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Study of ionization efficiencies for derivatized compounds in LC/ESI/MS and their application for targeted analysis

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III **Gornischeff, Artur**; Kruve, Anneli; Rebane, Riin (2020). Characterization of wines with liquid chromatography electrospray ionization mass spectrometry: quantification of amino acids via ionization efficiency values. Journal of Chromatography A, 1620:461012, 1–7. 10.1016/j.chroma.2020.461012.

Author's contribution

Paper I: Main person responsible for planning and writing the manuscript. Performed all experimental work.

Paper II: Performed part of the experimental work. Participated in the writing of the manuscript.

Paper III: Main person responsible for planning and writing the manuscript. Performed all experimental work.

ABBREVIATIONS

AQC	6-aminoquinolyl-N-hydroxysuccinimidyl carbamate
CID	collision-induced dissociation
DEEMM	diethyl ethoxymethylenemalonate
ESI	electrospray ionization
Ezfaast	reagent kit name for propyl chloroformate (PrCl)
HPLC	high performance liquid chromatography
IE	ionization efficiency
LC	liquid chromatography
LC/ESI/MS	liquid chromatography electrospray ionization mass spectrometry
LC/ESI/MS/MS	liquid chromatography electrospray ionization tandem mass spectrometry
LC/FL	liquid chromatography with fluorescence detection
LC/UV	liquid chromatography with ultraviolet detection
LoD	limit of detection
log*IE*	logarithm of ionization efficiency value in positive ion scan mode (also logIE_{scan})
logRF_{MRM}	logarithm of response factor in MRM mode
MS	mass spectrometry
MS1	first mass analyzer in tandem mass spectrometry
MS^n	multi-stage mass spectrometry
PrCl	propyl chloroformate
RF	response factor

INTRODUCTION

Liquid chromatography electrospray ionization (tandem) mass spectrometry (LC/ESI/MS/MS) in combination with derivatization has been a versatile and powerful tool for analyzing amino acids in many different matrices nearly 30 years for now. The backbone of accurate quantitation in LC/MS is the use of standard substances, since ionization efficiency (IE), i.e. to what extent different compounds ionize in the MS source, vary over several magnitudes. This is also why the aspects of IE have been thoroughly studied over the past ten years.

There is no absolute scale available for IE, but there is a relative logarithmic scale of IE-s (log*IE*) that has been developed into a practical tool for better characterization of different compounds and their IEs. These scales, which are measured in constant system, line up compounds according to their ability to generate gas-phase ions in the source. Values of *IE* for different compounds are anchored to reference compound, which gives practical way of comparing their ionization efficiencies.

Until now, majority of IE research has been related to non-targeted analysis, where no preselection of compounds is made, when screening analysis are performed. However, from the practical point of view, it would be beneficial, if measured (or predicted) log*IE* values could be used for estimating concentration also in targeted analysis. More importantly, since most practical applications are using MS/MS systems, since it provides much lower detection limits and enhanced selectivity, a quantitation based on the log*IE* values in the MS/MS would be beneficial. Essentially, this would give a possibility to measure hundreds of compounds without the use of standard substances.

Therefore, the aim of my doctoral thesis is to provide a standard substance free quantitation for targeted analysis using signal response factors (RF) from MS/MS. In order to develop such an approach, analysis of amino acids through derivatization is chosen as the model analysis. The reasons being that analysis on amino acids in various matrices are constantly used for different applications and therefore this approach would be applicable to fields from food to medical research. In addition, the use of derivatization reagent allows to somewhat control the structure of a molecule which is crucial for such a new approach.

In order to achieve the aim of this thesis, firstly, the method for measuring log*IE* of derivatives have to be developed that would take into account the aspects of LC/MS analysis and secondly, fragmentation patterns of different derivatized amino acids need to be examined to see if it would be possible to estimate response factors from MS/MS. The final goal of the thesis is to apply obtained log*IE* and log*RF* values of derivatized compounds for estimating concentrations in beer, wine and tea. This would be highly beneficial to estimate analyte concentration in the sample in very low concentrations and compare beverages and their origin without using standard substances.

REVIEW OF LITERATURE

LC/ESI/MS/(MS) analysis

Liquid chromatography (LC) coupled with mass spectrometry (MS) is a widely used analytical technique to quantitatively analyze different substances like amino acids, biogenic amines, pharmaceuticals etc. LC/MS provides low quantitation limits, structural elucidation and quite fast selective analysis compared to amino acid analyzers.[1] It is very common to use tandem MS (MS/MS) system for quantitative and qualitative analysis, since with MS/MS more sensitive and selective results are obtained. It involves two mass analyzers in conjugation with dissociation process, which changes the mass of an ion from first mass analyzer.[2] In general, one can choose between non-targeted (MS) and targeted (suspect) (MS/MS) screening when combing LC with MS. With MS, only a molecular mass (mass to charge ratio) of an analyte can be seen. On the other hand, it gives a possibility to scan for existence of large amount of different suspect compounds and gives a lot of information about the sample. In comparison, when MS/MS experiment is run, preselected ions from MS1 can be isolated, fragmented and detected in MS2, which gives more information about the molecular structure of the analytes and could identify the analyte more precisely. MS/MS also provides much lover limits of detection (LoD). The most used tandem MS system is triple quadrupole mass spectrometry, which consists of three consecutives quadrupoles, from which first is mass analyzer (Q1), second (Q2) is used for fragmenting the ions selected by the first mass analyzer and the third quadrupole (Q3) is mass analyzer for detecting ions generated in collision quadrupole. Hence the name – QqQ, or triple quadrupole.

In general, for LC/MS/(MS) analysis, standard substances are used for constructing calibration graphs to quantitatively measure concentration of analytes in question.[3–5] One of the biggest problems when developing the LC/MS/MS method for analyzing different analytes, including food authentication studies, is that the methods and models developed in one laboratory are not directly applicable in another laboratory.[6] This complication comes from the fact that authentication relies both on the compounds and their amounts present in the sample. Signal responses for analytes are not directly comparable between analytes and devices, since they give orders of magnitudes different signal intensities and different systems (devices) might give different response due to the structure of the device.[7] In case of the suspect, targeted and non-targeted screening, this is solved by comparing peak areas of the standard substances with analyte's peak areas from chromatograms to characterize the quantity of the compound.[8,9]

LC/MS is a powerful complex measurement system and many aspects should be considered before starting an analysis. Maybe one the most complex part of LC/MS analysis, when considering targeted or non-targeted analysis, is the link between LC and MS – the ion source – where analytes leaving the LC are directed into MS.

Ion source and ionization efficiency

A key prerequisite for MS experiments is the conversion of condensed-phase species into gaseous ions, which can be then transported into MS. This is usually done in the ion source. Among all different ion-sources, electrospray ionization (ESI) is most commonly used with LC/MS where solution-phased analytes are converted into gas-phase ions. It has several advantages like compatibility with LC, soft ionization and virtual absence of restrictions on the molecular mass.[10–12]

The exact mechanism of ESI is still a matter of debate and research remains active.[7,11] In ESI, analyte solution from LC is infused into capillary which holds electric potential about 2–5 kV and charged droplets of an analyte in the solution are generated through ion evaporation model (IEM).[13–17] This applies to small molecules (<1500 Da). In the case of large molecules, e.g. biomolecules, charge residue model (CRM) is used.[16] For IEM, schematic presentation is given in **Figure 1**. Both models are currently recognized.

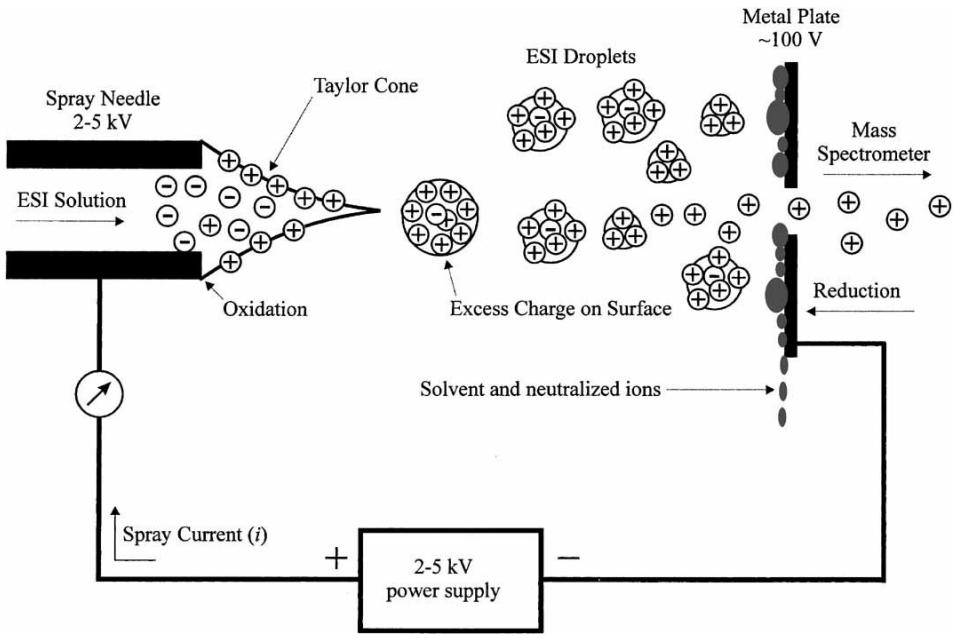

Figure 1. Schematic of the electrospray ionization process. Reprinted from ref[18].

Manufacturers have different ion sources which could lead to different ionization capabilities and substances with different properties could have different ionization efficiencies. Regards to ESI source, many configurations exist – different geometry and possibility of using extra drying gas are available. The

influence of the source design and configuration has been previously studied to conclude that source design could have an important influence on the IE.[7,12,19–22]

Besides ESI, atmospheric-pressure chemical ionization (APCI) source is also used, mostly for small and low polarity molecules. It is an ionization method, where gaseous charged ions are generated by corona discharge on a solvent spray to produce generally monocharged ions.[2] Its working principle is schematically shown on **Figure 2.**

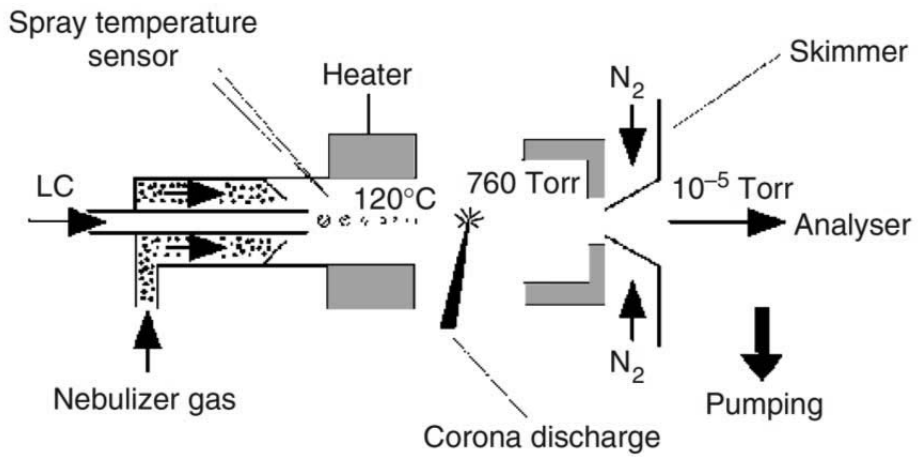

Figure 2. Schematic of the APCI process. Reprinted from ref[2].

Differently from ESI, in APCI, analytes do not have to be charged in a solution, but ionization takes place in gas phase. This is advantageous to generate ions from neutrals, which generally have low or medium polarity and are not charged in a solution. While the ionization occurs at atmospheric pressure with high collision frequency, which in turn thermalize the reactant species, rapid desolvatation reduce thermal decomposition of the analyte to produce predominantly molecular ions with very few source-fragmentation.[2] For both ionization methods, either positive ion mode via protonation or negative ion mode via deprotonation can be used.

Ionization

It is known that several factors influence the (electrospray) ionization process, such as analyte structure, solvent composition (pH, organic modifier type and content, buffer type), ion source parameters and design.[19,20,23–26] Hermans et al.[23] have shown that different physicochemical parameters like hydrophobicity, surface activity, molecular volume, and pK_a are affecting ESI efficiency in case of amino acids. Since these parameters are all related to each other, they showed

that molecular volume contributes the most affecting ionization, but pH and proton affinity may also affect ESI efficiency.

Liigand et al.[7,25] studied effects of mobile phase and the change of instruments on ionization. They concluded that in general, higher percentage of organic modifier enhances the ionization and that different instruments tend to change ionization in the same way, but errors between different instruments could be statistically significant for the same set of compounds. Kostiainen and Kauppila[24] reviewed different effects of eluent composition on the ionization. The review included comparison of solvents, additives and their pH which influence ionization in a different way for acidic or basic analytes. They included three source – ESI, APCI and APPI (atmospheric-pressure photoionization) – and compared their positive and negative mode ionization through changing parameters in eluent composition. They concluded that all three sources should be dealt with separately, as changing conditions could have diverse or enormous effects on ionization.

All these variables constitute to the term known as ionization efficiency (IE). It describes to what extent gas-phase ions are formed in the ESI source from the analyte molecules in a solution. Consequently, different analytes can have different IE when comparing their ionization in positive or negative ion mode.[27] Models have been created to predict IE on the basis of molecule structure which can take into account parameters of the molecule and eluent to predict IE.[13,20,27–29]

Ionization efficiencies of different compounds in ESI vary over several orders of magnitude and therefore it is not possible to compare and quantify compounds by simply evaluating their signal intensities.[27] Therefore, it would be beneficial, if there would be a universal IE scale which would give us more information about the IE of different molecules. This brings us to the log*IE* scale which tries to give IE some sort of a quantitative value.

log*IE* scale

As seen from the previous chapter, ionization is influenced by many factors. To compare two analytes and their ionization, many system parameters should be the same for the measurements. In order to quantitatively describe to what extent different substances form ionized particles in ionization source, IE scales for different ionization modes and compounds have been created.[27,29–32] These scales are tools for comparing compound's ionization efficiencies to each other and give users guidelines for selecting the best conditions for detecting analytes in interest.[33,34] Most of the time, it could be assumed, that compounds having similar structure to those in the scale, ionization efficiency could be predicted for these compounds from the scale values. Usually, log*IE* values covered by these scales are measured for vast choice of analytes of distinct properties. Different instruments and positive and negative ion mode have been also covered. The scales are usually constructed using relative values, because absolute log*IE* values are impossible to measure (we do not know a substance, which

would have IE of zero value). To make use of these values, all measured IEs should be linked to one compound e.g. anchored to an *anchor* substance to get relative ionization efficiencies (RIEs) for all the compounds in comparison to this anchor substance.[30]

Anchor substances which are usually used, have very high IE in order to anchor compounds to the scale and generate upper and lower limit of scale. Anchor compounds generally generate ions without any fragmentation or without forming Na-adducts and their ionization is reproducible, which allows to also anchor measured substances to the scale quite accurately.[27] IE scales are presented as logarithmic scale for easier presentation and analysis.

Knowing log*IE* values of different compounds, one has to measure only one compound from the scale as the standard to get reference point for this particular system. By then measuring analyte response and using its relative log*IE* value to this reference point, we can calculate concentration of an analyte. Knowing how good or poorly substance would ionize, i.e. knowing its log*IE* value, it would be possible to carry out quantitation without standard substances[13,35–38] or estimate the values of LoD[39].

While in the beginning, the log*IE* scale was established in positive ESI mode, in recent years scales for negative mode, sodium-adducts and even for APCI source have been developed.[29,31,32]

Negative and positive ionization mode

Both ESI and APCI can give positive and negative ions, but positive ions are far more used and studied than negative mode. It is preferred, since more compounds, mainly basic analytes, form positive ions and have better IE in positive mode.

