivatives of AAs in the samples (Figure 7a). It was concluded that other derivatized AAs and potentially also amines can cause ME for FMOC-derivatives. This is an important result, as derivatized matrix components can cause ME only during analysis of derivatized samples and these cannot be eliminated prior to the derivatization step. Furthermore, when comparing different infused FMOC-derivatives, it was observed that the signal of infused FMOC-Phe was less influenced than the signals of FMOC-Gly and -β-Ala, emphasizing that ME also depends on the structure of the AA part of the derivative.

In conclusion, in case of FMOC-Cl derivatives, ME caused by the derivatized compounds is similar to any ME caused by the co-eluting compounds: it cannot be easily reduced or eliminated and it needs attention during method development and validation. Additionally, the ME caused by other FMOC-derivatives

highlights the need for chromatographic separation in order to reduce the possible overlapping of the peaks causing ME with the analyte. Hence, chromatographic separation is essential even with MS detection.

The results of post-column infusion experiments with DEEMM-β-Ala were significantly different from FMOC-derivatives. All of the chromatographically analyzed samples, (1)–(3), caused the signal suppression of the infused DEEMM-β-Ala starting from the hold-up time at 3 min until about 31 min (Figure 7d). The cause could be the borate buffer which is used for both FMOC-Cl and DEEMM derivatization. The signal enhancement of FMOC-derivatives and suppression of DEEMM-derivatives due to the influence of borate buffer has been previously shown by our work group.[89,102] The considerable influence of borate buffer in case DEEMM could be due to 2.3 times higher relative concentration of the borate buffer in the derivatized sample compared to FMOC-Cl. Differently from ME caused by FMOC-derivatives, this type of general ME can be eliminated or reduced, e.g. by changing the type of buffer used or diverting first minutes of the chromatographic run into waste instead of the ESI source so that the compounds causing the suppression would contaminate the source [89].

In order to obtain more information about the nature of these signal suppressions, post-column infusion experiments were carried out at different concentrations of the infused analyte. Results showed that ME depends on the concentration of the analyte: the higher the concentration of analyte, the less ME was present in case of both derivatization reagents. This is in line with literature results – the increased analyte-to-matrix ratio can decrease ME [103] due to competition for excess charge between analyte and matrix compounds [104].

To sum it up, LC-ESI-MS analysis with both DEEMM- and FMOC-derivatives was affected by the sample matrix. Compounds causing ME emerge during derivatization for both DEEMM- and FMOC-derivatives, but the cause of the ME was different. In case of FMOC-Cl, matrix components having amine functional group react with FMOC-Cl and those derivatives cause ME. In case of DEEMM derivatives, the signal suppression was caused by the derivatization mixture itself, rather than the sample matrix components.

4.2.2. Post-column infusion experiments with APCI-MS [Paper V]

The post-column infusion experiments with APCI presented different results compared to ESI. In case of FMOC-derivatives, only the signal suppression at the hold-up time and at the retention time of FMOC-His (Figure 7e) were observed. This means that the signal suppression due other AA derivatives in the analyzed sample was not an issue during APCI ionization or the concentrations in chromatographically analyzed samples were not high enough for causing ME in case of APCI.

In contrast to results of DEEMM-derivatives with ESI, the signal suppression due to borate buffer was not present for APCI (Figure 7f). This means that borate buffer can be used for APCI applications without the need for any techniques to reduce possible ME.

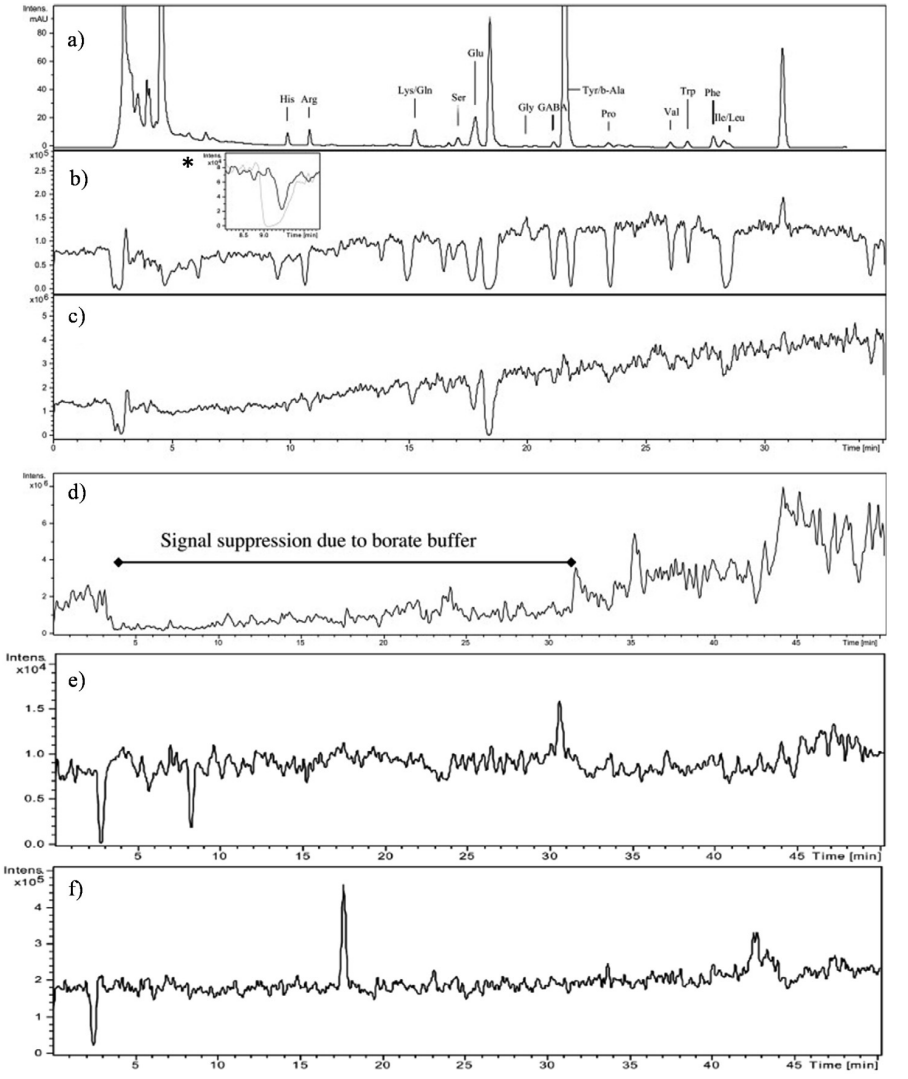

Figure 7. Post-column infusion experiments with ESI and APCI. (a) UV-chromatogram of tea derivatized with FMOC-Cl. Analysis in ESI positive mode: (b) and (c) are extracted ion chromatograms of infused FMOC-Gly at two concentration levels of pure standard ((b) 40 μg g^{-1} and (c) 1 mg g^{-1}) and (d) DEEMM β-Ala (40 μg g^{-1}) while the effluent from chromatographic analysis of derivatized tea (with respective derivatization reagent) is added. Analysis in APCI positive mode: (e) infused FMOC-Phe and (f) infused DEEMM-β-Ala (43–48 μg g^{-1}) when derivatized seven AA standard solution is analyzed chromatographically. In addition, (b)* shows FMOC-Gly signal when the derivatized water (grey) and sample (black) was analyzed.

Overall, the results of post-column infusion experiments show that there is a need for ME evaluation when using derivatization and it can depend on the derivatization reagents and ionization sources used. The qualitative experiments indicate that APCI is less prone to ME compared to ESI in case of FMOC-Cl and DEEMM. The advantage of the post-column infusion method is that it provides qualitative evaluation of ME over the entire chromatogram. However, quantitative evaluation of the matrix influence for each analyte is also needed, especially if the method is validated.

4.3. Evaluating ME – derivatization and ionization ME [Paper II]

The most widely used quantitative method for evaluation of ME is post-extraction spiking. In case of derivatization, this method results in ME (*ME*%*), which includes both the derivatization reaction yield and the ionization ME. A new method was proposed by our work group – post-derivatization spiking – which allows quantitative evaluation of ME without being affected by the derivatization reaction yield. The derivatization ME (*dME*%) is calculated as the ratio of the signal of derivatized analyte spiked into derivatized matrix to the signal of the derivatized analyte in standard solution, so that the analyte concentrations in both samples are equal. From the two ME values, *ME*%* and *dME*%, the relative yield of derivatization reaction can be calculated using Eq. 2–4 in Experimental section 3.5.2.

Comparison of four derivatization reagents (TAHS, DEEMM, DNS and FMOC-Cl) for analysis of two selenoamino acids in onion extract showed that the derivatization yield of FMOC-Cl, DNS and TAHS derivatization reactions were influenced by onion sample. In addition, no signal suppression was observed for TAHS and DEEMM. Although this ME evaluation method requires blank matrix and is therefore not suitable for all applications using derivatization, the results of two selenoamino acids emphasized the differences of derivatization reagents and the need for research from the ME point of view on a wider scale, i.e. more analytes, matrices and ion source variations.

4.4. Evaluating ME – sample dilution

Sample dilution experiments were applied for investigation of the matrix influence on analysis using derivatization, when neither blank matrix nor pure standards are available. The sample dilution influence was first evaluated on XCT mass spectrometer for seven AAs in green tea and honey samples with ESI and in green tea samples with APCI.

Sample dilution experiments, described in Experimental section 3.5.3, resulted in three main cases and examples of AA derivatives are presented in Figure 8. First case (Figure 8a) shows that sample dilution had no effect on the

results obtained as dilution factor does not influence the final concentrations (RSD < 20%). The second case (Figure 8b), where RSD was initially above 20% shows that after a certain dilution factor a plateau was reached, indicating that ME was eliminated (RSD < 20%). In the third case (Figure 8c), the RSD was above 20% and with every dilution a larger back-calculated concentration of the analyte was obtained, but no plateau was achieved, although the limit of quantitation has been reached and no further dilution is possible. In the latter case, the analyte concentration in the sample was evaluated using the extrapolative dilution approach [63] (Figure 8d).

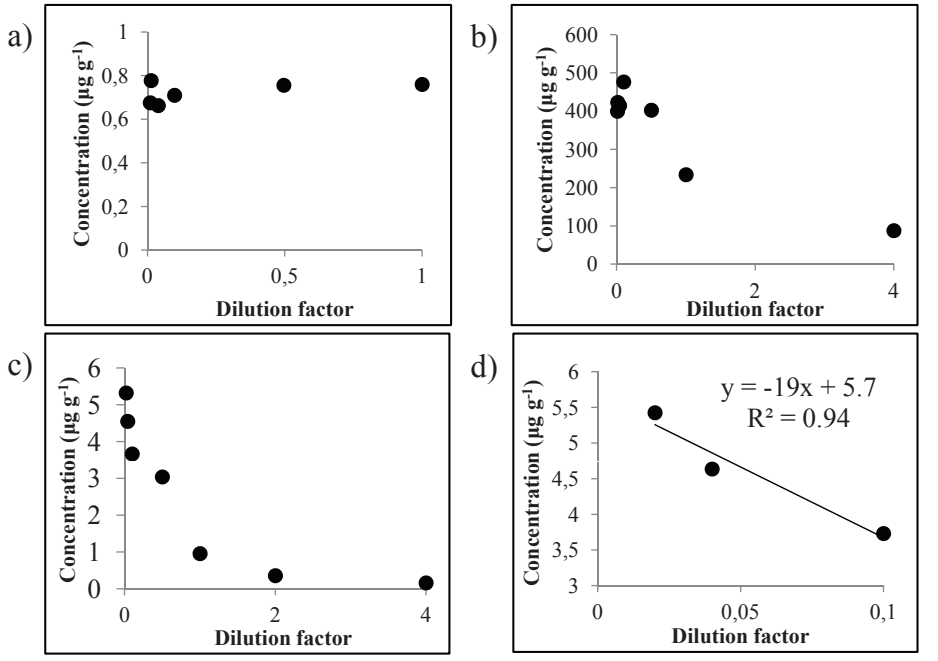

Figure 8. Examples of sample dilution approach for AA derivatives: (a) sample dilution had no significant influence on DNS-Pro in green tea; (b) sample dilution influenced analysis but a plateau of concentrations was reached for TAHS-Pro in honey; (c) strong ME on FMOC-β-Ala in honey and (d) extrapolative dilution approach was applied.