Positive ionization mode is mainly used for small molecules which are weak bases, hence easily protonated, like drugs.[40] For acidic functional group, which easily undergoes deprotonation, negative mode could also be used. Positive ionization mode is taking place via single or multiple protonation or adduct formation. In negative ionization mode, ions are generated via deprotonation, anion adduction or simultaneously via deprotonation and adduct formation with cations.[18]

In negative mode, same number or even more analytes have similar or even better IE compared to positive ion mode out of 33 compounds as shown by Liigand et al.[33] Major advantage for negative mode is lower background noise compared to positive mode, which also contributes to lower detection limits.[18,41] For direct comparison of ionization efficiencies and log*IE* values measured in positive and negative mode, Liigand et al[33] united these scales into a single system by using reference compounds which ionize to similar extent both in positive and negative mode.

While choosing ionization mode, one has to look, which molecular properties analyte has and usually, the choice of the working mode is done according to the analyte before analysis. For different modes, models have been created, which allows to estimate analyte's response in either mode according to mole-

cular structure properties.[29,35,36] It is a need for making the best choice between an ionization mode, since it is shown, that IE of the same compound could be very different for compounds compared with positive or negative ion mode. It is also important for structure elucidation and that quantitative data would be more feasible by enhancing IE and lower the detection limits.

Adduct formation in ESI

It is generally known that both positive and negative modes give adducts. Adduct formation is a result of mobile phase additives, solvent impurities etc. and it would be beneficial to know why and to what extent adducts are formed in the source and how to control their formation. It is also possible, that adduct formation could affect ionization through (de)protonation. Adduct formation in ESI source is very common and several research papers have been written about the possible mechanisms and applications.[18,31,42,43] However, its exact mechanism is not precisely known and possibilities to control its extent in ESI source are limited.[31,44] Some attempts have been made to describe possibilities to predict, control and use adduct formation for real samples.[31,45,46] In most cases it has been shown that control of solvent composition seems to be the easiest way to keep adduct formation under control. Also, structure of the compound may influence formation of adducts. In some cases, it is shown that adduct formation gives much higher signal response compared to protonation and it is possible to use adducts for quantitative analysis.[42,43] That is why in some cases it is more reasonable to use adduct ions for determining analytes concentration instead of protonated form. Kruve et al[31] have also compiled self-consistent scale of sodium adducts and later described how mobile phase additives influence this scale.[44] They also showed that measurements in some cases could give reproducible results between day to day measurements which in turn gives future possibilities to draw conclusions and decide, if it is reasonable to use adducts for quantitative analysis or not.

On the other hand, it is generally believed that quantitation via protonation (or deprotonation) is more feasible, since adducts behavior is unexpected or unstable. Reproducibility of adduct formation could be very irreproducible due to the fact that different batches of organic solvents could have different amount of salt content. Also, interpretation of adduct MS/MS spectra is more complex for identification of compounds, since only fragment ion generated collision cell could be sodium ion, which do not give any specific information about the structure of a compound.[47] Solutions have been reported to control adduct formation like adding small amounts of ammonia or ion-pairing reagent to eluent to suppress the formation of sodium adduct formation.[44,48,49] Some works are dealing with fragmentation of adducts.[47,50,51] Du et al[50] provided rearrangement mechanism of the sodium adducts of fluorenylmethyloxycarbonyl-(FMOC) derivatized amino acids and came to a conclusion that during fragmentation of these adduct ions in MS/MS, elimination of amino acid part occurs and hydroxyl group of C-terminal was transferred to the FMOC group. Dziadosz et

al[51] have successfully used sodium-adduct fragmentation for quantitative analysis of γ-hydroxybutyrate in human serum.

Fragmentation characteristics

Fragmentation is a process, where previously ionized charged particle (precursor ion) from the ionization source is isolated and decomposed (spontaneously or as a result of some activation technique) in a collision cell into a new (another) charged particle (ion) or many particles and neutral fragment. A fragmentation reaction may be written as follows:

$$M_1^+ \rightarrow M_2^+ + M_3 \qquad \text{(Eq. 1)}$$

Charged particle M_1^+ will lose neutral fragment M_3, after what a new ion M_2^+ with a new *m/z* is formed. Fragmentation usually occurs in tandem mass spectrometry (MS/MS) and in the context of current work, we consider fragmentation only between two mass analyzers in a so-called collision cell. To clarify, fragmentation in different regions of mass spectrometer is possible. We can distinguish between source and triple quadrupole fragmentation. It could be possible, that when increasing cone voltage in the source, an ion can be energized in the transport region. This promotes collision between solvent and gas molecules and so-called in-source collision induced dissociation (CID) is induced. This will produce fragment ions in mass spectra which therefore gives overlapped spectra for all compounds, when analyzing mixtures of different analytes. This is undesirable and therefore chromatographic separation is preferred since otherwise interpreting spectra could be very difficult.[52]

Another fragmentation mechanism, which is also under study in this work, is CID in triple-quadrupole MS (MS/MS or QqQ). This is usually applied to preselected daughter ions, which are transported into second quadrupole acting as a collision cell where they are subjected to dissociation by collision gas. All fragments are scanned by the third quadrupole or only previously defined fragments are being isolated and detected.[52]

The identification of the compounds largely relies on the prediction of the fragmentation spectra of the compounds detected.[53] The fragmentation spectra are relatively characteristic to the compounds and often reveal important information about the functional groups of the compound.[54–56] Fragmentation is influenced by different molecular properties like charge location, amino acid side chain etc. Knowing how to interpret fragmentation information from MS2, gives a possibility for structure elucidation. The suspect screening relies on the identification of compounds with the aid of chemical libraries using fragmentation information from the spectra, while non-targeted screening aims at identifying compounds detected from the sample without preselecting compounds.[57]

Using MS/MS, different methods could be employed which give possibility to study fragments generated in different processes. Four main possibilities are product ion scan, precursor ion scan, neutral loss scan and selected reaction monitoring or multiple reaction monitoring.[2]

Product ion scan or daughter ion scan consists of selecting precursor ion produced in the source and determined all product ions which are generated from CID.

Precursor ion scan consists of choosing product ion in the second mass analyzer and scanning all parent ions which produce ions through reactions in collision quadrupole.

Neutral loss scan consists of scanning all reactions which lead to a loss of selected neutral fragment. In this case, two mass analyzers are scanned together with a constant mass offset.

Selected reaction monitoring (SRM) or multiple reaction monitoring (MRM – many different reactions are monitored simultaneously) consists of selecting specific fragmentation reaction. For this type of scan mode, first and second mass analyzers are set to measure only selected masses.

Every scanning mode has its own purpose, but what unites them all, is that they give some sort of structure elucidation information. Some modes are essentially used for quantitation purposes, as they allow lower quantitation limits and more selectivity when it comes to identifying different compounds. Lower LoD comes from the fact that only parent and fragment ions are selected, which significantly lowers background noise. Examples of drawbacks of fixed position mass analyzers are lower flexibility and no possibilities to extract data after initial experiment.[2]

When it comes to fragmentation of derivatized compounds, many possibilities are available how derivatization reagents could change the fragmentation of derivates. To aid fragmentation and ease identification of MS/MS spectra, different special derivatization reagents have been designed as described below. Analysis of derivatives could lead to increased or decreased fragmentation, direct fragmentation to new bonds or enhance sensitivity and selectivity of the ionization process.[58,59]

LC/ESI/MS analysis of amino acid derivatives

Amino acids are important group of analytes while they exist in food, are used to synthesize proteins in our bodies, and furthermore, they can be used to diagnose some diseases and confirm authenticity of origin of foodstuff.[60–65] Amino acids possess characteristic information about the origin of the food or beverage like beer, wine and tea which are amongst the most consumed beverages in the world. All these amino acids origin from the raw material of specific drink, e.g. cereal grain, grapes and plant leaves. The content of amino acids in beverages varies depending on geographic origin, year of production, crops used in production etc. giving them specific taste and properties. Therefore, screening of

amino acids, both targeted and non-targeted mode is becoming more and more popular in food authentication and fingerprinting studies.[8,66–70]

Biogenic amines are another important group of analytes. They are found in food (meat, dairy, fruits, seafood and fermented products),[71] and can cause severe health effects, when consumed over certain limit.[72] Ordonez et al[73] has given exhaustive overview about recent trends in the determination of biogenic amines in fermented beverages.

Due to the abovementioned reasons, it is necessary to analyze amino acids and biogenic amines in various matrices. These can be analyzed by different analytical techniques but are still foremost analyzed by LC/MS methods, mostly due to the low concentration of amino acids and biogenic amines in different samples. Regarding their small structure and low molecular mass, they have poor IEs as well as poor retention on the reversed phase liquid chromatography (RPLC). In order to increase retention and enhance detection (IE), derivatization can be used.[3,74,75] If coupled with a LC/MS method, detectability,[76–79] sensitivity and selectivity[80,81] of a method can be enhanced to reach desired LoD,[82,83] as well as the stability of the analyte and retention in chromatographic separation by RPLC.[84,85] Derivatization can also reduce matrix effects which may occur when co-eluting compounds originated from the samples are suppressing or enhancing the signal of the analyte.[86] By increasing the molecular weight on an analyte by derivatization, it is also possible to decrease background noise, since the background is generally lower in mass range.[87]

For analysis of amino acid derivatives, for years, it was popular to use ultraviolet (UV) and fluorescence (FL) detectors coupled with LC, and thus derivatization reagents were designed originally for FL and UV detection. But in recent years, amino acids are analyzed more with LC/MS/MS systems since these systems provide lower detection limits and provide more information in a form of *m/z*.

Derivatization can lead to many positive outcomes when coupled with LC/MS analysis – it will stabilize the analyte, improve its retention time and peak shape in LC. Positive effects of derivatization regarding mass spectrometry detection are the following:

- Adding non-polar hydrophobic tag, ions are more readily going into gas phase, since they prefer droplet surface which gives higher response;[88]
- Introduces chargeable or easily ionizable moieties, which improve IE for neutral compounds;[78,89]
- Adding derivative moiety, it generates a bond between target compound and reagent that is easy to cleave in collision-induced dissociation (CID)[58] which in turn aid fragmentation for tandem mass spectrometry (MS/MS);[90]
- Helps to improve structure elucidation studies, because only certain functional groups could be derivatized;[58,91]
- Extend linear dynamic range.[92,93]

For amino acid derivatization, different derivatization reagents have been used and many review articles have been written about the derivatization methods and derivatization reagents for LC/MS.[87,90,94–96] Also, some studies bring out positive and negative aspects of different derivatization reagents comparing their reaction time, selectivity, ease of use etc. Uutela et al[63] have shown that by comparing three reagents, FMOC- and propyl chloroformate- (PrCl) derivates show best chromatographic retention compared to butanol-derivates. Butanol reacts with carboxyl group instead and leaves amino group unmasked which reduce retention under chromatographic conditions. They also showed that PrCl showed less ion source fragmentation than FMOC and butanol and in case of FMOC and PrCl, sodium-adduct formation was common.

Lkhagva et al[97] compared different amine-derivatization methods for metabolites with LC/MS/MS. They showed that optimal pH for elution could be different for reagents and concluded that molecules having higher hydrophobicity (FMOC and Dabsyl) are beneficial to separate from interfering compounds in LC. They also compared IE to show that FMOC and Marfey's reagent have lower IE compared with Dansyl, Dabsyl and o-phthalaldehyde (OPA). Also, all reagents except OPA, showed constant fragment ion in MS/MS regardless of analyte structure, which indicate similar fragment loss for different compounds.

Rebane et al[98] have done an exhaustive comparison of derivatization procedure, repeatability, LC separation, LoQ, LoD etc to conclude that DEEMM is most suitable for amino acid analysis out of TAHS, FOSF, DNS and FMOC for LC/ESI/MS/MS. Oldekop et al[99] on the other hand studied derivatization reagents for LC/APCI/MS by the example of seven amino acid. They concluded that only FOSF is not suitable for APCI source out of DEEMM, DBEMM, DNS and FMOC. All reagents differ from each other by giving different matrix effects and sensitivity.

But there are also some derivatization reagents which are specially designed to have good properties for LC/MS/(MS) analysis. This means that the structure of the derivative should be suitable for MS detection, i.e. allows sensitive analysis in LC/MS and LC/MS/MS. Therefore, firstly, the derivate must be in its ionic form in the solution phase or be chargeable trough adduct formation in gas-phase reaction.[18] Secondly, it should have non-polar sidechain or region because these compounds could be easily separated in RPLC from salts and other interfering compounds that could suppress signal in an ion source.[84] And thirdly, derivatization reagent should carry chargeable fragment which will induce fragmentation efficiently upon CID and generate an intense and specific product ion for the sensitive MS/MS detection.[84,87]

All abovementioned properties are important for derivatization for LC/MS analysis and one should keep these in mind when making the choice between reagents. Derivatization reagents are studied for different applications and sometimes no difference is made while choosing reagent specially designed for UV, FL or MS detection, e.g. originally designed reagents could be used for MS detection.

Derivatization reagents

As mentioned above, for amino group derivatization, different reagents for different purposes have been previously studied by others. **Table 1** shows different reagents and suitability for analytes. There have been specially developed reagents for different detection types, e.g. UV, FL or MS detection. When reagent is specially design for MS/MS analysis, often simple fragmentation pattern is expected, like producing common fragment from reagent side for all analytes in questions. There are several examples, where *m/z* 177,1 or 171,0 yields from derivatized amino acids breaking reagent skeleton from molecular ion.

Table 1. Overview of the derivatization reagents for LC/MS/MS analysis.

Reagent	Application	Specific fragment ion MS
Propyl chloroformate (PrCl)[63]	Amino acids	N/A
9-fluorenylmethyl chloroformate (FMOC)[5,63,98,100]	Amino acid	263,0
Dansyl chloride (DNS)[98,101]	Alcohols, amines, amino acids	252,0
Diethyl ethoxymethylenemalonate (DEEMM)[3,79,102–104]	Amino acids	N/A
2,5-dioxopyrrolidin-1-yl N-tri(pyrrolidino)phosphoranylideneamino carbamate (FOSF)[98]	Amino acids	298,0
p-N,N,N-trimethylammonioanilyl N′-hydroxysuccinimidyl carbamate iodide (TAHS)	Amino acids, amines	177,1
6-aminoquinolyl-N-hydroxysuccinimidyl carbamate (AccQ-Tag or AQC)[106–109]	Amino acids	171,0

Some of these reagents have been included in the standard amino acid analysis kits like 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate (AQC) by Waters[110], and propyl chloroformate by Phenomenex[111], providing all the necessary materials for fast, economic and time-saving analysis of free amino acids. These kits include all the reagents, often standard calibration solutions, materials and some even chromatographic columns for reproducible results between different laboratories. There are kits for direct amino acid analysis with derivatization,[112] kits designed for use with LC/FL[110] and LC/UV[113] and kits designed for use with LC/MS/MS systems.[111,114]

Two reagents, that are extensively used in this study were diethyl ethoxymethylenemalonate, shortly DEEMM and 6-aminoquinolyl-N-hydroxysuc-

cinimidyl carbamate (AQC). DEEMM has been tested for many aspects, including suitability for LC/MS/MS analysis, and several studies have been published.[65,79,86,98,99,102,104] It has many advantages like short reaction time, low LoD values, good chromatographic separation of the derivatives and wide linear range and compatibility with LC/ESI/MS method for separating and analyzing amino acids together with low matrix effects.[79,98] DEEMM has been used in several studies and in many matrixes, e.g. beer[3], cheese[115], honey[65,79,116] and wines[117].

AQC is widely used for different applications ranging from human plasma samples to amino acid analysis in beverages.[75,107,108,118,119] Although it was originally designed for fluorescence detection,[119,120] more and more studies are published using it for MS detection in different matrixes like skin[121], water[122] and tea[118]. Kabelova et al[123,124] have used AQC for determination of amino acids in cheeses from the Czech market with FL detection and for comparison of Czech and foreign beer brands with MS detection. Cizkova et al[125] have used AQC for determination of amino acids in beers. Fiechter et al[126] have used AQC reagent for free amino acid determination in wines.

For all these studies, standard substances were used to quantitatively measure amino acids in selected matrixes. One of the biggest time- and cost savings would be the elimination of standard substances from the analysis sequence which could be done using log*IE* values as described above to evaluate the concentrations of the analytes. For non-targeted scanning, log*IE* values can be used, but since most of the LC/MS experiments nowadays are run in MS/MS mode, there is a need to investigate if log*IE* values could be used to estimate response factor (RF) in MRM mode.

Benefits of knowing RF values for MRM mode

Since LC/MS signal intensities are unrepresentative of the compound concentration, standard intensities for every compound are needed to compare it with analyte signal in real sample for concentration determination. This comes from the fact that compounds have very different IEs in ESI source and since peak areas, which is the main output of signal intensity (directly related to analyte concentration), are used to measure analyte concentration. To overcome the need of standard substance, we should know to what extent each compound is ionized. When using MS/MS for quantitation, it is also important to consider fragmentation pattern and its efficiency, since this determines how many ions finally reach to the detector.

In this doctoral thesis I try to provide a solution to use previously mentioned IE to eliminate the need of standard substance. This approach would take into account derivatization, ionization and fragmentation characteristics to estimate amino acid concentration in different beverages. This could be done by using the response factor (RF) values for analyte in MRM mode. RF in MRM mode is

an ion count measured as signal intensity by the third mass analyzer for each analyte and besides ionization, it also covers fragmentation efficiency.

However, since all log*IE* measurements so far have been performed in the MS not in MS/MS systems[30] they cannot be directly applied to characterize the RF in the MRM mode, since MRM measurements also incorporates fragmentation which gives an extra variable that needs to be accounted for. Also, signal intensities in MS/MS are magnitudes of order lower than in MS, which automatically means that RF values are lower when anchored to the same compound as for log*IE* measurements in MS mode. This is due to the fact, that fewer ions reach the detector and cannot be compared to log*IE* values that are obtained on MS1 mode.

Though IE in ESI/MS has been thoroughly researched throughout the last decade,[18,27,29–32,127] none of these studies focus on the IE of derivatized compounds. There have been some preliminary studies relating to IE of the derivatized compounds,[26,82,128] but these results are not linked to the results of existing log*IE* scales. Furthermore, no attempts have been made to align different derivatized compounds into existing scales or tried to make self-consistence scale.

The practical advantage of this approach would be the possibility to perform targeted screening of compounds to authenticate and compare different beverages. Let's assume a wine producer wants to confirm whether particular batch of wine is within limits of quality. For that, he should evaluate amino acid concentrations or their ratios to each other in every batch they produce. To exactly determine analyte concentration in samples, he needs standard substances, but which is not time and cost effective. In order to overcome this problem, previously measured or estimated IE or RF values could be used to eliminate the need for standard substances. This will allow the producer to make estimation of analyte concentration with a certain error, which in many cases is actually satisfactory enough to make preliminary decisions about the quality of a product or further need of quantitative analysis.

EXPERIMENTAL

Chemicals and materials

HPLC grade methanol (MeOH) and acetonitrile (MeCN) were obtained from Sigma Aldrich, Germany. Borate buffer was made from boric acid (Sigma-Aldrich) and sodium hydroxide (Sigma-Aldrich). Derivatization reagent DEEMM was purchased from Fluka, Germany, FMOC-Cl was from Aldrich. Other derivatization reagents were purchased as commercially available amino acid analysis kits from Phenomenex (EZ:faast™, PN: KH0-7337)[111] and Waters (AccQ·Fluor™, cat no. 186003836).[110]

The compounds included in the studies with DEEMM and FMOC were mainly amino acids but also some metabolites and amines. The list is following: Cadaverine, spermidine, histamine, normetanephrine, metanephrine, s-methyl-L-cysteine (selenocysteine, Sec), selenomethionine, histidine (His), tryptophan (Trp), arginine (Arg), proline (Pro), asparagine (Asn), aspartic acid (Asp), (Aminomethyl)phosphonic acid (AMPA), leucine (Leu), serine (Ser), phenylalanine (Phe), cysteine (Cys), glutamic acid (Glu), lysine (Lys), glutamine (Gln), isoleucine (Ile), methionine (Met), valine (Val) and threonine (Thr), altogether 25 compounds. All chemicals were purchased either from Sigma or Fluka and were with purity of 97 % or higher. For reference compound tetraethylammonium (Et_4N^+) perchlorate salt from Alfa-Aesar (USA) was used. For eluent composition studies, in-house synthesized[86,129] pure DEEMM-β-Ala, DEEMM-Gly and DEEMM-Phe were used. Purity has been previously confirmed by LC/UV.[130]

Amino acid mixture from Phenomenex (part no. AL0-7500) with concentration 200 (2.0×10^{-4} M) nmol/mL in ultra-pure water was used for measurements for log*IE* and logRF_{MRM} with AQC, PrCl and DEEMM containing following amino acids: 1-methyl-histidine (1MHis), 3-methyl-histidine (3MHis), α-Aminoadipic acid (Aaa), α-Aminobutyric acid (Aba), alanine (Ala), arginine (Arg), asparagine (Asn), aspartic acid (Asp), β-Aminoisobutyric acid (βaib), Citrulline (Cit), Cystine (C-C), 4-Aminobutyric acid (Gaba), glycine (Gly), glutamic acid (Glu), glutamine (Gln), histidine (His), 4-Hydroxyproline (Hyp), isoleucine (Ile), leucine (Leu), lysine (Lys), methionine (Met), Ornithine (Orn), phenylalanine (Phe), proline (Pro), Sarcosine (Sar), serine (Ser), threonine (Thr), tryptophan (Trp), Tyrosine (Tyr) and valine (Val) altogether 30 compounds. For reference (anchor compound) compound tetraethylammonium (Et_4N^+) perchlorate salt from Alfa-Aesar (USA) was used.

For log*IE* measurements, 20 and 2 nmol/mL solutions were selected for scan mode measurements and 0.2 and 0.02 nmol/mL concentrations for logRF_{MRM} measurements. For constructing calibration graphs to measure amino acid concentration in beverage samples following concentrations: 10, 5, 2, 1, 0.5, 0.2, 0.1 and 0.02 nmol/mL both in scan and MRM measurement mode. From these solutions six dilutions were injected for calibration graph (1, 1.25, 1.67, 2, 2.5, and 5-fold) by autosampler.

Derivatization of amino acids

DEEMM. The procedure originally presented in reference[79] was used for derivatization, but volumes were reduced as in reference[86]. To 250 µL of sample solution in a chromatographic vial, 375 µL of DEEMM solution in methanol (1:50, 21.4 mg/L) and 875 µL of borate buffer solution (0.75 M, pH = 9) were added. Vials were mixed moderately and were kept in the dark at room temperature and the analysis was carried out after 24 h. Solutions were kept in the autosampler at 4 °C until injection, but no longer than 48 h. Derivatization completeness was checked by looking for the *m/z* of the underivatized compound from the chromatograms of injected derivatives. In all cases the original compound peak was absent from chromatograms and derivatization yield is expected 100 %. Derivatization reaction is presented in **Figure 3.**

Propyl chloroformate. EZ:faast™ kit consists of derivatization reagent (propyl chloroformate, hereinafter referred as PrCl), internal standard solution, eluting medium (mixture of sodium hydroxide and n-propanol), washing solution (n-propanol), organic solutions (I – chloroform; II – iso-octane) and amino acid standard mixture. Derivatization procedure including SPE was a little more complicated than other used reagents. Manufacturer procedure was followed. To 100 µL of the sample 100 µL of the internal standard solution was added. Mixture was pipetted through SPE tip into a syringe. 200 µL of the washing solution was pipetted into sample preparation vial and then passed through SPE tip into a syringe. 200 µL eluting medium was pipetted into sample preparation vial and then SPE was wetted and washed out into sampling vial from SPE tip. 50 µL of chloroform (including derivatization reagent) was added into sample preparation vial. Vial was vortexed for about 5–8 seconds. Vial was let to stand at least one minute and then vortexed again and let to stand one more minute. 100 µL of iso-octane was added into a sample vial and vortexed for about 5 seconds. From the upper organic layer, 50 µL of the aliquot was transferred into autosampler vial and evaporated into dryness under nitrogen. Sample was re-dissolved in 0.1 % formic and methanol mixture (98:2). Derivatization reaction is presented in **Figure 3.**

AQC. AccQ·Fluor™ kit consists of AQC reagent (2,5-Dioxopyrrolidin-1-yl quinolin-6-ylcarbamate; 6-Aminoquinolyl-N-hydroxysuccinimidyl carbamate, hereinafter referred as AQC), acetonitrile for dissolving the reagent powder and 0.2 mM sodium borate buffer, pH 8.8. To a 70 µL of borate buffer 10 µL of sample was added, followed by 20 µL of the derivatization reagent (10 mM in acetonitrile). Mixture was vortexed and heated for 10 minutes at 55 °C. Derivatization reaction is presented in **Figure 3.**

FMOC-Cl. The procedure from reference[86] was followed except reaction was ended with glycine, instead of histidine. To 300 µL of sample, 300 µL of borate buffer (0.75 M, pH 9) and 300 µL of FMOC-Cl solution (1 mg/L in MeCN)

were added. Derivatization reaction was ended after 30 min by adding 300 µL of glycine solution (8 mg/mL). Derivatization reaction is presented in **Figure 3.**

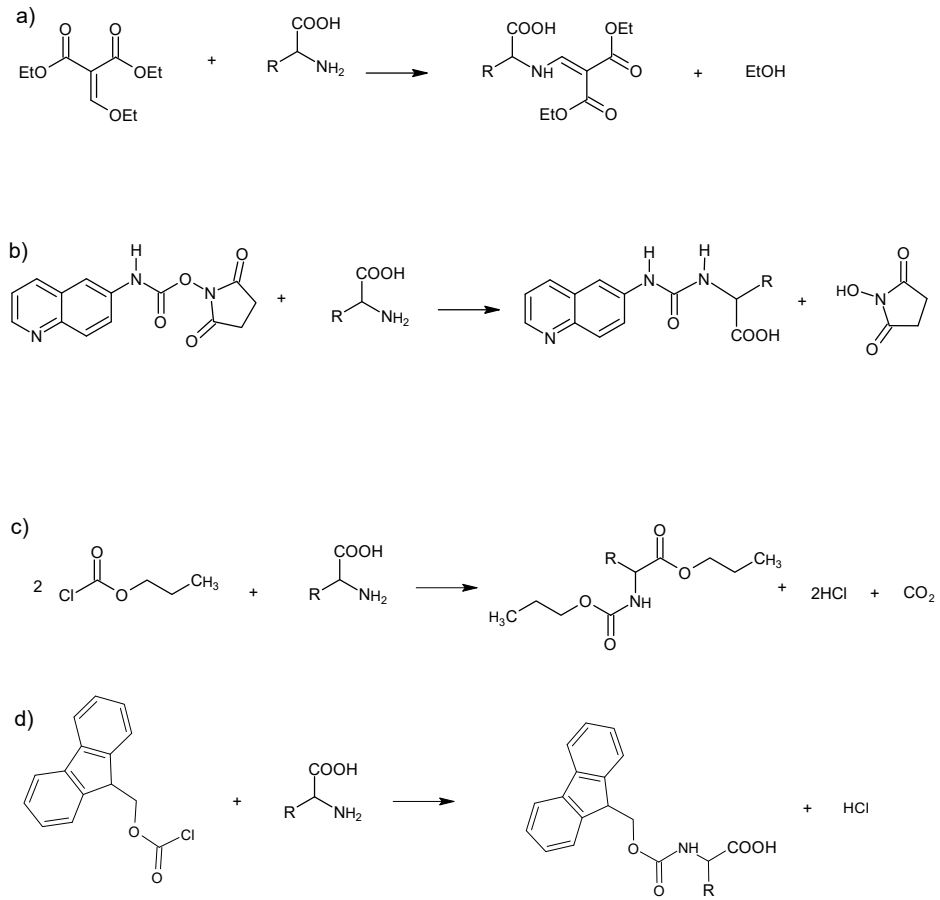

Figure 3. Reaction of amino acids with different derivatization reagents. a) DEEMM; b) AQC; c) PrCl; d) FMOC-Cl

LC-ESI-MS-MS conditions

For separating derivatized compounds, the following gradient profile was used: 0 – 3 min, 2 %; 3 – 10 min, 2 – 100 %; 10–12 min, 100 % B. Component A was 0.1 % formic acid in water and component B was methanol. Flow rate was 0.4 mL/min, column temperature was 40 °C and 10 µL of sample was injected. Agilent Zorbax C18 reversed phase column with dimensions 50x2.1 mm with particle size 1.8 µm and with a corresponding pre-column with dimensions 5x2.1 mm from Agilent was used to separate compounds. The same method was applied to all compounds under investigation.

Studied compounds analyzed without derivatization were injected separately (one analyte per injection), since they have short retention times (0.32 – 0.79 min for most compounds and ~5.1 min for tryptophan) and are therefore more prone to matrix effects from each other when injected together in a solution. Since without derivatization there are no additional compounds in the solvents, additional effects from other co-eluting components is not expected.

Automatic dilution

In the case of constructing calibration graphs in order to obtain slope values, derivatization was carried out in chromatographic vials as in ref[86]. Solution with the highest concentration was injected as prepared. All lower concentration points were prepared from the highest concentration solution by diluting them in the autosampler. Results showed that pooled standard deviation over both manual and automatic dilution measurements made on different days (n=2–3) was 0.05 log*IE* units when automatic dilution system was used.

Measuring effects of solvent composition in flow injection mode

For eluent composition effects, six different mobile phase compositions in flow injection mode were studied in isocratic mode:

1) 0 % (v/v) of methanol and 100 % (v/v) 0.1 % formic acid in ultra-pure water
2) 20 % (v/v) of methanol and 80 % (v/v) 0.1 % formic acid in ultra-pure water
3) 40 % (v/v) of methanol and 60 % (v/v) 0.1 % formic acid in ultra-pure water
4) 60 % (v/v) of methanol and 40 % (v/v) 0.1 % formic acid in ultra-pure water
5) 80 % (v/v) of methanol and 20 % (v/v) 0.1 % formic acid in ultra-pure water
6) 100 % (v/v) of methanol and 0 % (v/v) 0.1 % formic acid in ultra-pure water

Selection of compounds were also dissolved in respective mobile phase and injected with autosampler in flow injection analysis mode, to get respective solvent composition in the ESI source.

Comparing different sets of conditions

For comparing log*IE* values with previously obtained values published in the literature and in order to fit them in the existing log*IE* scales, method comparison measurements were carried out. During the same day, four different set of conditions were used to measure IE for different compounds in isocratic mode:

- 80 %(v/v) of methanol and 20 %(v/v) 0.1 % formic acid in ultra-pure water with chromatographic column
- 80 %(v/v) of acetonitrile and 20 %(v/v) 0.1 % formic acid in ultra-pure water without chromatographic column
- 80 %(v/v) of methanol and 20 %(v/v) 0.1 % formic acid in ultra-pure water without chromatographic column
- 80 %(v/v) of acetonitrile and 20 %(v/v) 0.1 % formic acid in ultra-pure water with chromatographic column

All dilutions for calibration graphs were made by autosampler.

Instrumentation

The IE measurements were carried out with Agilent 6490 series Triple Quadrupole LC/MS system coupled with Agilent Jetstream ESI source and Agilent liquid chromatographic system 1290, which was equipped with inline degasser, binary pump, column heater (40 °C) and autosampler cooled to 4 °C. ESI source is equipped with additional sheath gas. In the context of the current work, we consider it as an ESI source.

Another Agilent 6495 series Triple Quadrupole LC/MS system coupled with Agilent liquid chromatographic system 1290 was used to measure only PrCl reagent. For instrumental control Agilent MassHunter Workstation Software LC/MS Data Acquisition (v. B.08.00) and for data analysis Qualitative Analysis (v. B.08.00) were used. As described above, gradient elution was used to separate derivatized compounds from reagents. The following MS parameters were used: scan from 50 to 600 *m/z*, capillary voltage 3000 V, nozzle voltage 1500 V, nebulizer gas pressure 20 psi, drying gas flow rate 14 L/min and temperature 250 °C, sheath gas flow rate 11 L/min and temperature 400 °C. All chromatograms were recorded as total ion chromatograms (TIC) (two scan segments and 200 ms per segment) and extracted ion chromatograms (EIC) were extracted by software.

For MS/MS fragmentation measurements product ion scan was used: MS2 was set to scan 100 – $[M_1+H]^+$. Each precursor ion was scanned for 100 ms to produce 2.47 cycles per second. Collison energy parameters were set to use 6 steps from 5 to 30 V, with step size set to 5 V in case of DEEMM and 8 steps from 5 to 50 V, with step size set to 5V up to 30 V and additionally 40 and 50 V.