4.4.1. ME during LC-ESI-MS analysis of green tea and honey [Papers III and V]

For investigating the ME during LC-ESI-MS analysis, green tea and honey samples were derivatized with DEEMM, DNS, FMOC-Cl and TAHS and green tea with DBEMM and FOSF. The results with FOSF have not been published. Concentrations of seven AAs were determined in the original sample extract and five of its dilutions (up to 100-fold). Results showed that the concentrations obtained in original undiluted sample extract with different derivatization

reagents did not agree. Therefore, it was necessary to find conditions where the concentrations would agree and then evaluate the real concentrations in the samples. Adding SPE purification step to green tea extract sample preparation did not improve agreement between different reagents, implying that diluting the samples was necessary (Figure 9). The results are discussed below for all of the derivatization reagents.

The analysis of green tea and honey (Figure 9a,b,c) showed that most DNS-derivatives were affected by sample matrix in green tea, as RSD exceeded 50%, and was below 20% only for few AAs (Pro and Trp or Gly and β-Ala). Up to 50-fold dilution was needed to eliminate ME and extrapolative dilution approach was used for Arg, Gly and Phe. Analysis of honey was less influenced by the dilutions and ME was eliminated by sample dilution (a plateau was reached) for all analytes.

Analysis of green tea with FMOC-Cl was free from ME for Arg, Pro and Phe (RSD was below 20%), but extrapolative dilution approach was used for Asp, Gly and Phe. The matrix influence was stronger in honey and was eliminated by sample dilution for Asp and Trp derivatives, which means that extrapolative dilution approach was used for other derivatives. In comparison with DNS-derivatives, matrices appear to influence different AA derivatives to a different extent.

The analysis using DEEMM was least affected by the sample matrix. Depending on samples and chromatographic separation, RSD was below 21% for all AA derivatives or up to 4-fold dilution was needed for eliminating any matrix influences. In general, less influence from sample matrix could be associated with the stronger retention of DEEMM-derivatives on RP-LC compared to other reagents. This leads to better chromatographic separation from matrix components. As a result, there are less matrix components, which could co-elute and cause ME. Note that the influence of borate buffer (section 4.2.1) was accounted for as both the analyzed samples and the procedural standards used for calibration contained borate buffer.

From the novel derivatization reagents, analysis of green tea with TAHS was free from ME (RSD below 20%) for Asp, Trp and Phe, a plateau was reached for β-Ala and extrapolative dilution approach was used for Arg, Gly and Pro. Similarly to FMOC-Cl, honey matrix (especially the most concentrated sample) had stronger influence and sample dilution was necessary for all AAs. Analysis of green tea with FOSF showed less ME compared to TAHS and sample dilution eliminated ME in all cases. The influence of green tea matrix on DBEMM analysis showed that Gly- and β-Ala-derivatives were not affected by the ME. Sample dilution was needed for eliminating ME in case of Trp- and Phe- (2-fold), Arg- and Pro- (4-fold) and Asp-derivatives (20-fold).

In conclusion, amino acid derivatives differ markedly with respect to their susceptibility to matrix effects. Derivatization reagents exhibiting lower ME should be preferred for the applications where complex matrices are analyzed.

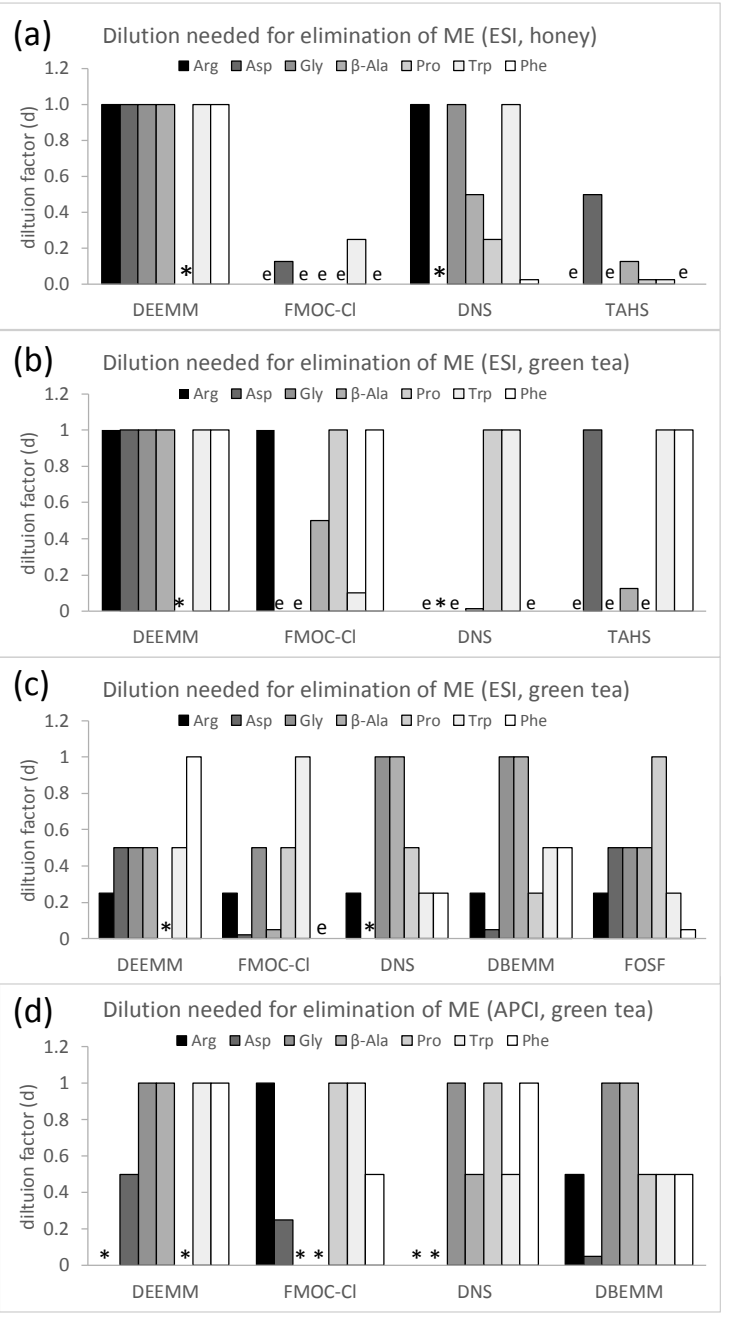

Figure 9. Dilution factors needed for elimination of ME in case of (a) honey and (b) green tea with ESI [Paper III] and green tea with (c) ESI and (d) APCI [Paper V]. The dilution factor 1 corresponds to undiluted sample (Eq. 6), "e" marks the cases when ME was not eliminated by sample dilution and extrapolative dilution approach was used for evaluating the analyte concentration, * – the analytes for which signals were not stable for quantitative analysis.

Agreement of concentrations obtained with four derivatization reagents [Paper III]

It was concluded that different derivatization reagents have different ME for the same sample and dilutions are necessary for achieving higher trueness. In order to evaluate the success of eliminating the ME with sample dilution, agreement of the concentrations obtained with different ionization reagents was evaluated. The agreement between concentrations obtained with DEEMM, DNS, FMOC-Cl and TAHS (Table 7) was evaluated based on the RSD for each AA over all reagents for different matrices (Figure 10). The concentration of each AA in green tea extract was 0.87–57 μg g^{-1} and in honeys 0.44–508 μg g^{-1}.

For green tea, the results were within the RSD of 21% for Arg, Asp, Gly, Trp and Phe. For β-Ala better agreement was obtained (RSD = 11%) when results of TAHS were excluded. This stresses the fact that analytes at low concentrations could be more prone to ME [105] and that TAHS-derivatives are more influenced by the sample matrix. The agreement of Pro concentrations did not improve when excluding results obtained with different reagents. However, as Pro is a secondary amine and has reaction problems with DEEMM, this could be the possible cause for poor agreement of concentrations found in tea.

Table 7. Final AA concentrations in tea (μg g^{-1} in extract) and honey (μg g^{-1}) using dilution experiments for LC-ESI-MS.

		DEEMM	DNS	FMOC-Cl	TAHS	Average
Tea	**Arg**	9.7 (15%)	7.7[b] (1)	11 (18%)	13[b] (0.84)	10 (21%)
	Asp	53.8 (17%)	–	70[b] (0.97)	48 (9%)	57 (20%)
	Gly	0.85 (13%)	1.0[b] (0.79)	0.80[b] (0.99)	0.83[b] (0.82)	0.87 (10%)
	β-Ala	0.36 (7%)	0.42[a] (11%)	0.40[a] (17%)	6.8[b] (0.82)	2.0 (161%)
	Pro	–	0.77 (18%)	3.2 (16%)	6.0[a] (14%)	3.3 (78%)
	Trp	5.9 (20%)	4.2 (14%)	4.6[a] (3%)	4.3 (22%)	4.7 (17%)
	Phe	8.0 (11%)	7.6[b] (1)	7.3 (14%)	6.6 (12%)	7.4 (8%)
Honey	**Arg**	5.3 (15%)	4.6 (17%)	5.5[b] (0.99)	6.2[b] (0.89)	5.4 (12%)
	Asp	11 (21%)	–	3.8[a] (14%)	15[a] (9%)	10 (57%)
	Gly	3.9 (20%)	6.1 (12%)	4.9[b] (0.99)	4.9 [b] (0.93)	4.9 (21%)
	β-Ala	7.5 (20%)	7.5[a] (12%)	5.7[b] (0.94)	8.7[a] (10%)	7.4 (17%)
	Pro	–	587[a] (21%)	529[b] (0.98)	408[a] (7%)	508 (18%)
	Trp	0.53 (18%)	0.52 (22%)	0.22[a] (4%)	0.43[a] (21%)	0.44 (32%)
	Phe	15 (19%)	16[a] (13%)	12[b] (0.95)	15[b] (0.99)	15 (10%)

[a] Estimated using plateau of AA concentration at lower dilution factors (RSD is presented).
[b] Estimated using extrapolative dilution approach (r^2 is reported in parenthesis).
All other concentrations were calculated as an average of all concentrations at all dilution factors (RSD is presented).

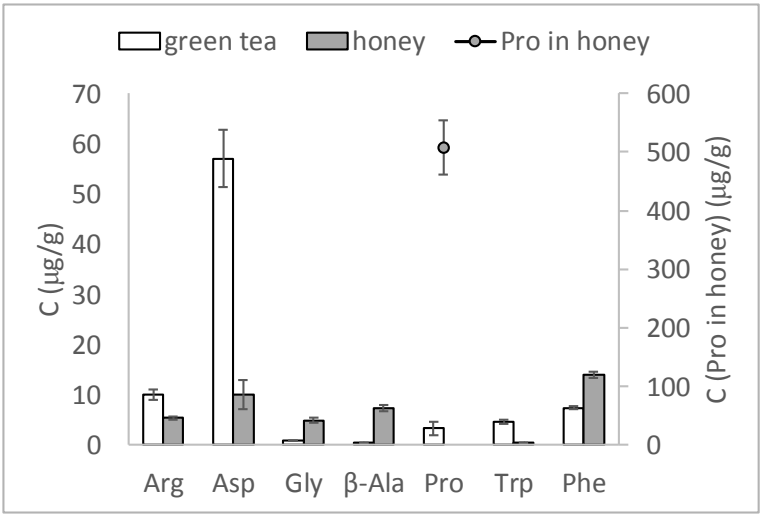

Figure 10. AA concentrations in green tea (µg g^{-1} in extract) and honey (µg g^{-1}) from LC-ESI-MS analysis. Average concentrations are obtained over concentrations obtained with four derivatization reagents (n = 4, except for β-Ala in tea with TAHS).