Calculation of log*IE* and log*RF* values

log*IE* for derivatized amino acids

Ionization efficiencies were studied in the positive ion mode. Only single protonation was taken into account and in all cases doubly-charged and sodium adduct ions were neglected if detected (because only ionization via protonation is considered, similar as in[27]).

For every compound, a calibration curve was constructed from six concentration levels and the quantitative estimation of ionization efficiencies using slopes of calibration curves were evaluated using an approach described by Kruve et al.[29] Equation for the model is:

$$RIE(\mathrm{M_1}/\mathrm{M_2}) = \frac{\mathrm{R}([\mathrm{M_1{+}H}]^+)}{\mathrm{R}([\mathrm{Et_4N}]^+)} = \frac{slope([\mathrm{M_1{+}H}]^+)}{slope([\mathrm{Et_4N}]^+)} \qquad \text{(Eq. 2)}$$

where *RIE* stands for relative IE, M_1 stands for studied compound, Et_4N^+ stands for tetraethylammonium and the slope of the analyte signal versus concentration is estimated via linear regression in the linear range of the signal-concentration plot. For better visualization of the data, logarithmic scale (log*IE*) was used as described by Leito et al.[131]

For anchoring the log*IE* values so that they would be comparable with previously published results,[27] every day Et_4N^+ was also measured in order to have a reference point for the particular day and the following anchoring method was used:

$$\log IE = \log\left(\frac{slope([\mathrm{M_1{+}H}]^+)}{slope([\mathrm{Et_4N}]^+)}\right) + \log IE([\mathrm{Et_4N}]^+)_{ref} \qquad \text{(Eq. 3)}$$

where $slope([\mathrm{Et_4N}]^+)$ is slope value in respective set of conditions in particular day and $\log IE(\mathrm{Et_4N^+})_{\mathrm{ref}}$ value is 3.95 in reference system.[25,27] All compounds were measured against the value obtained for Et_4N^+ in respective measurement system.

Measuring relative response factor logRF_{MRM} in MRM experiments

For AQC, MS/MS transition $[M+H]^+$ -> 171 was used to calculate response factor as indicated in **Table 2.** Collision energy of 25 V generated product ion *m/z* 171 originating from the AQC reagent moiety for all amino acids. For DEEMM and PrCl several MRM transitions were used, and their responses were summed together for calculating the response factors. For DEEMM, three most abundant transitions from **Table 2** were used. Collision energy was set to 10 V, which generated most abundant product ion *m/z* $[M+H-46]^+$. For PrCl, transitions from the manufacturer manual were selected.[111] Some common transitions are listed in Table 2 and all fragment ions for all amino acids are listed in Table S2 in appendix. Collision energy for PrCl was set to 5 V.

Response factors logRF_{MRM} in MRM mode were measured similarly to the logIE_{scan} values. The values were anchored to the logIE scale by using the calibration graph of Et_4N^+ measured in the full scan mode:

$$\log RF_{MRM} = \log\left(\frac{slope([fragment]^+)}{slope([Et_4N]^+)}\right) + \log IE([Et_4N]^+)_{ref} \quad \text{(Eq. 4)}$$

Table 2. The fragments formed in fragmentation experiments from glycine derivate with DEEMM, AQC and PrCl.

Amino acid	DEEMM	AQC	PrCl
Glycine, M =75 g/mol, derivatized *m/z* = 246 both DEEMM and AQC and *m/z* = 204 for PrCl	246 -> 200 ($-C_2H_5OH$) 246 -> 172 ($-C_2H_5OH$, -CO) 246 -> 156 ($-C_2H_5OH$,$-C_2H_5OH$) 246 -> 126 ($-C_2H_5OH$,$-C_2H_5OH$, $-CH_2O$) 246 -> 110 ($-C_2H_5OH$, $-C_2H_5OH$, $-CH_2O$, -O)	246 -> 171 ($-CH_2NH_2COOH$) 246 -> 145 ($-CH_2NH_2COOH$, -CO) 246 ->128 ($-CH_2NH_2COOH$, $-NH_3$)	Common neutral losses for majority of AA -60 ($-C_3H_7OH$) -88 (C_3H_7COOH)

Analysis of wine, beer and tea samples

Five different wines from different regions of the world were selected from the supermarket. Selection was following: 1) Põltsamaa Kuldne, Sweet apple wine, 2009, Estonia; 2) Barton & Guestier, Merlot, 2017, France; 3) Massai, Pinotage, South Africa; 4) Robertson, Cabernet Sauvignon, 2017, South Africa and 5) Calvet, Cabernet Sauvignon, 2017, France.

Four different beers from Estonia were selected as following: 1) A.LeCoq Premium, Pale lager 2) lehe Sunrise at the Secret Beach, New England IPA, craft beer 3) Óllenaut Amber ale, craft beer and 4) Põhjala Pilky, Pilsner, craft beer.

From all samples 100-fold dilution was made in MilliQ water; samples were filtered through Millipore 0.22 µm nylon membrane filter and further 10-fold dilution were made. Two samples from each wine and beer were obtained (100 and 1000-fold dilution) and derivatized with AQC reagent following the procedure described above.

Two tea samples were obtained as follows: 10 mL of Milli-Q was added to 0.25 g of tea sample and heated to 80 °C for 25 min. After that they were injected without dilution. Samples were Green tea (Ahmad Tea, London, Green Tea) and local chamomile tea (*Chamomilla recutita*).

RESULTS AND DISCUSSION

Knowing IE values of different compounds, including derivates, gives a possibility to evaluate concentration of compounds of interest in different samples without standard substances. In order to get better knowledge about the ionization efficiencies of derivatized compounds, a log*IE* measurement method suitable for derivatization mixtures is needed. IE measurement method without chromatographic column used so far is not feasible, because derivatized sample contains more components than only analyte and solvent, e.g. buffer salts, derivatization reagent, side products etc.

The focus in this study is amino acids, which have poor or no retention in reversed phase (RP) LC. Compounds having no retention in LC will elute in the beginning of the chromatogram and could cause matrix effect (ME), while matrix compounds co-eluting with analytes could suppress or enhance analytical signal. To overcome this, derivatization is widely used for amino acids to give polar analytes more retention and separate them from matrix. Derivatization also changes signal intensities of analytes i.e. providing lower detection limits. This work presents results for IE scale measurements regarding the derivatized compounds, mainly amino acids, in scan mode and a method for applying these values to estimate the response factors in MRM mode for the same compounds. The main goals of the current thesis were:

- To develop a method for measuring log*IE* values for derivatized compounds (paper I).
- To measure the IEs of the derivatized compounds and to widen the scope of the existing IE scale[27] (paper I and III).
- To study correlation between log*IE* and log*Slope* values measured in flow-injection mode and in real LC/MS conditions (paper II).
- To study correlation of log*IE* values between positive and negative mode and Na-adducts (unpublished results).
- To study fragmentation characteristics for some of the derivatization reagent in MS/MS (paper III).
- To put measured values into practice and evaluate amino acid concentrations in different drinks (paper III).

Method development of log*IE* measurement for derivatized amino acids (Paper I)

When developing a method for log*IE* measurements of derivatized compounds, couple of aspects have to be kept in mind. Firstly, the original log*IE* measuring approach which does not use chromatographic column and measures pure compounds one at the time[29] has to be modified because in case of derivatized compounds, the mixture obtained after derivatization contains also derivatization reagents, by-products, buffer components etc.[116,132] If these arrive in the

ionization source at the same time, i.e. are not separated with the chromatographic column, these compounds may generate matrix effects in the ion source leading to signal suppression (less often enhancement) in detector and influence the results.[86] Therefore, a chromatographic separation is necessary for measuring IE of derivatized compounds since different compounds need to be separated from each other chromatographically.

Secondly, solvent effect is one of the main concerns influencing ionization as discussed in literature overview. Until now, all log*IE* measurements have been made using a fixed solvent composition (80/20 acetonitrile/0.1 % formic acid[27,29–31]) without chromatography column (i.e. flow injection mode) and gradient elution. However, when using chromatographic column together with a gradient elution which is often necessary for analyzing derivatized compounds and is a common practice analyzing samples in real life, it is obvious that organic modifier content in the ionization source is different over the timespan of gradient elution and its exact content cannot be controlled at the time when an analyte reaches the ionization source. That is why it is a need to evaluate the effect of eluent composition on ionization.

And finally, taking all of this into account, it is necessary to evaluate the results and see, if there is a correlation with previously measured log*IE* results which are measured without column and also in different solvent compositions. To do that, it is necessary to anchor results obtained in this work to the previous log*IE* scales in order to get comparable results and also widen the scope of the previous scales.

To study the influence of these three effects, solvent composition and chromatographic method were studied. Results are discussed and compared in the following chapters.

Setting up chromatographic analysis for derivatized amino

An important consideration while planning this work was that the obtained results should be comparable to the previously published log*IE* scale.[27,33] Even though all previous measurements of log*IE* have been made using acetonitrile instead of methanol, it was not possible to use this solvent for the measurements in this study since the used derivatization procedures have been developed using methanol.[79] Methanol is also cheaper, less toxic and more readily available in a long-term perspective for different applications. Due to this reason, different solvent composition studies, with and without a column, were carried out in order to compare the effects of different conditions on IE.

For estimating how different organic modifiers and the use of chromatographic column affect IE values, four different sets of conditions (methanol vs acetonitrile without a column and methanol vs acetonitrile with column) and a selection of compounds (histamine, threonine, glutamine, Et_4N^+ and previously in-house synthesized pure DEEMM-Phe, DEEMM-β-Ala and DEEMM-Gly)

were chosen for comparative measurements. It was important to use pure compounds of derivatives as it was not possible to use derivatization mixtures without column due to the reasons described above.

Measurements were carried out during one day, keeping the MS conditions constant. All measurements were made using isocratic elution in order to only evaluate the effects originating from adding the chromatographic column or changing the organic phase of the eluent. Tetraethylammonium ($Et_4N^{+)}$ was chosen as the reference (*anchor*) compound. Its IE is one of the highest and is most reliably (reproducibly) measured. It has also been used as a anchor compound in previous works.[25] For evaluation of the effects on IE when using different measurement conditions, all log*IE* values were anchored to log*IE* value (3.95) of Et_4N^+ 80/20 acetonitrile and 0.1 % formic acid in ultrapure water.

Underivatized amino acid show 0.19 to 0.34 log*IE* unit change between different conditions (see graphical expression of results in **Figure 4**). Histidine is showing least variability and glutamine and threonine a little bit higher (0.33–0.34). For latter two, methanol shows best ionization and acetonitrile worst in flow injection mode. Considering the fact that pooled standard deviation for all log*IE* measurements for this work was 0.17 log*IE* units, the change for log*IE* for underivatized amino acids histidine, threonine, glutamine and Et_4N^+ was not considerable.

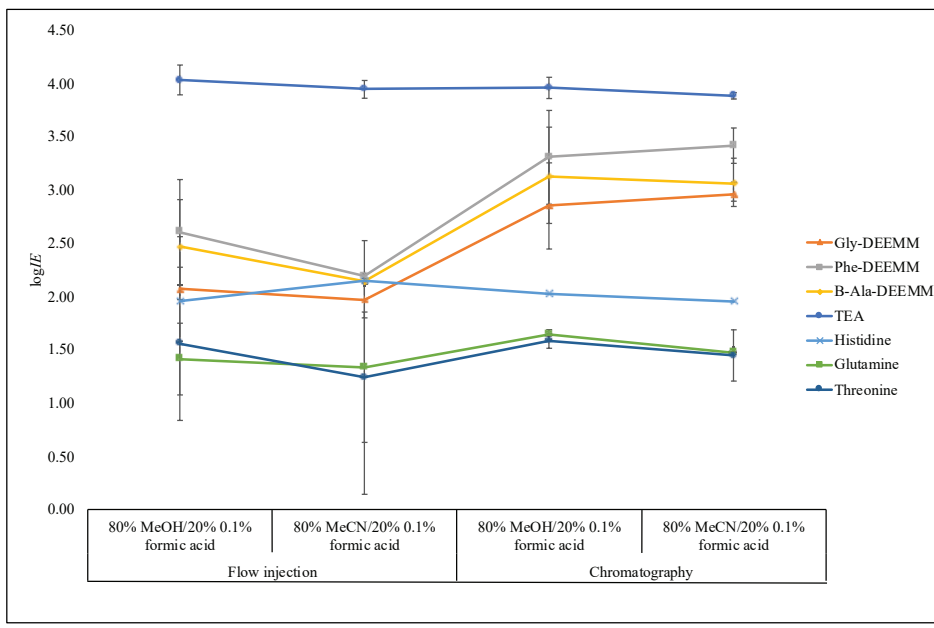

Figure 4. log*IE* values of different compounds in different conditions.

On the other hand, when comparing the results for DEEMM-Phe, DEEMM-β-Ala and DEEMM-Gly, there were clear differences between log*IE* values obtained with column and without column. Measurements with chromatographic separation gave up to 1.05 log*IE* units higher results compared to measurements without column. For methanol log*IE* values were 0.66 to 0.78 and for acetonitrile 0.92 to 1.22 log*IE* units higher with column measurements when compared to measurements in flow-injection mode. One of the explanations could be that although these in-house synthesized DEEMM-derivatives were purified, there still might have been some co-eluting impurities which suppress the signal. Derivatives have retention times 6.5 to 8.2 min with chromatographic column and therefore it is more likely that the separation from possible impurities and other interfering compounds is achieved while in flow-injection they co-elute and cause matrix effects. Additionally, the effect could be related to the DEEMM-derivatives themselves since their structure and polarity is different from other compounds tested. It can be that for that reason they are more influenced by the changing conditions.

It could be concluded that if compounds have a considerable retention in RPLC, then one can assume higher ionization in the source due to less influence from possible impurities eluting with column dead time.

Solvent composition studies

When log*IE* values are measured with chromatographic column and a gradient elution is used, compounds have different retention times and therefore the composition of eluent is different in ionization source at different times i.e. not all compounds have the same eluent composition at the time of the ionization. In this work, the retention times of the compounds of interest were between 5.1 and 8.5 minutes and the organic component percentage in the mobile phase in ion source was between 25 % and 70 %(vol), depending on the retention time. Since IE is known to be influenced by the percentage of organic modifier,[19,20,25,133] it is important to investigate the impact of solvent composition when log*IE* values of derivatized compounds are measured. Although Kruve[19] has shown that in thermally focused ESI source IE is not influenced by the organic solvent content as much compared to the conventional source in negative ion mode, it is generally known, that higher organic modifier content in eluent composition usually give higher IE values.[25] That is why the solvent composition was still studied in this work for positive mode. Besides, Liigand et al[7] have shown that different ion source designs could have different effect on IE, what regards experimental setups and that span of the log*IE* scale could be different on different instruments.

To investigate eluent composition influence, analyses of some underivatized and derivatized amino acids at different eluent compositions were studied. This cannot be done with a column and therefore these experiments had to be carried out with flow injection analysis in order to control the eluent composition for

compounds. This study included different DEEMM-derivatives (DEEMM-β-Ala, DEMM-Gly, DEEMM-Phe), some amino acids in underivatized form (arginine, leucine, phenylalanine) and Et_4N^+ at different organic (methanol) phase percentages (0, 20, 40, 60, 80 and 100 %(vol)).

For each compound, six concentration points were injected, calibration curves were constructed and finally log*IE*s were calculated. Results show (**Figure 5**) that when the mobile phase was 100 % of organic solvent, then the IE of all the compounds differ from those obtained with any other mobile phase compositions. In this case, no acidic additives were added to the mobile phase, therefore, the acidity of the solvent is lower and weak bases are expected to be protonated to a smaller extent.

Slopes of the calibration curves were also compared by a *t*-test. Results showed that the slopes of calibration curves obtained with different organic phases have some differences, but slopes corresponding to more similar organic phases have also more similar slope values. Results showed that for derivatized compounds and for Et_4N^+, IE differs from other compositions when eluent composition was 0 % organic phase. In most cases, change of methanol concentration from 20 to 80 % does not influence the IE and since previous log*IE* scales are constructed at 80 % acetonitrile, it reassures that the IE scales constructed using a new method proposed in this work with a chromatographic column are comparable to previously obtained ones without any correction.