The agreement of results was similar for honey sample, where the RSD was below 22% for Arg, Gly, β-Ala, Pro and Phe, but was higher for Trp (32%) and Asp (57%). In general, analysis of honey with DEEMM and TAHS was less affected by the ME compared to the analysis using FMOC-Cl and DNS. The stronger ME for FMOC-Cl is in accordance with the post-column infusion experiments (chapter 4.2.1) which revealed several signal suppression areas for FMOC-derivatives. Concentrations obtained with FMOC-Cl were in many cases lower than with other reagents. This shows that although the analytes are chromatographically separated from each other, both matrices contain other AAs, amines or similar compounds which could cause ME.

The sample dilution experiment with ESI showed that DEEMM-derivatives were least affected by the ME, as sample dilution was not necessary. Analysis using DNS was more affected in green tea and FMOC-Cl and TAHS overall, but especially in the concentrated honey samples. For the latter three, sample dilution and extrapolative dilution approach was needed in order to eliminate the ME.

4.4.2. ME during LC-APCI-MS analysis of green tea [Paper V]

In order to evaluate ME for LC-APCI-MS analysis, green tea sample and its four dilutions (up to 50-fold) with DEEMM, DNS, DBEMM and FMOC-Cl were analyzed.

Results in Figure 9d show that some ME was present also for APCI analysis, but compared to ESI analysis, less dilution was needed to eliminate ME (final concentrations in Table 8). Similarly to results with ESI, concentrations

obtained with DEEMM were also least influenced in case of APCI analysis. The RSD was below 20%, except for Asp analysis, which needed 2-fold dilution in order to eliminate the ME. This also confirms the results of post-column infusion experiments which did not reveal any signal suppression areas for DEEMM-derivatives with APCI (section 4.2.2).

Table 8. The final concentrations of seven AAs in green tea extract (μg/g) with DEEMM, DNS, DBEMM and FMOC-Cl found using APCI and ESI (RSD < 20%, n = 2–11)

AA	DEEMM		FMOC-Cl		DNS		DBEMM	
	APCI	ESI	APCI	ESI	APCI	ESI	APCI	ESI
Arg	- [a]	28	27	25	- [a]	24	28	27
Asp	129	126	109	93	- [a]	- [a]	166	161
Gly	0.9	1.0	- [b]	1,1	1.4	1.3	1.0	0.9
β-Ala	1.1	1.0	- [b]	0,9	0.9	1.6	1.7	1.1
Pro	- [a]	- [a]	9.6	6.5	13.3	9.5	10	8.7
Trp	12	17	11	12	15	12	9.6	9.2
Phe	28	36	28	29[*]	24	27	24	27

[a] – signals not obtained
[b] – signals below LLOQ
[*] – concentration evaluated using the extrapolative dilution approach

Compared to ESI, FMOC- and DNS-derivatives were less influenced by sample matrix with APCI. FMOC-Arg, -Pro and -Trp were not affected by ME, FMOC-Phe needed 2-fold and FMOC-Asp needed 4-fold dilution in order to eliminate ME. The concentrations of Gly and β-Ala in green tea were below LoQ and could not be evaluated. Although less ME was present in case of APCI, ESI should be preferred for FMOC-Cl analysis from the sensitivity point of view. For DNS-β-Ala and -Trp, a 2-fold dilution was needed, but other analytes were free from ME.

During analysis of DBEMM-derivatives with APCI, ME was similar to ESI. Gly- and β-Ala-derivatives did not have ME, 2-fold dilution was needed for Trp-, Phe-, Arg- and Pro-derivatives and 20-fold needed for Asp. As DBEMM is more sensitive compared to other derivatization reagents in ESI, and ME is eliminated with sample dilution, it could be considered suitable for both ESI and APCI.

To conclude, the sample dilution experiments with APCI showed that the analyses with DEEMM and DNS were less affected than analysis with DBEMM and FMOC-Cl. This is similar to ESI, where DEEMM was less affected by ME than FMOC-Cl and DNS. Compared to ESI, APCI was in general less affected by ME, which means that less sample dilution is needed for elimination of the ME. This stresses the need to choose suitable derivatization reagent and ionization source for specific application.

4.4.3. Agreement of concentrations in green tea obtained with ESI and APCI

As a result of sample dilution experiments, expected final concentrations were obtained with both ESI and APCI (Figure 11). This was achieved by eliminating ME by sample dilution or taking it into account using the extrapolative dilution approach. A comparison of these concentrations was carried out for seven AAs in green tea using DEEMM, DBEMM, DNS and FMOC-Cl. This comparison presents an additional confirmation of the reliability of the estimates of the AA concentrations with each derivatization reagent.

The agreement of concentrations obtained with ESI and APCI was evaluated using two-tailed *t*-test at confidence level 95% for comparison of two means with equal variances (checked using F-test: two-tailed, confidence level 95%) for each analyte. The differences were not statistically significant, except for analyses of β-Ala with DBEMM and DNS. The problems with β-Ala have been mentioned earlier and the low concentration could be one reason or there might be sample components that co-elute with β-Ala and cause ME. Nevertheless, the agreement of concentrations shows that sample dilution has successfully eliminated ME in both ESI and APCI.

Overall, the LC-APCI-MS methods provide quantitative analysis with DEEMM, DBEMM, DNS and FMOC-Cl. Matrix influence was successfully eliminated with sample dilution, improving the trueness of the method.

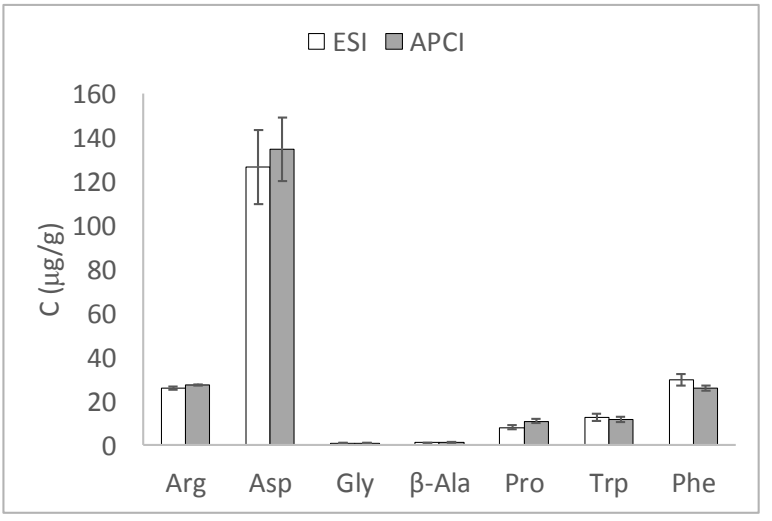

Figure 11. The average concentrations of seven AAs in green tea extract (μ g/g) found with four derivatization reagents using APCI and ESI.

4.5. Evaluating ME – dependence of ME on ESI polarity [Paper IV]

The previous comparison of derivatization reagents was carried out in positive ionization mode as it was suitable for all of the chosen derivatives, including novel derivatization reagents for ESI-MS carrying permanent positive charge (TAHS, FOSF). It has been shown that there can be less ME in negative ion mode.[44] Thus, the dependence of ME on ionization polarity was examined for LC-ESI-MS analysis which is more prone to ME compared to APCI. DEEMM was chosen for analysis with alternating polarity ionization mode since the sample matrix does not affect the derivatization yield of DEEMM derivatization and only IE of the derivatives is influenced. This would additionally test the hypothesis that DEEMM-derivatives have less ME on a wider scale.

The scope of ME investigation with DEEMM was widened in three aspects: analysis in the negative ion mode in addition to positive ion mode, 15 AAs were added to the previously analyzed seven AAs and, in addition to green tea and four honeys, 10 herbal extracts from Estonia were analyzed. The herbal extracts are used for recreational or healing purposes [106]: pine growth, yarrow, heather, linden (2 samples), chamomile (2 samples), cowslip, St. John's wort and peppermint. The analysis was carried out simultaneously in positive and negative ion mode, so that the matrix influence would be comparable and not dependent on, for example, variations in sample, injection repeatability, ion source parameters or the ion source contamination.

As the comparison of positive and negative ionization modes was carried out on QqQ mass spectrometer, a new LC-ESI-MS method for 22 AA DEEMM-derivatives using alternating polarity ESI mode was developed. Therefore, method optimization (section 4.1.3) and validation was carried out in both ionization modes.

4.5.1. Method validation

Method validation involved estimating the repeatability (sample preparation, injection), linearity, limit of detection (LoD) and limit of quantitation (LoQ). The injection repeatability was below 15% (n = 3) and the repeatability of the sample preparation was below 18% (n = 3) for all AAs. The latter also takes into account the repeatability of the derivatization reaction. The values of within day repeatability were acceptable for ME studies, which were carried out based on each sample and its dilutions prepared on one day.

The quantitative analysis requires estimating the linearity and linear range. As there were various matrix-analyte-concentration combinations, the working range spanned over 4 orders of magnitude. However, the data was not linear over the entire working range and two linear ranges were used instead, approximately 0.8–150 ng g^{-1} and 150–7000 ng g^{-1}.

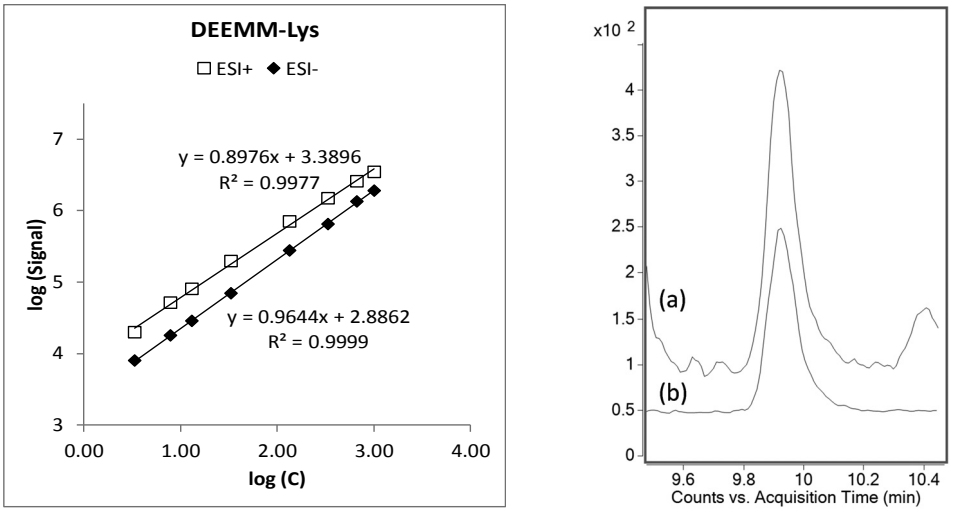

Figure 12. Linearity of DEEMM-Lys (4–7000 ng g^{-1}) shows higher signal in positive ion mode, but the S/N is (a) 14.4 in positive ion mode (C = 0.8 ng g^{-1}) and (b) 66.6 in negative ion mode (C = 4 ng g^{-1}).

The reason why positive ion mode is usually preferred is the higher signal intensity compared to negative ion mode. The LoD and LoQ values for 22 AAs in positive and negative ion modes are in Table 9. Although the signals were higher in positive ion mode, the signal-to-noise ratios (S/N) were higher in negative ion mode (see an example of linearity and S/N in case of DEEMM-Lys in Figure 12). The LoQ values were comparable in the two ionization modes for all AAs, being 3.0 to 32 fmol on column in positive ion mode and 4.5 to 57 fmol on column in negative ion mode.