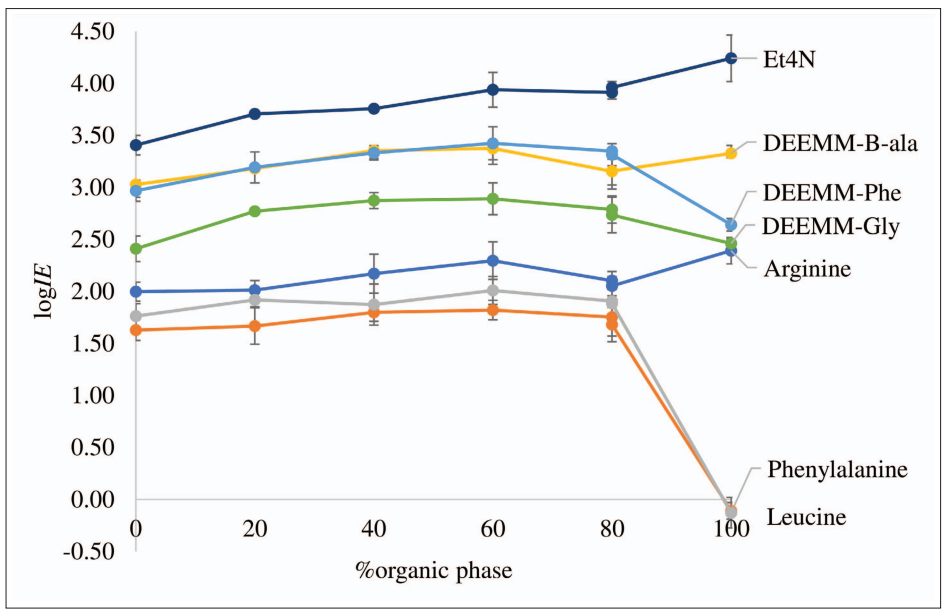

Figure 5. log*IE* values of different compounds on different organic modifier content relative to Et4N+ in 80 % MeCN and 20 % 0.1 % formic acid.

However, in current work, a model of the impact of the organic solvent content on IE could be derived for positive mode. 100 % organic modifier composition was left out from the calculation due to very large deviation and since different compounds do not agree well in this high organic percentage region. Considering, that only a small constant effect on IE occurred with change in organic modifier content, we fitted a quadratic function (**Figure 6**) between the average change in log*IE* (across all compounds) relative to mobile phase with 80 % of methanol as an organic modifier content. The following model was obtained:

$$\Delta \log IE_{\text{x-80\%}} = 1.03\times10^{-2} org\% - 9.62\times10^{-5} org\%^2 - 0.198 \qquad \text{(Eq. 5)}$$

Figure 6 shows that in average, IE is highest at about 55 % of organic modifier and lowest at 0 % of organic modifier. On the other hand, in the region of organic solvent content 0 % to 80 %, the solvent effect on IE is relatively small for all compounds.

The most comprehensive IE scales published so far are measured in 80/20 organic solvent/water phase mixture.[27] Therefore, to allow comparability to those published values it was best to recalculate all values measured under chromatographic conditions to 80 % of organic modifier.

As it is known, different ionization sources are somewhat differently influenced by the mobile phase content. For example, the influence is larger in case of conventional nebulizer without thermal focusing. The model together with mathematical coefficients cannot be directly applied on a different system for estimating organic modifier influence on IE, however, the approach itself can, because as described, different system could affect IE to a different extent.

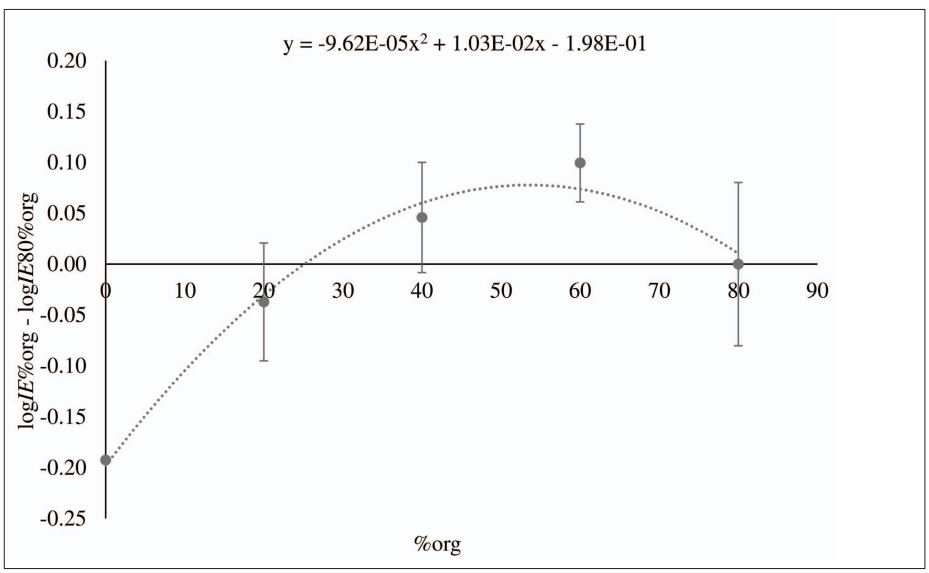

Figure 6. Quadratic fit between the average change in log*IE* relative to mobile phase with 80 % of methanol and organic modifier content.

Influence of derivatization on amino acid ionization efficiency

All measured log*IE* values were anchored to previously published results and are relative to methyl benzoate (log*IE* taken as 0) using one-point anchoring to the log*IE* value of tetraethylammonium 3.95. This anchoring enables direct comparison of the results obtained in current work to the previously published scales.

Firstly, log*IE* values of the underivatized amino acids measured in this work with column were compared to the previously published log*IE* values of amino acids measured in our group.[33] These results were measured in solvent composition 80/20 acetonitrile and 0.1 % formic acid in ultrapure water and without column. Correlation between the two scales (R^2=0.62 and root mean square error 0.60 excluding cysteine and histidine) for underivatized compounds is within the limits of transferability between different systems (0.24–0.74 log*IE* units) observed previously for log*IE* measurements carried out on different instruments.[7] Additionally, the solvent system used in this and in previous study were different.

Secondly, log*IE* values of 24 derivatized and underivatized compounds were compared (**Table 3, Figure 7** and **Table S1**). As seen in the previous chapter, during the gradient elution, organic mobile phase content is increasing and in general this also increases IE. To consider the fact that every compound elutes at different organic phase concentration, abovementioned modelling was applied to scale the log*IE* values into a reference solvent system (80/20 acetonitrile and 0.1 % formic acid in ultrapure water). This was done by using retention time of each derivatized and underivatized compound. This allows us to differentiate between the IE change due to (1) derivatization and (2) change in elution mobile phase content.

Table 3. Comparison of log*IE* values between underivatized compounds and DEEMM-derivatized compounds in 80/20 acetonitrile and 0.1 % formic acid in ultrapure water

	Underivatized (n=3, s=0.09)		Derivatized with DEEMM (n=3, s=0.05)		
Compound name	log*IE*	Retention time, min	log*IE*	Retention time, min	Difference, abs.
AMPA*	0.22	0.40	2.66	5.5	2.44
Asparagine	0.95	0.38	2.21	6.1	1.26
Aspartic acid	0.98	0.38	2.73	6.5	1.75
Histamine*	1.80	0.33	3.23	5.7	1.43
Cysteine	2.16	0.42	1.85	7.3	-0.31
Serine	2.17	0.41	2.87	6.3	0.70
Histidine	2.22	0.35	3.41	5.6	1.19
Proline	2.24	0.41	2.16	7.1	-0.08
Selenocysteine	2.28	0.46	3.41	7.5	1.13
Spermidine*	2.31	0.33	2.68	5.1	0.37
Threonine	2.47	0.42	3.08	6.7	0.60
Glutamine	2.51	0.42	3.02	6.3	0.51
Arginine	2.57	0.36	3.18	5.8	0.62
Lysine	2.58	0.37	3.55	8.5	0.97
Selenomethionine	2.60	0.77	3.03	8.2	0.43
Tryptophan	2.61	5.07	3.45	8.1	0.83
Glutamic acid	2.69	0.42	3.16	6.7	0.47
Valine	2.75	0.42	3.23	8.1	0.48
Methionine	3.10	0.44	3.39	8.0	0.29
Normetanephrine*	3.11	0.62	2.76	7.3	-0.35
Isoleucine	3.22	0.42	3.31	8.5	0.09
Leucine	3.27	0.40	3.42	8.5	0.15
Metanephrine*	3.60	0.81	2.87	7.3	-0.74
Phenylalanine	3.61	0.40	3.28	8.3	-0.33

*not amino acid

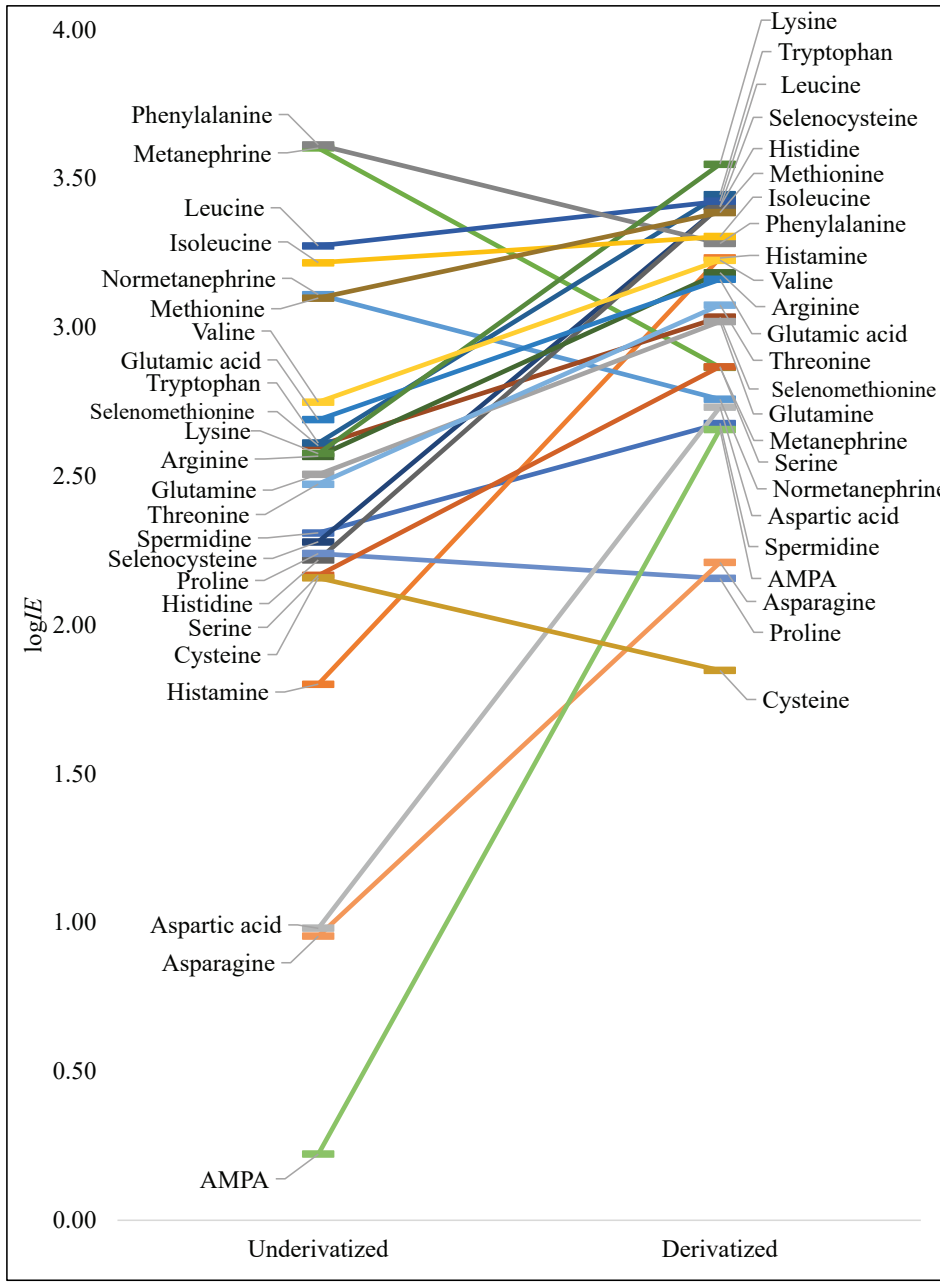

Figure 7. Comparison of log*IE* values between underivatized and DEEMM-derivatized compounds in 80/20 acetonitrile and 0.1 % formic acid in ultrapure water.

In most cases, derivatization improves ionization and gives more similar IE values for different compounds meaning that derivatization compresses the IE scale. The range of log*IE* values observed for underivatized compounds was 3.4 log*IE* units, while for derivatized compounds it was only 1.7 log*IE* units. This means that for underivatized compounds, the sensitivity varies almost 2500 times but for derivatized compounds only 50 times. This difference also proved to be statistically significant with *t*-test. This can be explained by the fact that derivatization with DEEMM adds similar molecular moiety to each amino acid which is most likely responsible for ionization. It also increases molecular hydrophobicity which in turn helps molecule to move closer to the droplet surface and further into gas phase. DEEMM adds molecule weight and it is believed generally, that larger molecule has better stabilization in the gas phase than small one. Considering the fact that DEEMM itself, though, has not very high IE, as stated by Rebane et al,[32] and taking into account different retention times of derivatized compounds, derivatization increased IE as expected.

In average, the rise in log*IE* was 0.9 units when comparing derivatized compounds with underivatized ones. Lowest increase was observed for proline, isoleucine, leucine and methionine whose log*IE* value changed only -0.08 – 0.29 logarithmic units. Spermidine, valine, glutamine and other several compounds (up to lysine) had IE rise due to derivatization about 0.37 – 0.97 log*IE* units. Highest increase was for compounds such as selenocysteine, histidine, asparagine, aspartic acid, histamine and AMPA. The latter one had 2.4 log*IE* units higher IE compared to underivatized form. It could be the reason that AMPA has phosphonic acid group in molecular structure and this rather ionizes in negative mode, via deprotonation, than in positive mode.[134,135]

Results show that largest changes are for molecules that either have low IE without derivatization (AMPA, Asp, Asn) or include imidazole sidechain (His and histamine) which could be the reason for such a high rise in IE. On the other hand, derivatization did not change IE to such an extent for molecules that already have quite high IE without derivatization, e.g. metanephrine, normetanephrine, phenylalanine, leucine and isoleucine. First three also have higher molecular weight compared to other studied compounds, which gives molecule better ionization properties. Proline and cysteine have both quite low IE and one could assume their IE rise after derivatization, but quite the opposite, derivatization lowers IE. This phenomenon could be explained by the fact that they have poorer derivatization properties. It has been shown that DEEMM-Pro has higher detection limits compared to other amino acids.[98]

To conclude, results show that in general, after modelling the results into comparable scales (considering the mobile phase differences in gradient elution mode) one can see, that derivatization increases IE. Additionally, results obtained in different IE measuring systems could be compared to each other.

Using log*IE* to predict sensitivity for LC/MS analysis (Paper II)

As already discussed, many things influence IE value of a compound in the ionization source and different sources could have different IE. Also, for sources like ESI or APCI, LC conditions could be vastly different, which in turn may influence signal intensities in LC/MS. Therefore, it is of interest to see if measured log*IE* values measured in flow-injection mode would correlate with calibration graph slopes obtained under LC gradient conditions. A high correlation would indicate that the determined log*IE* values can be used for predicting the sensitivity in LC/MS analysis.

For the comparison, the slopes of the calibration graphs measured with a gradient elution were expressed as log*Slope* and they were plotted against log*IE* values. It is seen from **Figure 8a**, that log*Slope* vs log*IE* in the case of ESI source, log*IE* values are useful in predicting sensitivities (R^2 values 0.7 and 0.8) for chromatographic elution. Despite the fact, that solvent composition in the source is different when measured with chromatographic column and that good correlation has been showed between log*IE* and organic modifier content,[136] log*IE*s were not influenced show much in this case. As shown by Kruve,[137] it might be due to the fact that ESI source with additional sheath gas was used, like ESI Agilent JetStream. This type of source is also used in this work, considered as ESI source, and it is believed to dry effluent much faster, which reduces effect of organic modifier content.