In conclusion, the developed alternating polarity mode LC-ESI-MS method was suitable for quantitative analysis of 22 AAs in 11 herbal extracts and 4 honeys using DEEMM-derivatization. As not all derivatization reagents allow analysis in both ion modes, it can be an advantage of DEEMM.

Table 9. Comparison of LoD and LoQ in positive and negative ion mode for analysis of 22 DEEMM-derivatized AAs using alternating ion mode LC-ESI-MS

	Positive ion mode		Negative ion mode	
AA	**LoD (fmol)**	**LoQ (fmol)**	**LoD (fmol)**	**LoQ (fmol)**
His	3.6	11	7.1	22
Arg	6.0	18	6.3	19
Asn	4.9	2.5	10	7.0
Gln	7.9	24	12	37
Ser	8.2	25	19	57
Asp	7.3	22	4.8	15
Gly	6.7	20	15	47
Thr	9.3	28	3.4	10
β-Ala	5.0	15	12	37
GABA	4.4	13	8.6	26
α-Ala	7.7	23	11	34
Pro	10	32	9.0	27
Glu	1.8	5.0	10	31
Tyr	6.2	19	6.4	19
Met	3.4	10	10	32
Val	4.8	15	15	45
Trp	1.0	3.1	1.5	4.5
Orn	5.3	16	2.7	8.1
Phe	4.7	14	5.7	17
Ile	8.9	27	5.1	16
Leu	5.8	18	6.8	20
Lys	7.4	22	6.4	19

4.5.2. Evaluation of ME

The concentrations of 22 AAs were determined in three dilutions (dilution factors 1, 0.1 and 0.01) of 11 herbal extracts and four honeys using DEEMM derivatization. Analysis was carried out simultaneously in ESI positive and negative ion modes and results are discussed separately for each ion mode.

ME in ESI positive ion mode for seven AAs in green tea and honey was previously discussed for DEEMM-derivatives in section 4.4.1. Analysis of 22 AAs showed the presence of ME for some AAs (RSD above 20%), but in general, herbal extracts were less influenced by the sample matrix. The average RSD of AA concentrations in all herbal extracts was 10% (SD = 11%, n = 498), while it was 20% (SD = 17%, n = 133) in honeys (Figure 13). Compared to previous experiments, the reason for ME in these experiments could be

explained with a wider range of analytes, but also differences in samples, in chromatographic conditions and in the ion source.

In honeys, ME could be observed for Asn, Asp, Gly, Thr, Val and Orn, and in some cases for Arg, α-Ala, Met, Trp, Leu and GABA. Due to the wide variety of herbal extracts, ME was observed in some cases with the RSD of 0%–47%, except for Val, for which the matrix influence was the strongest (RSD = 23%–129% in pine growth, heather and linden2). In addition, ME affected the analysis of Asp, Gly, Val, Trp, Phe, Tyr and Leu derivatives in several herbal extracts. AAs most affected by ME were Asp, Val and Leu. This could mean that either matrix components co-elute with them or the IE of these derivatives is more easily influenced by the matrix components in positive ion mode.

Negative ion mode was considerably less influenced by the ME. Taking into account all the matrices (herbal extracts and honeys), the average value of the RSD in the negative ion mode was 8% (SD = 7%, n = 641) (Figure 13). For honeys, the ME was present in honey1 and honey3 for Leu (RSD = 20%–76%) and in honey3 for Asp (RSD = 19%–28%), while ME was not present in herbal extracts.

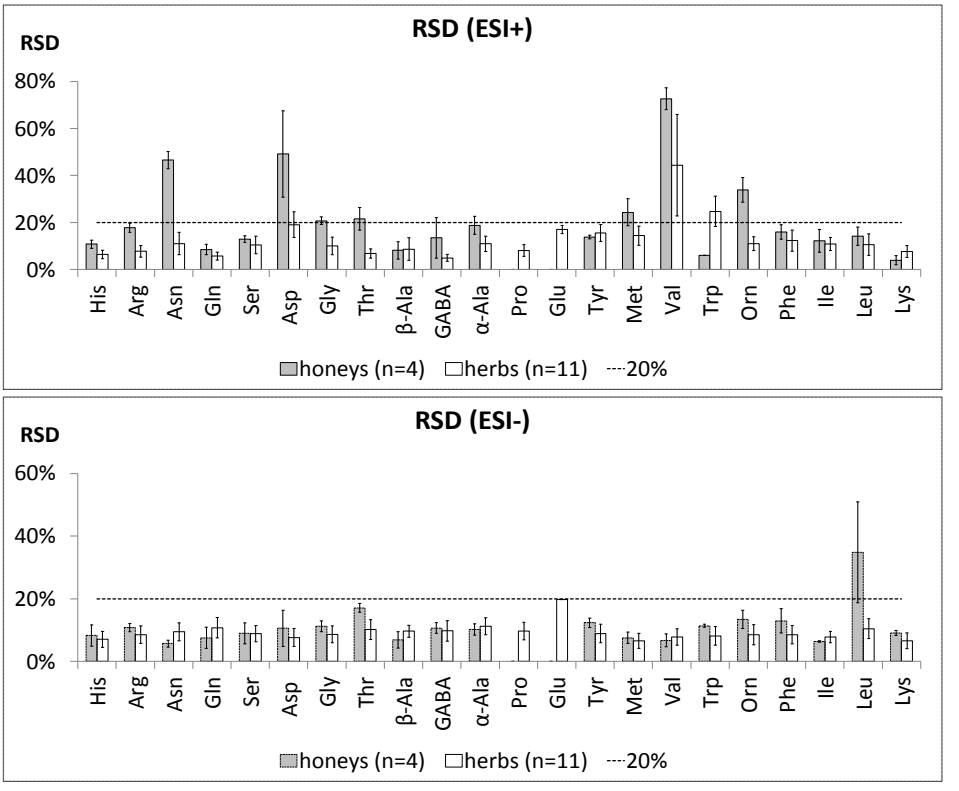

Figure 13. The dilution RSD values for 22 AAs in ESI positive (ESI+) and negative (ESI-) ion modes. The dashed line represents RSD = 20%, which is considered the threshold for presence of ME in this work.

The comparison of ionization modes shows that the negative ion mode is superior compared to positive ion mode in respect to the ME; at the same time the LoQ values are similar in the two modes. This proves that DEEMM in combination with the negative ion mode would be most suitable for analysis of complex matrices.

Agreement of concentrations obtained with alternating polarity ionization mode

ESI positive and negative ion modes had different ME and in order to evaluate the elimination of ME with sample dilution and its influence on the quantitative analysis, a comparison of concentrations of 22 AAs determined in 15 matrices in ESI positive and negative ion modes was carried out. Since ME was eliminated with sample dilution for positive ion mode, the concentrations from positive and negative ion mode agreed for most AAs.

Although AA analysis has been previously carried out in honey [101], green tea [80] and chamomile [107], several extracts were analyzed for the first time. Results in Table 10 showed that the total free amino acid (TAA) concentrations in herbs were higher than in honeys. This is expected, as the concentrations are given with respect to honey and dry herbs, but honeys have high content of sugars and water. In addition, the relative content of essential AAs (Phe, Val, Thr, Trp, Met, Leu, Ile, Lys, His) from the TAA was 10%–19% in herbs and up to 73% in honey1.

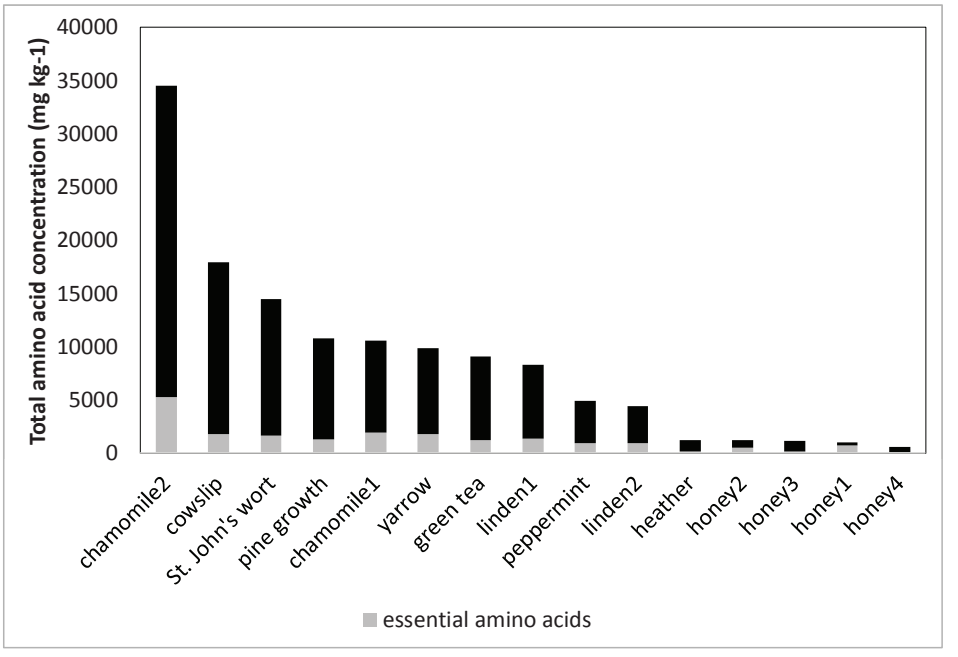

Figure 14. Total free AA concentration in herb and honey samples (mg kg^{-1}).

Table 10. The concentrations of 22 AAs in herbal extracts and honeys (negative ion mode) with RSD of concentrations from three dilutions in positive and negative ion mode. ME was considered present when the RSD was above 20% (marked with grey background). a – concentration in the sample was below LoQ; b – concentration in the sample was outside the calibration range; NA – the RSD could not be estimated

	pine growth			yarrow			heather			linden1			linden2		
	C (mg kg-1)	RSD (pos)	RSD (neg)	C (mg kg-1)	RSD (pos)	RSD (neg)	C (mg kg-1)	RSD (pos)	RSD (neg)	C (mg kg-1)	RSD (pos)	RSD (neg)	C (mg kg-1)	RSD (pos)	RSD (neg)
His	154	5%	12%	196	7%	13%	14	3%	6%	111	6%	7%	85	2%	1%
Arg	1198	9%	11%	224	7%	16%	51	10%	7%	80	8%	2%	48	2%	5%
Asn	3620	0%	5%	1980	35%	10%	123	15%	16%	3012	NA	NA	389	7%	11%
Gln	1481	10%	15%	1595	10%	20%	210	4%	18%	770	9%	7%	1008	6%	4%
Ser	327	20%	10%	455	24%	8%	41	3%	20%	393	19%	9%	108	7%	11%
Asp	47	16%	19%	260	34%	12%	31	21%	12%	318	33%	6%	77	10%	5%
Gly	58	24%	1%	52	8%	14%	22	2%	15%	41	3%	5%	23	17%	1%
Thr	294	13%	15%	292	9%	19%	47	4%	4%	113	10%	13%	135	9%	8%
β-Ala	54	4%	10%	53	23%	10%	6.4	2%	12%	29	3%	8%	23	4%	1%
GABA	1122	7%	8%	821	9%	15%	380	10%	15%	643	3%	16%	394	2%	3%
α-Ala	166	21%	17%	289	19%	14%	127	20%	1%	234	14%	10%	266	3%	5%
Pro	1145	6%	18%	2134	11%	11%	69	12%	7%	1281	NA	NA	997	5%	6%
Glu	5.4	NA	NA	2.7	NA	NA	- a	NA	NA	3.1	NA	NA	NA	13%	NA
Tyr	177	12%	9%	165	19%	14%	22	21%	18%	161	17%	9%	103	28%	0%
Met	0.7	NA	NA	22	25%	5%	0.3	NA	NA	0.4	NA	NA	1.1	20%	6%
Val	231	100%	11%	363	0%	15%	34	129%	4%	205	33%	4%	120	49%	8%
Trp	79	26%	1%	140	28%	19%	9	NA	NA	43	37%	15%	71	19%	5%
Orn	78	22%	9%	5.9	4%	9%	3.0	13%	0%	3.0	7%	6%	2.1	15%	10%
Phe	130	27%	10%	224	26%	15%	14	5%	4%	664	23%	10%	340	11%	0%
Ile	76	12%	6%	231	26%	7%	26	12%	5%	69	7%	8%	90	8%	14%
Leu	83	27%	20%	202	23%	17%	18	1%	9%	142	16%	7%	85	5%	6%
Lys	269	9%	7%	145	3%	11%	12	11%	15%	26	2%	5%	48	4%	4%