On the other hand, in the case of the APCI source, the log*IE* values are not useful for predicting sensitivity in LC/MS analysis (R^2 values around 0.4) as seen in **Figure 8b**. This may result from a number of factors. Firstly, APCI ionization process may be much more complicated what regards the organic solvent content and secondly, the influence of APCI source design may have an effect, as the log*IE* and log*Slope* values have been measured on different instruments. This also means that when designing a derivatization reagent suitable for ESI/MS or APCI/MS detection, then the log*IE* values measured in one solvent system with flow injection can be used as a useful guidelines for possible structures of the reagents and will be relevant for real LC/MS conditions when ESI source is used.

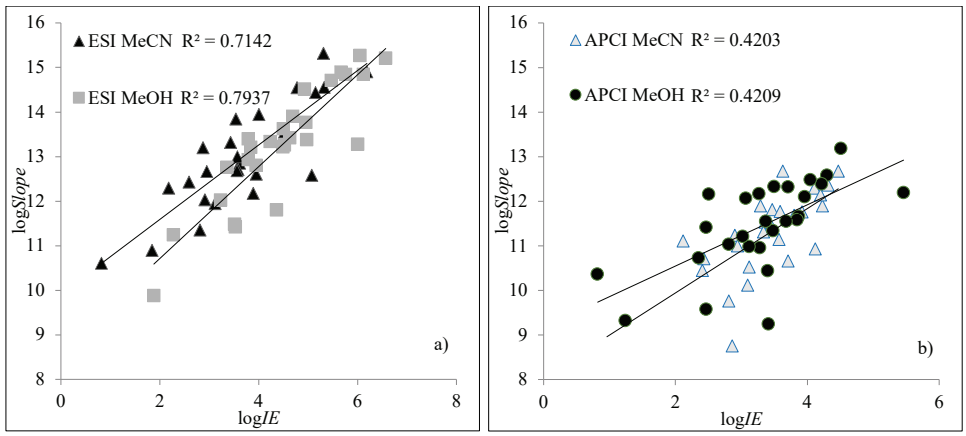

Figure 8. Correlation of log*IE* and log*Slope* values in case of a) ESI and b) APCI.

Sodium-adduct formation and negative mode ionization efficiency

It was also under interest to measure IE in negative mode and using Na-adduct information besides protonation since these have been less investigated and could provide interesting insight to the IE mechanisms. In some cases, it has been shown, that some compounds give higher signal intensities in negative ion mode or using sodium-adducts, although probably some experimental conditions like solvent pH or sodium content should be modified accordingly.[29,33,98,138] In addition, there are applications where adducts and negative ions are used for quantitation purposes.[51,139,140] It is also interesting to see how (and if) ionization efficiencies in negative and positive mode and sodium-adduct correlates to each other. DEEMM-, AQC-, FMOC- and PrCl derivates were selected for comparison since these have been used the most in various applications.

Alongside positive ion scan mode, negative ion scan was also studied alternating positive and negative in short timespan in the source. This allowed to gather data for both ionization modes. Although in this study special modifications were not made for negative mode, like using basic eluent composition (ammonia solution), the aim was to see if, and to what extent, negative ions will generate from derivatized compounds and how (if at all) they correlate to positive mode ionization.

In case of **DEEMM**-derivates, it was seen, that in negative and positive ion mode intensities of signal responses in scan mode were in the same order of magnitude and differ only marginally. DEEMM sodium-adducts showed ~1.5 times lower abundance than positive ion mode, which confirm the fact that they could possibly be used for quantitation purposes in case of a controlled eluent composition. Although sodium-adducts and protonation both showed similar

intensities, there was no correlation between them whatsoever. This also apply for positive vs negative ion mode and negative ion mode vs sodium-adduct, which means that mechanisms of generation of adducts is unpredictable under given conditions.

For **AQC**-derivates, Na-adduct ions were observed in all cases, along with $[M+H]^+$ in mass spectra. Inversely to DEEMM, Na-adduct showed very low, about 22 times lower signal intensities compared to positive ion mode. It could be explained, that AQC moiety includes easily chargeable nitrogen in its structure, which allows weak Na-adduct formation. In negative ion mode, signal intensities were about 15 times lower than in positive ion mode. Similarly, as for DEEMM, no correlation between Na-adduct vs positive mode were noticed. This also apply for positive vs negative ion mode and negative ion mode vs sodium-adduct, which means that mechanisms of generation of adducts is unpredictable under given conditions.

FMOC gave average about four times higher signal intensities for Na-adducts compared to singly protonated form, which could be explained by the effect of chloroformate ester group existing in its moiety forming carbamate group when reacting with amino acids. Carbamate group is very attractive for forming ion-dipole with Na^+, since oxygen atoms are as a rule more negative than nitrogen. This part of a derivate most probably compete with protonation mechanism. And it has been seen in the literature, that analytes containing carbamates, yielding sodium adducts is common, which also supports this theory. Negative ion mode for FMOC-derivates gave lowest signal intensities compared to Na-adducts and singly protonated form, which could be explained by the use of acidic eluent, and generation of positively charged particles are far more favorable than negative ion formation in such medium. It was seen that in negative mode, average signal intensities were about five times lower than in positive mode and ~21 times lower than Na-adducts signal intensities. Although FMOC-derivatized amino acids have been used for quantitation in MRM negative ion mode,[5] it wouldn't be optimal case under conditions in this work due to low signal intensity. In case of FMOC-derivates, no correlation between negative-positive, Na-adduct-positive nor negative-Na-adduct formation were found.

In case of **PrCl**, Na-adducts were observed in all cases and their intensities were average two times higher compared to protonated form in positive ion mode. Very weak correlation between Na-adducts and protonated form were observed (R^2=0,52). That might be explained by the fact that Na-adducts are not competing to the same site within the molecule as proton, which give equal opportunities for generation of both protonated and Na-adduct form. Interestingly, no signal intensities were observed for PrCl in any case. This could be explained with the fact, that PrCl is the only reagent out of these four, which reacted both amino and carboxy group of the amino acid, eliminating easily deprotonating site.

As seen from the results, negative ion mode and Na-adduct formation need special method development to have best conditions for using these measure-

ments modes. For current studies, it is best to use singly protonated forms to evaluate log*IE* values, since current compounds under study gave less predictable values for Na-adducts and in negative ion mode, which cannot be used for prediction of log*IE*.

In general, results of studies concluded, that mechanisms of formation of sodium-adducts and deprotonation do not correlate to each other and probably complicated mechanisms are involved, which give no universal possibilities for practical use.

Measurements in MRM mode

Majority of the suspect and non-targeted analysis data are collected in the data-dependent or data-independent mode that is run alongside scan mode. MS/MS is widely used for different application like structure elucidation and quantitation of environmentally hazardous trace substances in very low concentrations. MS/MS provides a lot more information about the structure of the compounds and has lower quantitation limits; therefore, it is of need to make standard substance-free quantification possible also for methods utilizing MS/MS data accusation modes.

As seen in previous chapters, when using scan mode for screening, solely ionization in ESI source could be complicated and sometimes unpredictable process. If we add extra dimension to that, which would be MS/MS together with fragmentation, things can get even more complicated. In addition to variation of IEs in orders of magnitude in case of different compounds, those ionized particles could each fragment in their own way depending on their properties. Fortunately, it could be assumed already before experiments that similarities in fragmentation patterns could be seen, when using one type of derivatization reagent, which should ease interpretation of results.

Derivatization of analytes with different reagents could produce different fragmentation patterns in MS/MS. Some reagents fragment only by breaking off small functional group (in case of DEEMM, PrCl), some break between reagent and the target analyte and yield reagent-specific product ion (in case of AQC), when used in MS/MS experiments. The latter fragmentation pathway gives easier and simpler quantitation opportunities, since fragmented product ion is also most abundant one since almost no other fragmentation, if all, is taking place which is straightforward for selecting precursor and product ions.

To measure response factors of derivatized compounds after ionization and fragmentation in MS/MS, some fragmentation-related aspects should be considered. Firstly, it is necessary to know fragmentation pattern of compounds and secondly, its relation to collision energy which is applied to ionized molecule leaving from ionization source and isolated in MS1. Appling correct fragmentation energy on molecular ion and selecting correct fragment ion(s) for quantitation are important prerequisite for correct analytical determination. Besides, instruments from different manufacturers could have differences in optimal

system parameters with the same value due to the constructional peculiarities. In this work, fragmentation patterns for amino acids derivatized with different reagents were studied. This also covered changing fragmentation energy to see, which fragments are formed in MS/MS system.

The outcome of these studies should indicate if IE values measured in the scan mode could be used to estimate the response of the derivatized amino acids analyzed with LC/ESI/MS/MS. DEEMM- and AQC-derivatized amino acids were selected as these allow exploring two different fragmentation mechanisms (different types of bond cleavage, rearrangement reactions, etc.) for a series of compounds. Different derivatization reagents induce different fragmentation mechanisms (pathways) and allow, therefore, to systematically evaluate the applicability of the IE predictions on the response in MS/MS experiments.

Fragmentation patterns of derivatized amino acids

It was seen that different derivatization reagents fragment differently and fragmentation patterns were influenced by the structure of the derivate.

AQC reagent

AQC fragmentation follows strict mechanism of charge retention fragmentation pathways. For this reason, a very simple fragmentation pattern was observed where for all amino acids (AA) derivatized with AQC (AA-AQC, for short) a charge retention fragmentation occurs where the charge carrying fragment (m/z = 171) is formed from the reagent side. Example of fragmentation is shown on **Figure 9.**

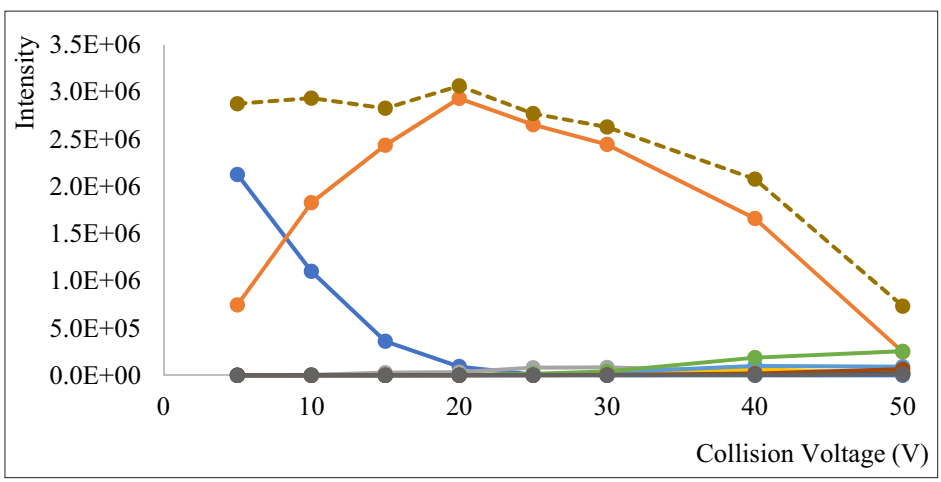

Figure 9. Intensity of AA-AQC fragments and sum (dotted line) of total ion count depending on collision voltage. Orange line indicates m/z 171 fragment and blue line $[M+H]^+$.

Fragmentation yield for *m/z* 171 for AA-AQC is presented in **Figure 10** and it can be seen that also the fragmentation yield vs fragmentation energy follows a very similar pattern for all of the AA-AQC. This is expected, as for all AA-AQC the same bond is broken in the MRM experiments, followed by the formation of *m/z* 171.

Observing the same fragment ion for a large number of compounds is unfavorable from the selectivity point of view; all compounds with the same nominal mass containing amino group would yield both molecular ion and fragment ion with the same *m/z*. Still, the retention time predictions and ion mobility can be used to add selectivity for this type of cases. However, from the quantitation point of view this is highly beneficial. The *IE* values predicted for the molecular ion can be correlated with the response factors observed in the MRM mode (see discussion below).

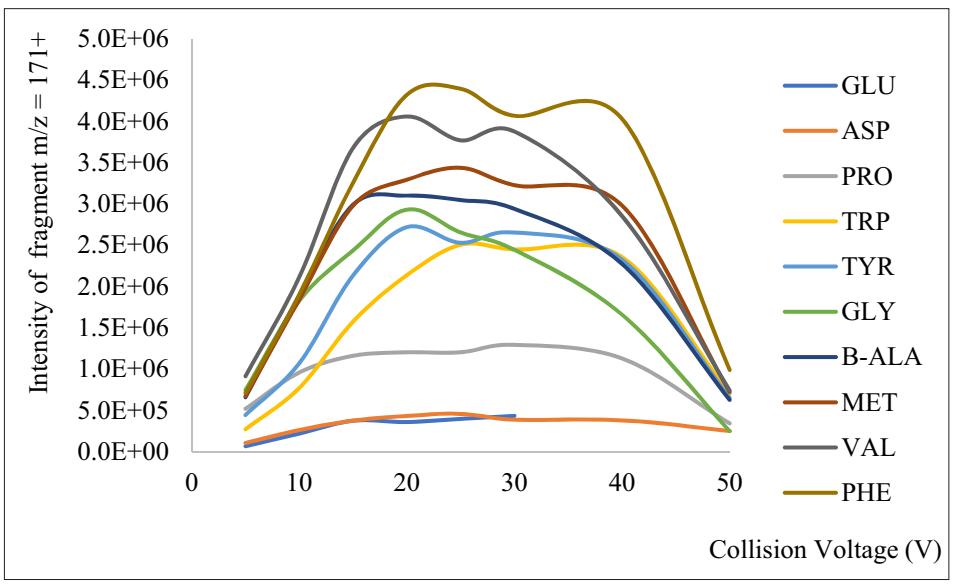

Figure 10. The fragmentation yield of *m/z* = 171 for AA-AQC at different fragmentation voltages. All measurements were done with 2 µM solution of AA-AQC and chromatographic separation was used.

DEEMM

DEEMM derivatization shows characteristic fragmentation from reagent side, which subsequentially loses neutral parts. Vu et al[141] has provided diagnostic fragment ions obtained from DEEMM-derivatized methionine and selenomethionine. In case of DEEMM derivatized amino acids, the fragmentation spectra is dominated by the $[M+H\text{-}C_2H_5OH]^+$ ions for most of the amino acids. Also, the further fragmentation producing following fragments: $[M+H\text{-}C_2H_5OH\text{-}CO]^+$, $[M+H\text{-}C_2H_5OH\text{-}\ C_2H_5OH]^+$, $[M+H\text{-}C_2H_5OH\text{-}\ C_2H_5OH\text{-}CH_2O]^+$,

[M+H-C_2H_5OH, -C_2H_5OH, -CH_2O, -O]$^+$ is very similar for all amino acids. This shows that in the case of DEEMM, the fragmentation pattern is also largely dominated by the fragmentation of the derivatization reagent part of the molecule: a number of neutral losses from the reagent side occur consecutively. Example of fragmentation is shown on **Figure 11.**

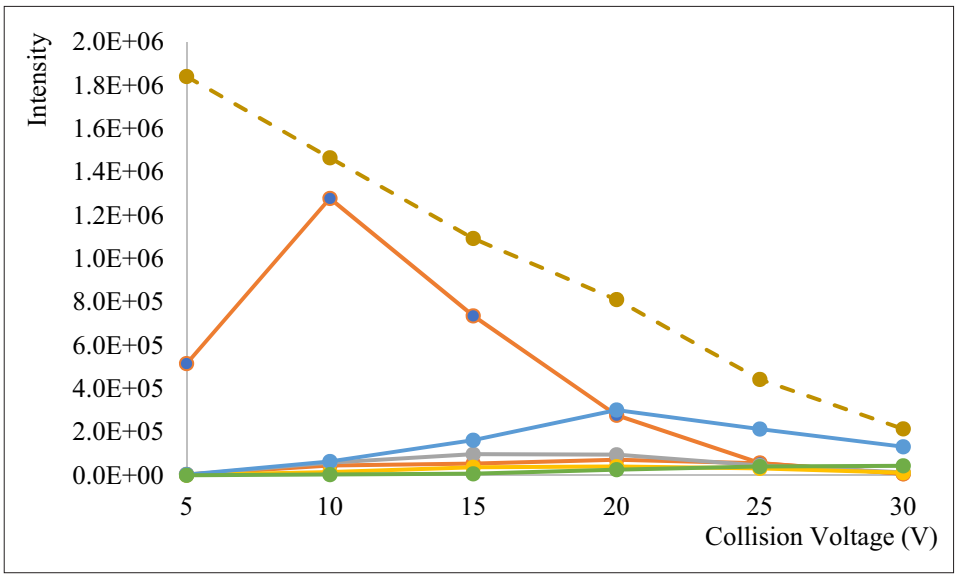

Figure 11. Intensity of most of the AA-DEEMM fragments and sum (dotted line) of total ion count depending on collision voltage. Orange line indicates [M+H-46]$^+$ fragment.