Table 10. Continued

	chamomile1			chamomile2			cowslip			St. John's wort			peppermint		
	C (mg kg-1)	RSD (pos)	RSD (neg)	C (mg kg-1)	RSD (pos)	RSD (neg)	C (mg kg-1)	RSD (pos)	RSD (neg)	C (mg kg-1)	RSD (pos)	RSD (neg)	C (mg kg-1)	RSD (pos)	RSD (neg)
His	138	5%	16%	431	7%	3%	118	5%	5%	180	5%	2%	57	10%	9%
Arg	171	3%	20%	684	0%	2%	692	12%	7%	203	6%	11%	89	15%	7%
Asn	476	9%	16%	4334	11%	8%	1818	15%	9%	616	7%	1%	479	8%	2%
Gln	2837	3%	19%	11629	8%	10%	10522	1%	13%	7615	0%	8%	1344	6%	3%
Ser	474	10%	1%	1113	6%	6%	309	13%	11%	484	0%	2%	104	3%	9%
Asp	292	9%	13%	977	8%	2%	302	36%	6%	79	15%	1%	271	21%	2%
Gly	123	3%	17%	158	13%	8%	53	19%	9%	44	3%	7%	34	8%	10%
Thr	282	1%	20%	648	0%	0%	183	5%	5%	396	6%	9%	114	11%	5%
β-Ala	65	7%	7%	109	8%	8%	20	9%	9%	29	2%	12%	30	3%	15%
GABA	1648	5%	17%	1684	5%	0%	1324	0%	11%	1549	4%	3%	623	6%	15%
α-Ala	739	9%	20%	675	3%	10%	396	7%	12%	503	7%	12%	325	11%	14%
Pro	1600	10%	9%	7620	9%	2%	678	1%	16%	1486	NA	NA	634	3%	14%
Glu	- a	NA	NA	8.3	19%	NA	- a	NA	NA	2.6	NA	NA	2.6	NA	NA
Tyr	195	9%	15%	228	5%	9%	75	10%	12%	160	17%	1%	67	8%	2%
Met	3	4%	14%	48	7%	6%	1.5	NA	NA	4.1	14%	1%	1.1	NA	NA
Val	358	2%	14%	1103	3%	1%	391	56%	4%	389	12%	10%	190	79%	2%
Trp	91	11%	3%	410	8%	5%	156	30%	9%	118	47%	5%	77	33%	7%
Orn	8	12%	10%	15	11%	4%	9.1	17%	6%	5.5	11%	1%	12	1%	20%
Phe	169	12%	18%	718	2%	1%	205	6%	8%	208	15%	4%	146	4%	15%
Ile	389	5%	7%	707	8%	9%	332	10%	1%	136	10%	6%	178	13%	8%
Leu	251	8%	16%	631	1%	1%	260	17%	8%	165	4%	5%	117	2%	8%
Lys	291	8%	14%	555	1%	0%	126	6%	2%	96	12%	0%	48	17%	6%

Table 10. Continued

	green tea			honey1			honey2			honey3			honey4		
	C (mg kg-1)	RSD (pos)	RSD (neg)	C (mg kg-1)	RSD (pos)	RSD (neg)	C (mg kg-1)	RSD (pos)	RSD (neg)	C (mg kg-1)	RSD (pos)	RSD (neg)	C (mg kg-1)	RSD (pos)	RSD (neg)
His	58	15%	4%	3.2	7%	5%	3.7	8%	9%	4.5	13%	2%	4.4	14%	18%
Arg	289	13%	7%	4.2	16%	8%	2.9	19%	10%	13	23%	14%	6.0	13%	10%
Asn	555	3%	16%	3.7	48%	5%	9.4	36%	4%	18	49%	6%	8.0	54%	9%
Gln	4117	6%	2%	1.9	4%	13%	6.7	7%	0%	36	15%	3%	8.2	8%	13%
Ser	325	10%	12%	4.4	9%	15%	4.7	16%	5%	9.7	15%	2%	8.2	12%	14%
Asp	1768	6%	5%	7.8	20%	6%	11	27%	7%	9.5	48%	28%	13	101%	2%
Gly	16	9%	8%	8.0	16%	16%	4.5	23%	9%	9.5	22%	9%	7.4	22%	10%
Thr	162	7%	13%	1.7	34%	20%	2.8	24%	13%	6.9	11%	17%	5.8	18%	18%
β-Ala	16	31%	15%	12	19%	4%	10	7%	1%	13	4%	10%	9.2	2%	13%
GABA	244	3%	2%	5.6	8%	9%	3.7	6%	10%	11	1%	16%	7.4	39%	9%
α-Ala	186	7%	8%	8.6	30%	8%	17	15%	15%	12	17%	7%	10	13%	11%
Pro	137	17%	4%	- b	NA	NA	593	NA	NA	901	NA	NA	404	NA	NA
Glu	2.4	19%	20%	- a	NA	NA	- a	NA	NA	- a	NA	NA	4.0	NA	NA
Tyr	146	24%	10%	213	NA	NA	10	14%	11%	16	15%	16%	7.4	12%	10%
Met	0.8	16%	NA	0.02	NA	NA	314	16%	10%	0.3	19%	3%	0.7	37%	10%
Val	191	25%	14%	4.7	79%	12%	4.5	81%	6%	8.6	61%	6%	6.9	70%	3%
Trp	188	10%	13%	0.04	NA	NA	0.04	NA	NA	2.3	6%	11%	0.02	6%	12%
Orn	2.5	7%	19%	1.0	23%	16%	0.8	42%	11%	1.0	27%	20%	0.6	44%	7%
Phe	226	5%	9%	705	NA	NA	30	20%	7%	71	NA	NA	28	12%	18%
Ile	157	9%	13%	3.4	21%	6%	181	NA	NA	27	2%	6%	8.0	14%	7%
Leu	114	11%	19%	3.5	22%	42%	4.7	5%	14%	7.3	11%	44%	5.7	19%	5%
Lys	171	10%	9%	16	1%	8%	14	0%	8%	22	6%	10%	19	8%	11%

4.6. Principles for designing novel derivatization reagents

Based on previously discussed results, conclusions can be made about the suitability of these derivatization reagents for LC-MS analysis. There are several aspects to keep in mind when choosing a suitable derivatization reagent for an application or designing a new one: reaction with the analyte, compatibility with the ion source and mode, ionization efficiency of derivatives, matrix influence, etc. The characteristics from the results obtained in this work and in our work group have been summed up for six derivatization reagents (Table 11 and Table 12). Following, the main conclusions drawn about designing novel derivatization reagents are discussed.

The structure of the derivatization reagent determines the ionization efficiency and fragmentation patterns, which are important for sensitive MS detection. From the sensitivity point of view, the log*IE* values have been the starting point when designing novel derivatization reagents for LC-MS analysis. For example, log*IE* values indicate that one aspect providing high IE and low LoD is the permanent charge on the analyte. However, results show that once the chromatographic system is added and analysis of samples is carried out, such derivatives might not be superior to other reagents and similar LoQ values can be obtained for reagents without permanent charge as well. Moreover, due to the permanent positive charge, novel reagents are not suitable for analysis in negative ion mode, which has been proved to be less influenced by ME and is superior when analysis with minimal ME is sought. Therefore, the other aspect from log*IE* scales, the large non-polar structure, like DBEMM, should be suitable for derivatization reagents. Structure wise, structures similar to DEEMM-derivatives provide sensitivity that was similar in both positive and negative ion modes.

Comparison of different ion sources showed that during method optimization it is important to re-evaluate the precursor and product ions. Some derivatives ionize via both protonation and adduct formation and the latter depends on the ionization source and instrument, as well as eluent composition. From the reagents used, DEEMM, DBEMM, DNS and FMOC-Cl, derivatives are suitable for both ESI and APCI ionization. ESI ionization can produce both protonated ions, as well as Na-adducts, while in APCI, protonation was observed for DEEMM, FMOC-Cl, DNS and DBEMM.

When a design of sensitive APCI derivatization reagents is targeted, then structures similar to DEEMM and DNS serve as useful starting points as their derivatives provided APCI LLoQ values comparable to ESI. In addition, FOSF did not provide stable signal in APCI possibly due to the permanent positive charge of the derivatives, which was designed for enhancing IE in ESI. Consequently, non-charged derivatives should be preferred for APCI reagents.

Characteristic transition is one of the aims of derivatization for LC-MS. For most of the derivatives studied (TAHS, FMOC-Cl, FOSF and DNS), the product ion is the same for all analytes and depends only on the derivatization reagent. On the other hand, DEEMM- and DBEMM-derivatives differ from others as the

same neutral fragment is lost during fragmentation, resulting in characteristic product ions for each analyte. As the fragmentation involves derivatization reagent moiety of the molecule, all the DEEMM-derivatives have similar MS/MS fragmentation parameters. This would considerably reduce time required for MS parameter optimization. It also enables use of neutral loss scan mode for non-target screening of amino group containing compounds.

From method validation point of view, accuracy (trueness and precision) is often one of the most important parameter that needs to be evaluated. In turn, ME is the main component of trueness evaluation for LC-API-MS analysis. From the ME point of view, both endogenous and exogenous compounds can affect the ionization efficiency, and in addition, new compounds forming during the derivatization reaction should be considered. On the one hand, ME caused by the exogenous compounds (e.g. derivatization reaction components) should be determined already in the method development step and the cause of ME might be eliminated by choosing the suitable components, e.g. buffer components. On the other hand, eliminating endogenous compounds, including derivatives of non-analytes formed during derivatization, can be more complex. This emphasizes the need for good chromatographic separation even if MS detection is used, and here the derivatives without permanent positive charge (DBEMM, FMOC-Cl, DEEMM and DNS) have an advantage.

Regardless of the cause of ME, different derivatization reagents can be differently affected by ME. In ESI, derivatives of DEEMM and DBEMM have the least ME, followed by FMOC-Cl and DNS depending on the matrix. TAHS, designed for high ionization efficiency and carrying a positive charge, was affected by ME the most. In APCI, less ME was present for all the derivatization reagents, while DEEMM and DNS were least affected by the ME, followed by DBEMM and FMOC-Cl.

It was demonstrated that the matrix influence depends also on the analyte and not only the derivatization reagent. This could be connected to the retention time and co-eluting components causing the ME, but also to the structure of the AA. Therefore, it is desirable that the structure of the derivatization reagent dominates and allows robust and ME free analysis. Based on these results it can be concluded that the derivatization reagents designed for APCI positive and/or negative ion detection should be targeted for robust and ME-free analysis.

While striving towards higher *IE*, chemical aspect – yield of derivatization reaction – must also be kept in mind. All studied reagents react rapidly with amines with the only significant exception of Pro reactions with DEEMM and DBEMM. Compared to standard solutions, derivatization procedures used for FMOC-Cl and TAHS and FOSF needed modification for more complex samples, and DNS reaction was also affected by the sample matrix. At the same time, the derivatization reaction yield of DEEMM proved to be unaffected by the sample matrix. This stresses that optimizing derivatization procedure with standard solutions is not sufficient. Therefore, reactive moieties of derivatization reagents similar to those of DEEMM and DBEMM are promising candidates for application with new derivatization reagents.

In addition, from practical point of view, automatization of the derivatization procedure can save resources (e.g. reagents, time, labor). Moreover, DBEMM [94], FMOC-Cl [108] and DNS [109] have already been used with automated derivatization procedure. Addition of a specific reagent to eliminate excess of derivatization reagent is an inconvenient step especially for automated derivatization. Preferably, novel derivatization systems shall be developed to not require stopping stage.

In conclusion, using the log*IE* scale as the starting point for developing novel derivatization reagents for LC-API-MS is suitable, when also taking into account other aspects besides IE, i.e. derivatization reaction, chromatographic retention, matrix influence on the reaction and on the IE, ionization and fragmentation mechanisms. Possible causes of ME from derivatization reaction should be monitored from the beginning of the method development. When analyte free matrix and pure standards are not available, then sample dilution method can be used for evaluating ME of derivatization LC-MS.

Table 11. Summary of comparison of six derivatization reagents for LC-API-MS analysis: derivatization reaction and chromatography

Reagent	Analytes	Sample size	Reagents added to sample	Reaction time	Matrix influence on reaction yield[1]	$M_{derivative}$ [g/mol]	Retention in RPLC	LoQ [fmol on column] [Paper I]
DEEMM	primary and secondary amines, *Pro	250 µl	1) 375 µl DEEMM (1:50 solution in MeOH) 2) 875 µl borate buffer (0.75 M, pH=9)	few minutes, 24 h for Pro	–	AH+170	**	**
DBEMM	primary and secondary amines	50 µl	1) 300 µl DBEMM (1:150 in isopropanol) 2) 200 µl HFIP (0.56 M, pH=9.0)	few minutes, 2h for Pro	N/A	AH+294	***	*** [94]
DNS	primary and secondary amines, *Asp	100 µl	1) 20 µl NaOH (2 M) 2) 500 µl DNS solution (5 mg mL−1 for tea and 10 mg mL^{-1} for honey) 3) reaction is ended with 10 µl conc NH_4OH	45 min (in the dark at 4 ºC)	+	AH+233	**	*
FMOC-Cl	primary and secondary amines	300 µl	1) 300 µl borate buffer (0.75 M, pH=9) 2) 300 µl FMOC-Cl (1 mg mL^{-1}) 3) 300 µl His (4 mg mL^{-1})	30 min for tea and honey	+	AH+222	***	*
TAHS	primary and secondary amines	10 µl	1) 30 µl borate buffer (0.2 M, pH= 8.8) 2) 20 µl TAHS (20 mg mL^{-1} in acetonitrile) 3) reaction is ended with 200 µL acetic acid (0.2%)	30 min	+	AH+177	*	**
FOSF	primary and secondary amines	10 µl	1) 30 µl borate buffer (0.2 M, pH= 8.8) 2) 20 µl FOSF (20 mg mL^{-1} in acetonitrile) 3) reaction is ended with 200 µL acetic acid (0.2%)	30 min	N/A	AH+298	*	**

[1] "+" problem, "–" not a problem;
***strong or good, ** medium, *poor

Table 12. Summary of comparison of six derivatization reagents for LC-API-MS analysis: positive ion mode.

Reagent	ESI					APCI				
	Ionization	**Frag-mentation**	**log*IE* (MeOH)** [95]	**log*IE* (MeCN)** [95]	**Resis-tance to ME[a]**	**Ionization**	**Fragmentation**	**log*IE* (MeOH)** [95]	**log*IE* (MeCN)** [95]	**Resis-tance to ME**
DEEMM	[M+H]+, [M+Na]+	–EtOH, –2EtOH	DEEMM 2.36, Phe-DEEMM 3.79, Gly-DEEMM 3.36	DEEMM 2.04, Phe-DEEMM 0.78, Gly-DEEMM 2.73	***	$[M+H]^+$ $[M+H–46]+$	–2EtOH, $–H_2O$, $–CO_2$	DEEMM 2.57, Phe-DEEMM 2.47, Gly-DEEMM 2.35	DEEMM 1.18, Phe-DEEMM 3.59, Gly-DEEMM 2.14	***
DBEMM	[M+H]+, [M+Na]+	–PhOH, –2PhOH	DBEMM 2.64	DBEMM 3.56	***	$[M+H]^+$	$–H_2O$, $–CO_2$, –PhOH, –2PhOH	DBEMM 2.51	DBEMM 2.43	*
DNS	[M+H]+	–AA, $–CO_2$, $–SO_2$	Problems with stable signal	Problems with stable signal	**	$[M+H]^+$	–(AA and SO_2), –(DNS and H_2O), –(H_2O and CO)	DNS 3.28	Problems with stable signal	**
FMOC-Cl	[M+H]+, [M+Na]+	m/z 263 is monitored	Phe-FMOC 3.53, Gly-FMOC 3.23	Phe-FMOC 2.95, Gly-FMOC 2.18	*	$[M+H]^+$	m/z 179 is monitored	Phe-FMOC 3.28, Gly-FMOC 3.12	Phe-FMOC 2.94, Gly-FMOC 2.41	*
TAHS	[M]+	m/z 177 is monitored	N/A	N/A	*	N/A	N/A	N/A	N/A	N/A
FOSF	[M]+	m/z 298 is monitored	N/A	N/A	**	N/A	N/A	N/A	N/A	N/A

***strong or good, ** medium, *poor

[a] based on sum of dilutions needed for Ar, Gly, b-Ala and Trp (amino acids evaluated in all cases) in green tea

5. SUMMARY

Present work focused on the evaluation of matrix effect (ME) during derivatization LC-API-MS analysis. ME affects method's accuracy and sensitivity and therefore needs to be assessed during method validation. This can be challenging for derivatization LC-API-MS analysis of amino acids, as the matrix can influence both derivatization reaction and ionization, moreover, pure analytes and analyte free matrices are in most cases not available.

In present work three methods were used for evaluating the influence of sample matrix on LC-API-MS analysis of derivatized amino acids: post-column infusion, post-derivatization spiking and sample dilution. Six amino acid derivatization reagents, from which two have been developed in our work group, were used for analyses involving ESI, APCI and alternating polarity ESI ion sources in different experiments.

Post-derivatization spiking, a method developed in our work group, enabled to reveal that in case of analysis of selenoamino acids in onion the sample matrix did not affect the derivatization reaction yield for TAHS and DEEMM derivatives. For DEEMM derivatives also ionization matrix effects were neglible. However, this approach requires blank matrix which is not always available.

The post-column infusion experiment showed that ME of the analysis depended on the derivatization reagent. Signal suppression of DEEMM-derivatives was caused by buffer solution used for derivatization reaction, while other derivatives supressed the signal of FMOC-derivatives. While the former underlines the concern for possible ME already in the method development step (e.g. optimizing the derivatization procedure), the latter stresses the need for chromatographic separation in case of ESI-MS detection.

The sample dilution approach was used for quantifying the ME. DEEMM was found to be least affected by the sample matrix followed by DBEMM, FOSF, DNS and FMOC-Cl. TAHS was the most problematic, especially in honey matrix, indicating that high ionization efficiency is not neccessarily accompanied by low ME. It was found that APCI is less affected by ME than ESI and therefore, LC-APCI-MS analysis was considered as an option to reduce ME in case of derivatization. In the early steps of method development, it appeared that FOSF was not a suitable reagent for APCI, which could be due to the permanent charge of the derivatives. LC-APCI-MS methods were development for DEEMM, DBEMM, DNS and FMOC-Cl derivatives. Although some sample dilution was needed in some cases, APCI was significantly less affected by ME than ESI.

Analysis with DEEMM proved to be most suitable for sensitive and ME-free analysis based on the LC-MS analyses of both standard solutions and samples. This was further tested in alternating polarity ionization mode for herbal extracts and honeys for 22 amino acids. Although some of the derivatized analytes suffered from ME in positive ion mode, negative ion mode was in general ME-free. Compared to conventional positive ion ESI mode, negative

ion ESI and positive ion APCI modes were demonstrated to be less prone to ME, while still providing LoQs comparable to positive mode ESI. This provides a new principle for developing novel derivatization reagents – derivatization reagent with a structure which enhances detectability in these modes could pave the road to ME-free LC-MS analysis.

In conclusion, three methods for evaluating ME in case of derivatization LC-MS analysis of amino acids were utilized and their usefulness for characterization of different derivatization reagents was demonstrated. Based on the ME characteristics, a justified selection of derivatization reagent for analysis of low concentrations of amino acids in complex matrices can be made. The conclusions made and methods used in this work can be used for developing and designing novel derivatization reagents for sensitive and ME-free LC-MS analysis.

6. SUMMARY IN ESTONIAN

Aminohapete LC-MS analüüsiks kasutatavate derivatiseerivate reagentide iseloomustamine

Käesoleva töö eesmärgiks oli maatriksiefektide hindamine analüüdi derivatiseerimist kasutava vedelikkromatograafia massispektromeetria (LC-MS) analüüsimeetodi korral. Maatriksiefektid halvendavad metoodika tõesust, täpsust ja tundlikkust ning seetõttu on neid oluline hinnata metoodika valideerimise käigus. Derivatiseeritud aminohapete LC-MS analüüsi maatriksiefektide hindamise teeb keeruliseks maatriksi mõju nii derivatiseerimisreaktsiooni saagisele kui ka analüütide ionisatsioonile MS ioonallikas. Samuti on takistuseks puhaste analüütide (aminohappe derivaadid) kättesaadavus ja analüüdivaba maatriksi puudumine enamikul juhtudel.

Proovi maatriksi mõju hindamiseks derivatiseeritud aminohapete LC-MS analüüsil kasutati kolme meetodit: kolonnijärgne infuseerimine, derivatiseerimisjärgne lisamismeetod ja proovi lahjendamine. Uuriti kuut aminohapete derivatiseerivat reagenti, millest kaks on väljatöötatud meie töörühmas Tartu Ülikoolis. Töös uuriti nende derivaatide omadusi ESI ja APCI ionisatsiooni positiivsete ioonide režiimis ning ESI vahelduva polaarsuse režiimis.

Meie töörühmas väljatöötatud derivatiseerimisjärgne lisamismeetod võimaldas kindlaks teha, et sibula maatriks ei mõjutanud TAHS-i ja DEEMM-i derivatiseerimisreaktsioone. Lisaks sellele ei mõjutanud maatriksiefektid DEEMM-derivaatide analüüsi. Siiski, derivatiseerimisjärgne lisamismeetod ei ole rakendatav kõigil juhtude, kuna vajab analüüdivaba maatriksit.

Kolonnijärgse infuseerimismeetodi katsed näitasid, et maatriksiefektid mõjutavad erinevaid derivatiseerivaid reagente erineval määral. Kui DEEMM-derivaatide signaal oli maha surutud derivatiseerimisel kasutatava puhverlahuse tõttu, siis FMOC-derivaatide signaali surusid maha teised, proovi maatriksi komponentidest tekkinud FMOC-derivaadid. Esimest maatriksi mõju on võimalik vähendada metoodika väljatöötamise käigus sobivate reagentide valikuga ning teine rõhutab kromatograafilise lahutuse vajalikkust ka massispektromeetrilisel analüüsil.

Maatriksiefekti kvantitatiivseks hindamiseks kasutati proovi lahjendamise meetodit. Selgus, et analüüs DEEMM derivaatidega oli kõige vähem maatriksi poolt mõjutatud, järgnesid DBEMM, FOSF, DNS ja FMOC-Cl. TAHS oli kõige problemaatilisem, eriti meeproovide korral. Probleemid TAHS derivaatidega näitavad, et kõrge ionisatsiooniefektiivsusega ei kaasne tingimata maatriksiefektivaba analüüs. Näidati, et APCI ionisatsioon on maatriksiefektidest vähem mõjutatud kui ESI ja rakendati LC-APCI-MS analüüsi maatriksiefektide vähendamiseks derivatiseerimisega aminohapete analüüsil. Juba metoodika väljatöötamisel selgus, et FOSF-derivaadid ei andnud APCI ionisatsioonil stabiilset signaali, mille üheks põhjuseks võib olla püsiv laeng derivaatidel. LC-APCI-MS metoodikad töötati välja DEEMM-i, DBEMM-i, DNS-i ja FMOC-Cl-i derivaatide analüüsiks. Kuigi proovi lahjendamine oli vajalik ka

APCI meetodis, oli APCI analüüs maatriksi poolt vähem mõjutatud kui ESI analüüs.