Fragmentation of proline, on the other hand, showed a little difference in fragmentation patterns. As seen on **Figure 12**, there is no single most abundant fragment, but different fragments contribute to sum of intensity equally. This could be explained by the fact, that nitrogen in the structure of proline is attached directly to the main chain and is counted as secondary amine.

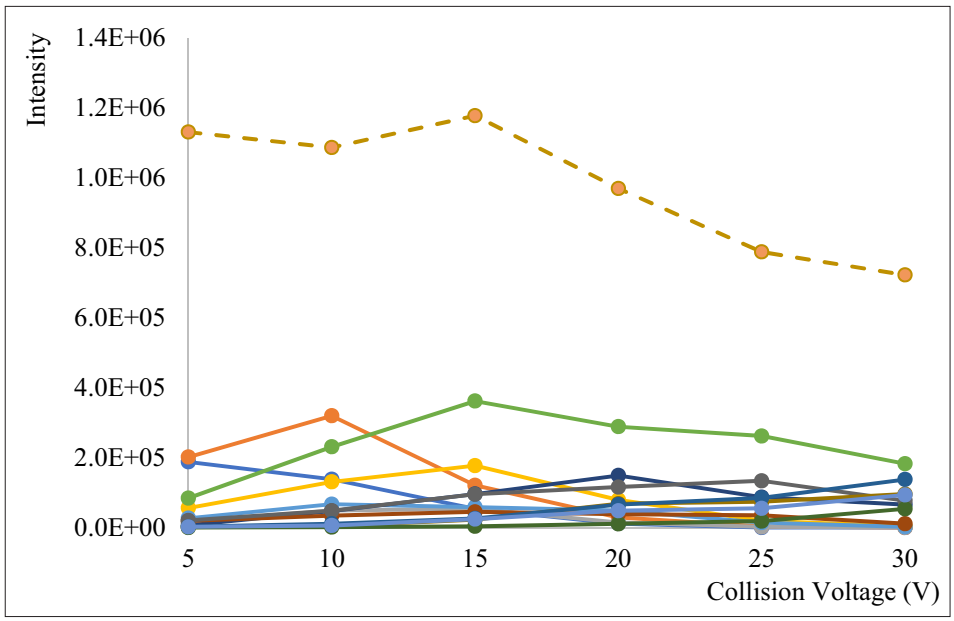

Figure 12. Intensity of the Pro-DEEMM fragments and sum (dotted line) of total ion count depending on collision voltage. Orange line indicates $[M+H-46]^+$ fragment.

On the other hand, the fragmentation yield dependence on the collision voltage for different AA-DEEMM is relatively similar. Fragmentation yield for AA-DEEMM is presented in **Figure 13**. Smaller differences occur at the lower collision voltage values but in general the profiles are similar. Similarly to AQC, this was expected since all AA-DEEMM follow the same fragmentation pattern. It is also noted, that half of that AA-AQC collision energy is needed to generate most intense fragment ion for AA-DEEMM.

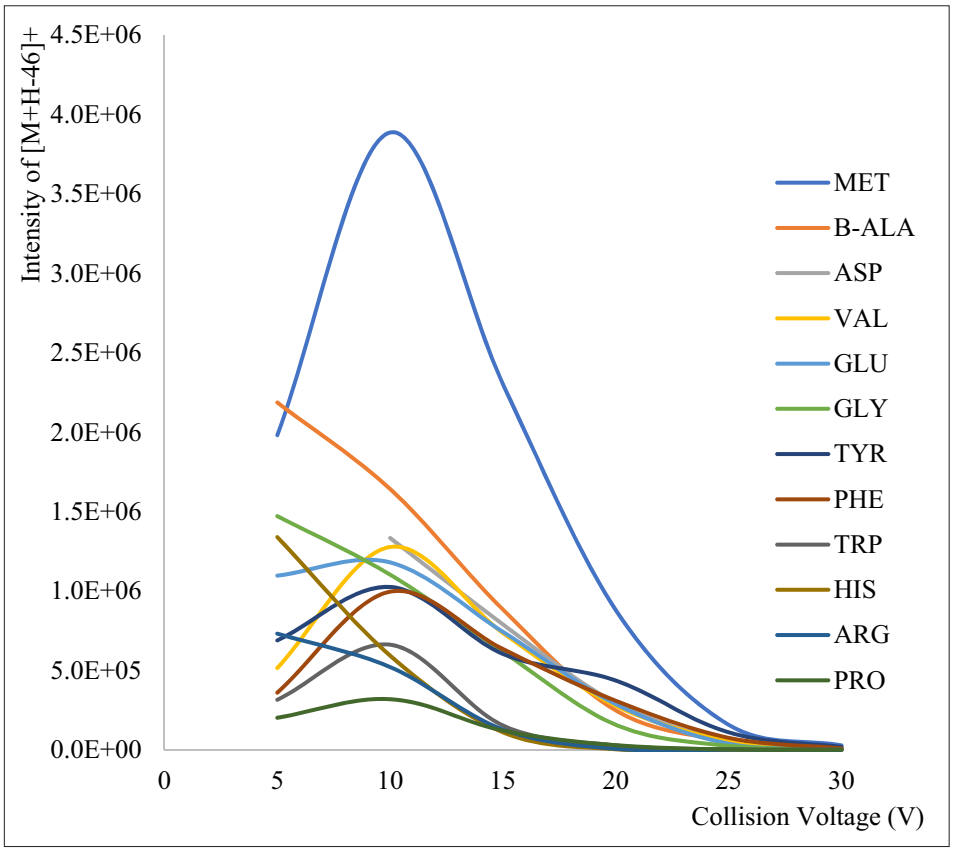

Figure 13. The fragmentation yield of [M+H-C_2H_5OH]+ for AA-DEEMM at different fragmentation voltages. All measurements were done with 2 µM solution of AA-DEEMM and chromatographic separation was used.

Propyl chloroformate

Compared to AA-AQC and AA-DEEMM, a very different fragmentation pattern was observed in the case of PrCl. For all AA-PrCl different fragments were observed (listed in Table 2 and Table S2).[111] This indicates that the fragmentation pattern strongly depends on the amino acid structure and in case of each AA-PrCl a different covalent bond is broken. For this reason, PrCl was discarded from the further studies. This could be explained by the fact that usually amino acid derivatization reagents react with amino group, but in case of PrCl, both amino and carboxylic acid group are taking part of derivatization reaction. Fragmentation mechanism for PrCl is very complex and involves hydrogen rearrangement from other part of the molecule.

Correlation between ionization efficiency and response factor in MRM mode for derivatized amino acids

To find a suitable reagent, which could be used for estimating response in MRM mode, correlation plot of log*IE* and logRF_{MRM} is necessary. A high correlation would indicate that all ions generated in the source would fragment to the same extent and no differences would occur between amino acids. In addition to developing a new experimental log*IE* measurement approach with chromatographic column, IE and RF values for DEEMM-, AQC- and PrCl-derivatized compounds and their comparison with each other is presented.

For calculating logRF_{MRM}, two approaches were used – for AQC-derivates, only single transition with a product ion *m/z* 171 was used. For DEEMM-derivates several transitions were monitored and the sum of intensities of all transitions were used instead. Moreover, for DEEMM, single transition to $[M+H-46]^+$ and sum of all transitions, very high (R^2=0.97) correlation was observed, since the primary transition is also the most abundant one as seen in previous chapter.

In general, the IE values measured in scan mode varied more than 10 times for all derivatization reagents (more than 1 logIE_{scan} unit). For the AA-AQC, the log*IE* values were from 1.2 to 2.9, for AA-DEEMM from 2.6 to 4.0, and for AA-PrCl from 3.0 to 1.1, see **Table 4**. The response factors measured in MRM mode (logRF_{MRM}) are influenced by two factors, the IE (logIE_{scan}) and the fragmentation yield. The response factors logRF_{MRM} measured in MRM mode varied from 2.0 to -0.7, from 2.9 to 0.8, and from 3.25 to -1.4 logarithmic units for AA-AQC, AA-DEEMM, and AA-PrCl, respectively.

Table 4. The retention times, ionization efficiency values measured in full scan mode, and response factors measured in MRM mode for amino acids derivatized with DEEMM, PrCl, and AQC, respectively.

		DEEMM			PrCl			AQC		
Abbreviation	Compound	t_R	logIE_{scan}	logRF_{MRM}	t_R	logIE_{scan}	logRF_{MRM}	t_R	logIE_{scan}	logRF_{MRM}
GLY	Glycine	6.79	2.99	1.88	7.53	1.46	1.04	4.73	2.39	1.95
ALA	Alanine	7.42	3.01	1.95	7.93	1.56	0.57	5.34	2.27	1.51
SAR	Sarcosine				8.09	1.77	-0.28	4.87	2.35	1.14
ABA	α-Aminobutyric acid	8.11	3.98	1.87	8.32	1.64	1.98	5.87	2.46	1.34
GABA	4-Aminobutyric acid	7.30	3.00		8.10	1.43	-0.14	5.37	2.32	1.26
ßAIB	β-Aminoisobutyric acid	7.49	3.02	1.94	8.20	1.52	0.49	5.57	2.36	1.30
SER	Serine	6.48	2.81	1.90	7.34	1.56	0.62	4.65	2.14	1.28
PRO	Proline	7.22	3.50	1.53	8.43	1.76	0.76	5.48	2.13	1.17
VAL	Valine	8.27	3.27	2.58	8.68	1.24	0.60	6.37	2.46	1.46
THR	Threonine	6.83	3.06	2.02	7.68	1.58	0.42	5.17	2.27	1.32
HYP	4-Hydroxyproline				5.93		0.51	6.94	2.85	1.86
ILE	Isoleucine			1.46	8.90	1.72	0.72		2.30	
LEU	Leucine	8.66	3.09	2.08	7.60	1.63	0.18			
ASN	Asparagine	6.22	2.74	1.66	7.44	1.56	0.17	6.93	2.17	0.81
ASP	Aspartic acid	6.66	2.89	1.94	8.67	1.42	0.50	4.93	1.60	0.76
GLN	Glutamine	6.46	2.80		7.17	1.63	-0.03	4.65	1.92	0.90
GLU	Glutamic acid	6.93	3.00	2.03	8.76	1.08	-0.12	5.07	1.64	0.64
MET	Methionine	8.14	3.64	2.85	8.52	1.05	1.19	6.21	2.26	1.31
HIS	Histidine	5.78	3.27	2.27	8.70	2.23	-0.69	6.03		
AAA	α-Aminoadipic acid	7.21	3.22	2.01	8.94	1.73	0.04	5.51	1.74	-0.67

Abbreviation	Compound	DEEMM			PrCl			AQC		
		t_R	logIE_{scan}	logRF_{MRM}	t_R	logIE_{scan}	logRF_{MRM}	t_R	logIE_{scan}	logRF_{MRM}
PHE	Phenylalanine	8.47	2.86	2.13	8.98	1.39	0.56	7.00	2.36	1.43
1MHIS	1-Methyl-histidine	5.75	3.65	2.46	6.27	2.52	-1.42	4.12	1.20	-0.74
3MHIS	3-Methyl-histidine	5.82	3.27	1.95	6.25		-0.53	4.26	1.36	-0.64
ARG	Arginine	6.16	3.65	2.46	6.56	2.56	-1.05	4.25–4.8	1.32	-0.27
CIT	Citrulline	6.68	2.75		7.34	1.44	0.60	4.98	1.75	0.51
TYR	Tyrosine	7.60	3.23	2.00	9.30	1.12	-0.42	6.17	2.10	1.21
TRP	Tryptophan	8.29	3.35	2.64	8.82	1.26	-0.13	6.98	2.19	1.20
ORN	Ornithine			1.02	8.56	1.30	0.52		1.74	
LYS	Lysine			2.00	8.75	1.39	0.83	6.00	1.72	
C-C	Cystine	8.43	2.56	0.79	9.26	1.36	-0.46	6.00	1.52	

All in all, the logIE_{scan} values and logRF_{MRM} are in good correlation for AQC (**Figure 14**), with R^2 of 0.80. This good correlation is expected, as the fragmentation pattern for AA-AQC is dominated by the charge retention fragmentation, where a single fragment is observed for all of the amino acids. A lower R^2 value was observed in case of AA-DEEMM (0.30). This is expected as the range of the logRF_{MRM} values is much narrower. For AA-DEEMM, the fragmentation spectra are dominated by the derivatization reagent side. Actually, three outliers in case of DEEMM, Pro, C-C and Aba could be explained as follows. Pro has different fragmentation pattern, C-C has previously also shown difficulties in measurements due to possible instability of the molecule and in case of Aba, three compounds with the same m/z were in the mixture and it could be identification error. If we remove these outliers, R^2 for AA-DEEMM is as high as 0.68. This gives future perspective for further studies. However, no correlation between logIE and logRF_{MRM} values is observed for PrCl (R^2 is 0.01). This is expected as the fragmentation pattern strongly depends on the amino acid; therefore, the fragmentation yield component in the response factor strongly varies from amino acid to amino acid. It was also no difference in case of PrCl, if only single or multiple transition(s) were used for logRF_{MRM} calculations.

logRF_{MRM} vs logIE_{scan} for amino acids derivatizised with PrCl, **AQC**, and **DEEMM**. All measurementsa have been carried out under chromatogrphic separation.

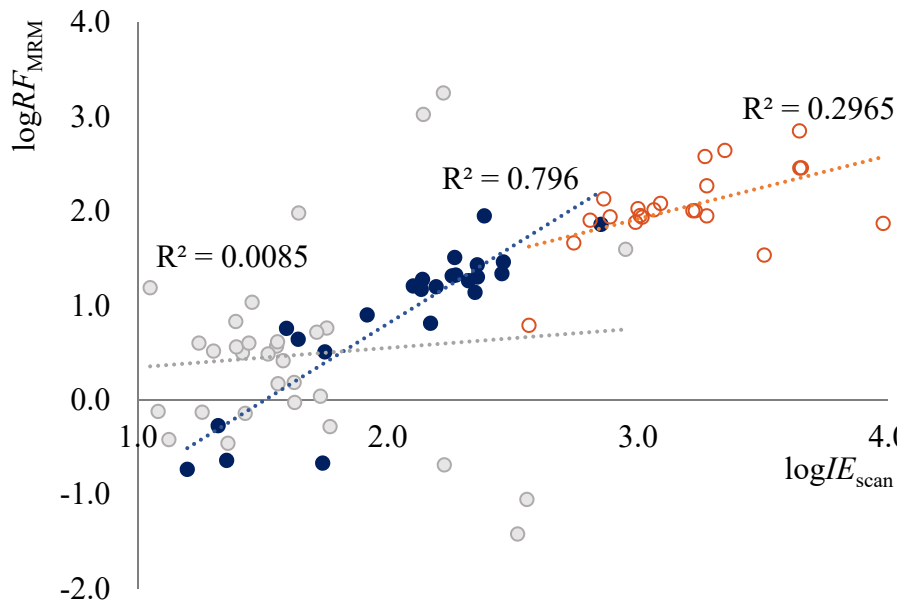

Figure 14. The correlation between the measured logIE and logRF_{MRM} values for AA derivatized with EZ fast, AQC, and DEEMM. The best correlation is observed for AA-AQC.