Analüüs DEEMM-iga oli nii tundlikkuse kui ka maatriksiefektide poolest kõige sobivam LC-MS analüüsiks. DEEMM-derivaate testiti ka vahelduva polaarsusega ESI režiimis 22 aminohappe analüüsil mee- ja taimeekstraktides. Kuigi positiivses režiimis esines mõnel juhul maatriksiefekte, oli negatiivne režiim maatriksiefektidest vaba. Võrreldes tavapärase ESI positiivse režiimiga olid maatriksiefektid positiivses APCI ja negatiivses ESI režiimis oluliselt väiksemad pakkudes võrreldavaid määramispiire. See avab uue suuna derivatiseerivate reagentide disainis – just nendes režiimides kõrget ionisatsiooniefektiivsust omavad derivaadid võiksid olla teerajajad maatriksiefektivabale LC-MS analüüsile.

Kokkuvõttes, maatriksiefektide hindamiseks derivatiseerimisega aminohapete LC-MS analüüsil kasutati kolme meetodit ning näidati nende rakendatavust erinevate derivatiseerivate reagentide iseloomustamiseks. Maatriksiefektide mõju põhjal on võimalik valida sobiv derivatiseeriv reagent madalate sisalduste määramiseks keerulistes proovides. Käesoleva töö järeldusi ja meetodeid saab rakendada uute derivatiseerivate reagentide väljatöötamisel tundliku ja maatriksiefektivaba LC-MS analüüsi jaoks.

7. REFERENCES

[1] Thakare, R., Chhonker, Y. S., Gautam, N., Alamoudi, J. A., Alnouti, Y., *J. Pharm. Biomed. Anal.* 2016, 128, 426–437.

[2] Holčapek, M., Jirásko, R., Lísa, M., *J. Chromatogr. A* 2012, 1259, 3–15.

[3] Konermann, L., Ahadi, E., Rodriguez, A. D., Vahidi, S., *Anal. Chem.* 2013, 85, 2–9.

[4] Reemtsma, T., *J. Chromatogr. A* 2003, 1000, 477–501.

[5] Covey, T. R., Thomson, B. A., Schneider, B. B., *Mass Spectrom. Rev.* 2009, 28, 870–897.

[6] Thurman, E. M., Ferrer, I., Barceló, D., *Anal. Chem.* 2001, 73, 5441–5449.

[7] Fenn, J. B., *J. Am. Soc. Mass Spectrom.* 1993, 4, 524–535.

[8] Dole, M., Mack, L. L., Hines, R. L., Mobley, R. C., Ferguson, L. D., Alice, M. B., *J. Chem. Phys.* 1968, 49, 2240–2249.

[9] Ahadi, E., Konermann, L., *J. Phys. Chem. B* 2012, 116, 104–112.

[10] Carroll, D. I., Dzidic, I., Stillwell, R. N., Haegele, K. D., Horning, E. C., *Anal. Chem.* 1975, 47, 2369–2373.

[11] Huang, E. C., Wachs, T., Conboy, J. J., Henion, J. D., *Anal. Chem.* 1990, 62, 713–725.

[12] Kolakowski, B. M., Grossert, J. S., Ramaley, L., *J. Am. Soc. Mass Spectrom.* 2004, 15, 311–324.

[13] Herrera, L., Grossert, J., Ramaley, L., *J. Am. Soc. Mass Spectrom.* 2008, 19, 1926–1941.

[14] Garcia-Ac, A., Segura, P. A., Viglino, L., Gagnon, C., Sauvé, S., *J. Mass Spectrom.* 2011, 46, 383–390.

[15] Leinonen, A., Kuuranne, T., Kostiainen, R., *J. Mass Spectrom.* 2002, 37, 693–698.

[16] Guo, T., Shi, Y., Zheng, L., Feng, F., Zheng, F., Liu, W., *J. Chromatogr. A* 2014, 1355, 73–79.

[17] Leito, I., Herodes, K., Huopolainen, M., Virro, K., Künnapas, A., Kruve, A., Tanner, R., *Rapid Commun. Mass Spectrom.* 2008, 22, 379–384.

[18] Deng, P., Zhan, Y., Chen, X., Zhong, D., *Bioanalysis* 2012, 4, 49–69.

[19] J.M. E. Quirke, C.L. Adams, G. J. Van Berkel, *Anal. Chem.* 1994, 66, 1302–1315.

[20] Cech, N. B., Enke, C. G., *Mass Spectrom. Rev.* 2001, 20, 362–387.

[21] Santa, T., Al-Dirbashi, O. Y., Fukushima, T., *Drug Discov Ther* 2007, 1, 108–118.

[22] Henriksen, T., Juhler, R. K., Svensmark, B., Cech, N. B., *J. Am. Soc. Mass Spectrom.* 2005, 16, 446–455.

[23] Xu, F., Zou, L., Liu, Y., Zhang, Z., Ong, C. N., *Mass Spectrom. Rev.* 2011, 30, 1143–1172.

[24] Dams, R., Benijts, T., Günther, W., Lambert, W., Leenheer, A. D., *Rapid Commun. Mass Spectrom.* 2002, 16, 1072–1077.

[25] Ehrmann, B. M., Henriksen, T., Cech, N. B., *J. Am. Soc. Mass Spectrom.* 2008, 19, 719–728.

[26] Oss, M., Kruve, A., Herodes, K., Leito, I., *Anal. Chem.* 2010, 82, 2865–2872.

[27] Kruve, A., Kaupmees, K., Liigand, J., Oss, M., Leito, I., *J. Mass Spectrom.* 2013, 48, 695–702.

[28] Rebane, R., Kruve, A., Liigand, P., Liigand, J., Herodes, K., Leito, I., *Anal. Chem.* 2016, 88, 3435–3439.
[29] Kostiainen, R., Kauppila, T. J., *J. Chromatogr. A* 2009, 1216, 685–699.
[30] Gao, S., Zhang, Z., Karnes, H., *J. Chromatogr. B* 2005, 825, 98–110.
[31] Maragou, N. C., Thomaidis, N. S., Koupparis, M. A., *J. Am. Soc. Mass Spectrom.* 2011, 22, 1826–1838.
[32] Nishio, T., Higashi, T., Funaishi, A., Tanaka, J., Shimada, K., *J. Pharm. Biomed. Anal.* 2007, 44, 786–795.
[33] Shimbo, K., Yahashi, A., Hirayama, K., Nakazawa, M., Miyano, H., *Anal. Chem.* 2009, 81, 5172–5179.
[34] Shimbo, K., Oonuki, T., Yahashi, A., Hirayama, K., Miyano, H., *Rapid Commun. Mass Spectrom.* 2009, 23, 1483–1492.
[35] Higashi, T., Takido, N., Yamauchi, A., Shimada, K., *Anal. Sci.* 2002, 18, 1301–1307.
[36] Qi, B.-L., Liu, P., Wang, Q.-Y., Cai, W.-J., Yuan, B.-F., Feng, Y.-Q., *TrAC Trends Anal. Chem.* 2014, 59, 121–132.
[37] Guilbault, G. G., Hjelm, M., *Pure Appl. Chem.* 1989, 61, 1657–1664.
[38] Taylor, P. J., *Clin. Biochem.* 2005, 38, 328–334.
[39] Matuszewski, B. K., Constanzer, M. L., Chavez-Eng, C. M., *Anal. Chem.* 2003, 75, 3019–3030.
[40] Kruve, A., Rebane, R., Kipper, K., Oldekop, M.-L., Evard, H., Herodes, K., Ravio, P., Leito, I., *Anal. Chim. Acta* 2015, 870, 8–28.
[41] Tang, L., Kebarle, P., *Anal. Chem.* 1991, 63, 2709–2715.
[42] Trufelli, H., Palma, P., Famiglini, G., Cappiello, A., *Mass Spectrom. Rev.* 2011, 30, 491–509.
[43] Ghosh, C., Shinde, C. P., Chakraborty, B. S., *J. Chromatogr. B* 2012, 893–894, 193–200.
[44] Ghosh, C., Shinde, C. P., Chakraborty, B. S., *J. Anal. Bioanal. Tech.* 2010, 01, DOI 10.4172/2155-9872.1000106.
[45] King, R., Bonfiglio, R., Fernandez-Metzler, C., Miller-Stein, C., Olah, T., *J. Am. Soc. Mass Spectrom.* 2000, 11, 942–950.
[46] Keski-Rahkonen, P., Huhtinen, K., Desai, R., Tim Harwood, D., Handelsman, D. J., Poutanen, M., Auriola, S., *J. Mass Spectrom.* 2013, 48, 1050–1058.
[47] Beltrán, E., Ibáñez, M., Sancho, J. V., Hernández, F., *Food Chem.* 2014, 142, 400–407.
[48] Matuszewski, B. K., *J. Chromatogr. B* 2006, 830, 293–300.
[49] Pizzutti, I. R., de Kok, A., Zanella, R., Adaime, M. B., Hiemstra, M., Wickert, C., Prestes, O. D., *J. Chromatogr. A* 2007, 1142, 123–136.
[50] Zhuang, X. M., Yuan, M., Zhang, Z. W., Wang, X. Y., Zhang, Z. Q., Ruan, J. X., *J. Chromatogr. B* 2008, 876, 76–82.
[51] Eggink, M., Wijtmans, M., Ekkebus, R., Lingeman, H., Esch, I. J. P. de, Kool, J., Niessen, W. M. A., Irth, H., *Anal. Chem.* 2008, 80, 9042–9051.
[52] Van Eeckhaut, A., Lanckmans, K., Sarre, S., Smolders, I., Michotte, Y., *J. Chromatogr. B* 2009, 877, 2198–2207.
[53] Uutela, P., Ketola, R. A., Piepponen, P., Kostiainen, R., *Anal. Chim. Acta* 2009, 633, 223–231.
[54] Bonfiglio, R., King, R. C., Olah, T., Merkle, K., *Rapid Commun. Mass Spectrom.* 1999, 13, 1175–1185.

[55] Kruve, A., Künnapas, A., Herodes, K., Leito, I., *J. Chromatogr. A* 2008, 1187, 58–66.
[56] Zhao, X.-E., Zhu, S., Yang, H., You, J., Song, F., Liu, Z., Liu, S., *J. Chromatogr. B* 2015, 995–996, 15–23.
[57] Hayama, T., Sakaguchi, Y., Yoshida, H., Itoyama, M., Todoroki, K., Yamaguchi, M., Nohta, H., *Anal. Chem.* 2012, 84, 8407–8414.
[58] Jia, S., Kang, Y. P., Park, J. H., Lee, J., Kwon, S. W., *J. Chromatogr. A* 2011, 1218, 9174–9182.
[59] Dams, R., Huestis, M. A., Lambert, W. E., Murphy, C. M., *J. Am. Soc. Mass Spectrom.* 2003, 14, 1290–1294.
[60] Cappiello, A., Famiglini, G., Palma, P., Pierini, E., Termopoli, V., Trufelli, H., *Anal. Chem.* 2008, 80, 9343–9348.
[61] Zaikin, V. G., Halket, J. M., *Eur. J. Mass Spectrom.* 2006, 12, 79–115.
[62] Stahnke, H., Kittlaus, S., Kempe, G., Alder, L., *Anal. Chem.* 2012, 84, 1474–1482.
[63] Kruve, A., Leito, I., Herodes, K., *Anal. Chim. Acta* 2009, 651, 75–80.
[64] Kloepfer, A., Quintana, J. B., Reemtsma, T., *J. Chromatogr. A* 2005, 1067, 153–160.
[65] Gangl, E. T., Annan, M., Spooner, N., Vouros, P., *Anal. Chem.* 2001, 73, 5635–5644.
[66] Stahnke, H., Reemtsma, T., Alder, L., *Anal. Chem.* 2009, 81, 2185–2192.
[67] Ferrer, C., Lozano, A., Agüera, A., Girón, A. J., Fernández-Alba, A. R., *J. Chromatogr. A* 2011, 1218, 7634–7639.
[68] Hernando, M. D., Suárez-Barcena, J. M., Bueno, M. J. M., Garcia-Reyes, J. F., Fernández-Alba, A. R., *J. Chromatogr. A* 2007, 1155, 62–73.
[69] Ito, S., Tsukada, K., *J. Chromatogr. A* 2002, 943, 39–46.
[70] Chin, C., Zhang, Z. P., Karnes, H. T., *J. Pharm. Biomed. Anal.* 2004, 35, 1149–1167.
[71] Liang, H. R., Foltz, R. L., Meng, M., Bennett, P., *Rapid Commun. Mass Spectrom.* 2003, 17, 2815–2821.
[72] Wu, G., *Amino Acids* 2009, 37, 1–17.
[73] Douglas, A. E., *J. Exp. Bot.* 2006, 57, 747–754.
[74] Wahl, O., Holzgrabe, U., *Talanta* 2016, 154, 150–163.
[75] Piraud, M., Vianey-Saban, C., Petritis, K., Elfakir, C., Steghens, J.-P., Morla, A., Bouchu, D., *Rapid Commun. Mass Spectrom.* 2003, 17, 1297–1311.
[76] Callejón, R. M., Troncoso, A. M., Morales, M. L., *Talanta* 2010, 81, 1143–1152.
[77] Gómez-Ariza, J. L., Villegas-Portero, M. J., Bernal-Daza, V., *Anal. Chim. Acta* 2005, 540, 221–230.
[78] Rebane, R., Herodes, K., *Anal. Chim. Acta* 2010, 672, 79–84.
[79] Casal, S., Alves, M. R., Mendes, E., Oliveira, M. B. P. P., Ferreira, M. A., *J. Agric. Food Chem.* 2003, 51, 6495–6501.
[80] Alcázar, A., Ballesteros, O., Jurado, J. M., Pablos, F., Martín, M. J., Vilches, J. L., Navalón, A., *J. Agric. Food Chem.* 2007, 55, 5960–5965.
[81] Gabel-Jensen, C., Odgaard, J., Skonberg, C., Badolo, L., Gammelgaard, B., *J Anal Spectrom* 2009, 24, 69–75.
[82] Yang, W.-C., Mirzaei, H., Liu, X., Regnier, F. E., *Anal. Chem.* 2006, 78, 4702–4708.

[83] You, J., Zhao, W., Liu, L., Zhao, X., Suo, Y., Wang, H., Li, Y., Ding, C., *Talanta* 2007, 72, 914–925.
[84] Santa, T., *Biomed. Chromatogr.* 2011, 25, 1–10.
[85] Rebane, R., Advanced Method Development Strategy for Derivatization LC/ESI/MS, University of Tartu, 2012.
[86] Iwasaki, Y., Nakano, Y., Mochizuki, K., Nomoto, M., Takahashi, Y., Ito, R., Saito, K., Nakazawa, H., *J. Chromatogr. B* 2011, 879, 1159–1165.
[87] Ziegler, J., Abel, S., *Amino Acids* 2014, 46, 2799–2808.
[88] Beaudry, F., Guénette, S. A., Winterborn, A., Marier, J.-F., Vachon, P., *J. Pharm. Biomed. Anal.* 2005, 39, 411–417.
[89] Rebane, R., Herodes, K., *J. Chromatogr. A* 2012, 1245, 134–142.
[90] Chan, E. C. Y., Wee, P. Y., Ho, P. Y., Ho, P. C., *J. Chromatogr. B* 2000, 749, 179–189.
[91] Lien, G.-W., Chen, C.-Y., Wang, G.-S., *J. Chromatogr. A* 2009, 1216, 956–966.
[92] Inagaki, S., Tano, Y., Yamakata, Y., Higashi, T., Min, J. Z., Toyo'oka, T., *Rapid Commun. Mass Spectrom.* 2010, 24, 1358–1364.
[93] You, J., Ming, Y., Shi, Y., Zhao, X., Suo, Y., Wang, H., Li, Y., Sun, J., *Talanta* 2005, 68, 448–458.
[94] Rebane, R., Rodima, T., Kütt, A., Herodes, K., *J. Chromatogr. A* 2015, 1390, 62–70.
[95] Rebane, R., Kruve, A., Liigand, J., Liigand, P., Leito, I., *manuscript* 2017.
[96] Salazar, C., Armenta, J. M., Shulaev, V., *Metabolites* 2012, 2, 398–428.
[97] Timperio, A. M., Fagioni, M., Grandinetti, F., Zolla, L., *Biomed. Chromatogr.* 2007, 21, 1069–1076.
[98] Carpino, L. A., Han, G. Y., *J. Org. Chem.* 1972, 37, 3404–3409.
[99] Alaiz, M., Girón, J., Hidalgo, F. J., de la Maza, M. P., Millán, F., Zamora, R., Vioque, E., *Synthesis* 1989, 7, 544–547.
[100] SANTE/11945/2015, Guidance Document on Analytical Quality Control and Method Validation Procedures for Pesticides Residues Analysis in Food and Feed, 2015.
[101] Rebane, R., Herodes, K., *J. Agric. Food Chem.* 2008, 56, 10716–10720.
[102] Rebane, R., Herodes, K., *Eur. J. Mass Spectrom.* 2012, 18, 71–75.
[103] Backe, W. J., Field, J. A., *Environ. Sci. Technol.* 2012, 46, 6750–6758.
[104] Kebarle, P., Tang, L., *Anal. Chem.* 1993, 65, 972–986.
[105] Wang, L.-Q., Zeng, Z.-L., Su, Y.-J., Zhang, G.-K., Zhong, X.-L., Liang, Z.-P., He, L.-M., *J. Agric. Food Chem.* 2012, 60, 6359–6363.
[106] Sõukand, R., Kalle, R., *J. Ethnopharmacol.* 2013, 150, 162–174.
[107] Kováčik, J., Grúz, J., Klejdus, B., Štork, F., Hedbavny, J., *Food Chem.* 2012, 131, 55–62.
[108] van Eijk, H. M. H., Suylen, D. P. L., Dejong, C. H. C., Luiking, Y. C., Deutz, N. E. P., *J. Chromatogr. B* 2007, 856, 48–56.
[109] Molins-Legua, C., Campins-Falco, P., Sevillano-Cabeza, A., *Analyst* 1998, 123, 2871–2876.

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CURRICULUM VITAE

Name:	Maarja-Liisa Oldekop
Born:	15.12.1988, Rapla, Estonia
Citizenship:	Estonian
Address:	University of Tartu, Institute of Chemistry, Ravila 14a, Tartu, 50411, Estonia
E-mail:	maarja-liisa.oldekop@ut.ee

Education

2013– …	University of Tartu, doctoral studies
2011–2013	University of Tartu, master's studies, MSc
2008–2011	University of Tartu, bachelor's studies, BSc

Professional employment

January 2013– … University of Tartu, Testing Centre, quality manager

Professional self-improvement

Seminar for validation and validation software VaLChrom, ValChrom, 30.06.2015

SFC – Principles, Instrumentation, Method Development, and Applications, Geneva, Switzerland, 21.06.2015

Noorte liidrite juhtimiskool, DD Akadeemia, 12–14.06.2015

Estimation of Measurement Uncertainty in Chemical Analysis, Lifelong Learning Centre of the University of Tartu, 02.03.–12.04.2015

Training for quality management systems internal auditor, Koolituskeskus Reiting, 20.01.2014

Biohit's Pipetting Academy, 04.04.2013

Scientific publications

1. 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. Journal of Chromatography B, 2012, 904, 99–106.
2. Rebane, R.; Oldekop, M-L.; Herodes, K. Matrix influence on derivatization and ionization processes during selenoamino acid liquid chromatography electrospray ionization mass spectrometric analysis. Journal of Chromatography B, 2014, 955–956, 34–41.
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7. Oldekop, M-L.; Herodes, K.; Rebane, R. Dependence of matrix effect on ionization polarity during LC-ESI-MS analysis of derivatized amino acids in some natural samples. European Journal of Mass Spectrometry. First published June, 6, 2017, DOI: 10.1177/1469066717711026
8. Oldekop, M-L.; Herodes, K.; Rebane, R. Comparison of amino acid derivatization reagents for liquid chromatography atmospheric pressure chemical ionization mass spectrometric analysis of seven amino acids in tea extract. *Submitted to International Journal of Mass Spectrometry.*

ELULOOKIRJELDUS

Nimi:	Maarja-Liisa Oldekop
Sünniaeg:	15.12.1988, Rapla, Eesti
Kodakondsus:	Eesti
Aadress:	Tartu Ülikool, keemia instituut, Ravila 14a, Tartu, 50411, Eesti
E-mail:	maarja-liisa.oldekop@ut.ee

Haridus

2013– …	Tartu Ülikool, doktoriõpe
2011–2013	Tartu Ülikool, magistriõpe, MSc
2008–2011	Tartu Ülikool, bakalaureuseõpe, BSc

Teenistuskäik

Jaanuar 2013– …	Tartu Ülikool, Katsekoda, kvaliteedijuht

Erialane enesetäiendus

Valideerimise ja valideerimistarkvara VaLChrom seminar, ValChrom, 30.06.2015

SFC – Principles, Instrumentation, Method Development, and Applications, Genf, Šveits, 21.06.2015

Noorte Liidrite Juhtimiskool 2015, DD Akadeemia, 12–14.06.2015

Estimation of Measurement Uncertainty in Chemical Analysis, Tartu Ülikooli elukestva õppe keskus, 02.03.–12.04.2015

Kvaliteedijuhtimissüteemi siseaudiitori koolitus, Koolituskeskus Reiting, 20.01.2014

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2. Rebane, R.; Oldekop, M-L.; Herodes, K. Matrix influence on derivatization and ionization processes during selenoamino acid liquid chromatography electrospray ionization mass spectrometric analysis. Journal of Chromatography B, 2014, 955–956, 34–41.
3. Oldekop, M-L.; Herodes, K.; Rebane, R. Study of the matrix effects and sample dilution influence on the LC-ESI-MS/MS analysis using four derivatization reagents. Journal of Chromatography B, 2014, 967, 147–155.
4. Kruve, A.; Rebane, R.; Kipper, K.; Oldekop, M-L.; Evard, H.; Herodes, K.; Ravio, P.; Leito, I. Tutorial review on validation of liquid chromatography–

mass spectrometry methods: Part I. Analytica Chimica Acta, 2015, 870, 29−44.
5. Kruve, A.; Rebane, R.; Kipper, K.; Oldekop, M-L.; Evard, H.; Herodes, K.; Ravio, P.; Leito, I. Tutorial review on validation of liquid chromatography–mass spectrometry methods: Part II. Analytica Chimica Acta, 2015, 870, 8−28.
6. Kivirand, K.; Sõmerik, H.; Oldekop, M-L.; Rebane, R.; Rinken, T. Effect of spermidine and its metabolites on the activity of pea seedlings diamine oxidase and the problems of biosensing of biogenic amines with this enzyme. Enzyme and Microbial Technology, 2016, 82, 133−137.
7. Oldekop, M-L.; Herodes, K.; Rebane, R. Dependence of matrix effect on ionization polarity during LC-ESI-MS analysis of derivatized amino acids in some natural samples. European Journal of Mass Spectrometry. First published June, 6, 2017, DOI: 10.1177/1469066717711026
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