Application of the predicted ionization efficiency values in the MRM mode for concentration predictions for different beverage samples (Paper III)

In this work, a method to quantify the derivatized amino acids from beverages without the standard substances with both LC/ESI/MS and LC/ESI/MS/MS was developed. It was applied to distinguish between different types of wine, beer and tea of different origin.

For each compound, the measured log*IE* values and response factors logRF_{MRM} were used to evaluate concentrations in real samples. To calculate concentration units from these values in real samples, the following calculation method was used:

$$\mathrm{c,\,mg/L} = \frac{A_{sample}}{10^{\log IE - 3.95} * slope([\mathrm{Et_4N}]^+)} * [\mathrm{M(AA)}] * dil * 1000\ \mathrm{mg/g} \quad \text{(Eq. 6)}$$

where A_{sample} is intensity (peak area) of injected sample for selected substance, $\mathrm{slope}([\mathrm{Et_4N}]^+)$ is slope of tetraethylammonium measured together with sample series, M(AA) is a molecular weight of selected amino acid, *dil* is sample dilution factor and 1000 is conversion factor. These obtained concentrations were cross-checked against measured values with classical multipoint calibration graph method. Results are presented in Table 5 for beer, Table 6 for wine and **Table 7** for tea.

This approach could be used for fast and high-throughput sample screening for amino acid concentrations in different beverages. It will give an opportunity to compare batches of same kind of beverage to rule out counterfeits like dilution or syrup drinks etc. As seen from the equation 6, all that is needed, is anchor compound which will measured together with sample series.

Table 5. Amino acid concentration in different Estonian beers.

		ALeCoq				LEHE				Õllenaut				Põhjala			
		MRM		Scan		MRM		Scan		MRM		Scan		MRM		Scan	
		Measured via calibration curve c, mg/L	c, mg/L, log*RF*	Measured via calibration curve c, mg/L	c, mg/L, log*IE*	Measured via calibration curve c, mg/L	c, mg/L, log*RF*	Measured via calibration curve c, mg/L	c, mg/L, log*IE*	Measured via calibration curve c, mg/L	c, mg/L, log*RF*	Measured via calibration curve c, mg/L	c, mg/L, log*IE*	Measured via calibration curve c, mg/L	c, mg/L, log*RF*	Measured via calibration curve c, mg/L	c, mg/L, log*IE*
GLY	Glycine	47.4	7.1	40.8	29.7	50.3	7.6	42.7	31.0	34.7	5.2	30.9	22.5	59.1	8.9	51.5	37.5
ALA	Alanine	99.0	46.3	176.0	105.3	77.4	36.2	140.4	84.0	39.8	18.6	11.9	7.1	203.1	95.0	337.0	201.6
SAR	Sarcosine	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
ABA	α-Aminobutyric acid	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
GABA	4-Aminobutyric acid	60.8	42.6	58.6	40.1	87.6	61.3	88.9	60.7	9.0	6.3	9.6	6.6	94.8	66.4	105.4	72.0
ßAIB	β-Aminoisobutyric acid	1.6	1.3	4.7	3.8	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
SER	Serine	3.1	1.9	7.0	5.9	29.7	18.3	30.2	25.6	2.9	1.8	0.7	0.6	15.1	9.3	14.7	12.5
PRO	Proline	551.5	362.3	605.2	408.1	590.6	388.0	608.8	410.5	613.3	402.8	677.6	456.9	760.1	499.3	829.4	559.2
VAL	Valine	53.9	37.2	55.1	38.1	20.8	14.3	17.4	12.0	3.2	2.2	3.7	2.5	96.5	66.7	93.7	64.8
THR	Threonine	1.2	0.8	0.1	0.1	14.5	9.7	15.3	10.0	1.2	0.8	0.3	0.2	10.0	6.7	8.4	5.5
HYP	4-Hydroxyproline	15.1	11.0	11.3	8.5	16.5	12.0	14.0	10.6	2.2	1.6	0.5	0.4	48.3	35.3	44.5	33.6
ILE	Isoleucine	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
LEU	Leucine	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
ASN	Asparagine	19.2	11.3	13.3	9.8	19.6	11.6	25.4	18.8	2.4	1.4	ND	ND	46.3	27.3	56.0	41.4
ORN	Ornithine	ND	ND	ND	ND	ND	ND	308.3	325.2	ND	ND	ND	ND	ND	ND	71.0	74.9
ASP	Aspartic acid	4.6	4.0	3.5	4.0	19.1	16.7	14.7	16.5	2.2	1.9	1.6	1.7	22.9	20.0	23.0	25.8
GLN	Glutamine	2.1	1.7	ND	ND	14.8	11.7	10.5	8.2	0.5	0.4	0.0	0.0	26.5	21.1	20.3	15.7
GLU	Glutamic acid	20.2	24.7	ND	ND	31.3	38.3	27.8	26.9	4.9	6.0	3.9	3.8	52.2	63.9	51.3	49.8
MET	Methionine	1.3	1.0	0.5	0.3	4.5	3.4	4.2	2.7	0.3	0.3	0.0	0.0	11.8	8.8	12.1	7.7
HIS	Histidine	ND	ND	ND	ND	12.3	0.4	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
AAA	α-Aminoadipic acid	ND	ND	ND	ND	ND	ND	5.4	5.3	ND	ND	ND	ND	ND	ND	2.5	2.4
PHE	Phenylalanine	56.9	35.4	46.3	30.7	18.8	11.7	15.2	10.0	3.5	2.1	165.2	109.4	84.3	52.4	75.7	50.2
1MHIS	1-Methyl-histidine	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
3MHIS	3-Methyl-histidine	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
ARG	Arginine	53.0	50.2	48.1	33.6	59.4	56.3	56.8	39.8	1.3	1.3	ND	ND	96.9	91.8	99.6	69.6
CIT	Citrulline	2.0	1.4	0.3	0.2	2.0	1.4	ND	ND	2.5	1.9	ND	ND	ND	ND	0.5	0.3
TYR	Tyrosine	51.6	39.0	50.8	35.9	43.8	33.1	41.3	29.2	2.6	2.0	2.3	1.6	63.5	48.1	66.4	47.0
TRP	Tryptophan	27.7	19.8	24.6	15.1	28.5	20.3	20.7	12.7	0.9	0.6	0.4	0.2	46.4	33.1	46.0	28.2
C-C	Cystine	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
LYS	Lysine	ND	ND	22.3	14.1	ND	ND	5.0	3.2	ND	ND	ND	ND	ND	ND	ND	ND
square of correlation coefficient. R^2																	
	w/ PRO	0.989		0.999		0.988		0.949		0.998		1.000		0.980		0.995	
	w/o PRO	0.786		0.994		0.705		0.959		0.703		0.999		0.727		0.969	

Table 6. Amino acid concentration in different wines.

		Põltsamaa Kuldne				Merlot. France				Pinotage. South Africa				Cabernet Sauvignot. South Africa				Cabernet Sauvignot. France			
		MRM		Scan		MRM		Scan		MRM		Scan		MRM		Scan		MRM		Scan	
		Measured via calibration curve c. mg/L	c. mg/L. logRF	Measured via calibration curve c. mg/L	c. mg/L. logRF	Measured via calibration curve c. mg/L	c. mg/L. logRF	Measured via calibration curve c. mg/L	c. mg/L. logRF	Measured via calibration curve c. mg/L	c. mg/L. logRF	Measured via calibration curve c. mg/L	c. mg/L. logRF	Measured via calibration curve c. mg/L	c. mg/L. logRF	Measured via calibration curve c. mg/L	c. mg/L. logRF	Measured via calibration curve c. mg/L	c. mg/L. logRF	Measured via calibration curve c. mg/L	c. mg/L. logRF
GLY	Glycine	24.1	3.6	22.9	16.6	38.2	5.7	30.5	22.2	40.9	6.1	39.6	28.8	41.5	6.2	35.6	25.9	38.8	5.8	31.8	23.2
ALA	Alanine	36.7	17.2	53.4	31.9	59.1	27.6	82.1	49.1	105.7	49.5	172.1	103.0	56.8	26.6	91.8	54.9	55.5	26.0	91.3	54.6
SAR	Sarcosine	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
ABA	α-Aminobutyric acid	0.1	0.1	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
GABA	4-Aminobutyric acid	3.2	2.2	ND	ND	12.2	8.5	11.1	7.6	93.7	65.7	85.3	58.3	11.1	7.8	9.8	6.7	13.8	9.7	11.6	8.0
ßAIB	β-Aminoisobutyric acid	1.0	0.8	ND	ND	1.3	1.0	ND	ND	9.2	7.3	11.1	9.1	0.6	0.5	0.4	0.3	1.5	1.2	1.4	1.1
SER	Serine	3.7	2.3	0.7	0.6	9.6	5.9	9.3	7.9	18.3	11.3	15.7	13.3	13.1	8.1	8.8	7.4	11.5	7.1	11.9	10.1
PRO	Proline	8.0	5.3	13.4	9.1	2403	1578	2327	1569	1448	951	1436	968	3048	2002	3089	2083	2124	1395	2212	1491
VAL	Valine	2.4	1.7	1.0	0.7	7.1	4.9	6.7	4.6	8.2	5.7	14.3	9.9	8.6	6.0	8.5	5.9	9.2	6.4	8.8	6.1
THR	Threonine	0.9	0.6	0.2	0.1	7.0	4.7	4.2	2.7	23.6	15.9	24.0	15.7	8.0	5.3	6.9	4.5	7.7	5.2	7.7	5.0
HYP	4-Hydroxyproline	1.5	1.1	1.9	1.5	6.3	4.6	5.7	4.3	12.1	8.8	12.5	9.4	6.4	4.7	5.4	4.1	10.0	7.3	11.5	8.7
ILE	Isoleucine	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
LEU	Leucine	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
ASN	Asparagine	2.0	1.2	0.5	0.4	7.4	4.3	5.4	4.0	13.0	7.7	5.4	4.0	7.3	4.3	3.4	2.5	11.9	7.0	3.9	2.9
ORN	Ornithine	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	8.6	9.0	4.1	32.1	1.7	1.7	ND	ND	1.9	2.0
ASP	Aspartic acid	4.5	3.9	1.6	1.8	8.3	7.3	7.1	8.0	21.2	18.5	13.1	14.7	10.2	8.9	9.9	11.1	12.1	10.6	12.6	14.2
GLN	Glutamine	0.0	0.0	ND	ND	0.1	0.1	ND	ND	0.1	0.1	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
GLU	Glutamic acid	2.7	3.2	ND	ND	23.4	28.6	20.0	19.4	46.3	56.6	38.7	37.6	24.6	30.1	23.4	22.7	33.9	41.5	19.1	18.6
MET	Methionine	0.2	0.1	ND	ND	1.9	1.4	ND	ND	4.6	3.5	5.2	3.3	1.3	1.0	1.8	1.1	2.8	2.1	2.6	1.6
HIS	Histidine	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
AAA	α-Aminoadipic acid	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
PHE	Phenylalanine	2.7	1.7	1.0	0.7	7.1	4.4	5.7	3.8	12.6	7.9	13.4	8.9	7.5	4.6	6.7	4.4	10.6	6.6	10.1	6.7
1MHIS	1-Methyl-histidine	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
3MHIS	3-Methyl-histidine	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
ARG	Arginine	0.7	0.7	0.6	0.4	11.1	10.5	ND	ND	251.8	238.7	196.8	137.6	4.8	4.6	ND	ND	11.9	11.3	3.2	2.3
CIT	Citrulline	0.3	0.2	ND	ND	ND	ND	0.7	0.5	1.9	1.4	1.2	0.8	1.6	1.2	ND	ND	ND	ND	ND	ND
TYR	Tyrosine	2.2	1.7	ND	ND	3.1	2.4	ND	ND	7.4	5.6	14.0	9.9	4.6	3.4	7.5	5.3	5.0	3.8	8.8	6.3
TRP	Tryptophan	0.1	0.0	ND	ND	2.0	1.4	ND	ND	2.4	1.7	1.3	0.8	2.1	1.5	0.5	0.3	2.2	1.6	4.7	2.9
C-C	Cystine	ND	ND	ND	ND	ND	ND	1.5	1.1	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
LYS	Lysine	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	13.0	8.2	ND	ND	7.5	4.7	ND	ND	17.6	11.2
square of correlation coefficient. R^2																					
	w/ PRO	0.799		0.993		1.000		1.000		0.992		1.000		1.000		1.000		1.000		1.000	
	w/o PRO	0.805		0.993		0.579		0.973		0.938		0.986		0.540		0.970		0.501		0.970	

Table 7. Amino acid concentration in different tea.

		Chamomile tea				Green tea			
		MRM		Scan		MRM		Scan	
		Measured via calibration curve c. mg/L	c. mg/L. log*RF*	Measured via calibration curve c. mg/L	c. mg/L. log*IE*	Measured via calibration curve c. mg/L	c. mg/L. log*RF*	Measured via calibration curve c. mg/L	c. mg/L. log*IE*
GLY	Glycine	2.6	0.3	3.0	2.8	0.4	0.0	0.5	0.4
ALA	Alanine	9.6	9.1	9.7	10.9	3.4	3.2	4.5	5.0
SAR	Sarcosine	ND	ND	ND	ND	ND	ND	ND	ND
ABA	α-Aminobutyric acid	0.1	0.1	ND	ND	ND	ND	ND	ND
GABA	4-Aminobutyric acid	10.0	7.5	10.2	12.6	1.0	0.8	1.1	1.3
ßAIB	β-Aminoisobutyric acid	0.2	0.1	ND	ND	ND	ND	ND	ND
SER	Serine	8.6	3.7	11.8	9.6	7.3	3.2	10.5	8.6
PRO	Proline	19.7	13.9	24.3	25.3	2.5	1.7	3.0	3.2
VAL	Valine	7.2	4.8	7.9	8.5	5.0	3.3	5.9	6.3
THR	Threonine	6.0	4.1	8.4	8.6	3.8	2.6	5.6	5.7
HYP	4-Hydroxyproline	4.4	2.6	4.2	4.6	2.9	1.7	3.1	3.3
ILE	Isoleucine	ND	ND	ND	ND	ND	ND	ND	ND
LEU	Leucine	ND	ND	ND	ND	ND	ND	ND	ND
ASN	Asparagine	4.9	2.2	6.6	6.7	2.8	1.2	4.5	4.6
ASP	Aspartic acid	7.6	3.3	8.2	6.2	6.8	2.9	45.1	34.1
GLN	Glutamine	9.7	5.8	12.6	11.3	23.9	14.3	33.5	30.1
GLU	Glutamic acid	13.3	4.7	22.8	3.6	26.0	9.1	46.7	7.3
MET	Methionine	ND	ND	ND	ND	ND	ND	ND	ND
HIS	Histidine	ND	ND	ND	ND	ND	ND	ND	ND
AAA	α-Aminoadipic acid	ND	ND	ND	ND	ND	ND	ND	ND
PHE	Phenylalanine	3.9	2.3	4.2	5.4	4.2	2.5	5.3	6.9
1MHIS	1-Methyl-histidine	ND	ND	ND	ND	ND	ND	ND	ND
3MHIS	3-Methyl-histidine	0.3	0.3	0.7	0.5	0.3	0.3	0.8	0.5
ARG	Arginine	10.0	12.8	30.2	23.2	20.0	25.6	55.0	42.2
CIT	Citrulline	ND	ND	ND	ND	ND	ND	ND	ND
TYR	Tyrosine	6.8	3.3	9.2	8.7	3.1	1.5	3.2	3.0
TRP	Tryptophan	2.5	1.5	3.0	3.4	3.7	2.3	5.6	6.4
ORN	Ornithine	ND	ND	ND	ND	ND	ND	ND	ND
LYS	Lysine	ND	ND	25.9	ND	ND	ND	10.9	ND
C-C	Cystine	ND	ND	ND	ND	ND	ND	ND	ND
Square of correlation coefficient. R^2									
	w/ GLU	0.741		0.617		0.663		0.736	
	w/o GLU	0.816		0.932		0.806		0.991	

Validation of the ionization efficiency-based quantification method (Paper III)

The trueness of the method can be evaluated based on the analysis of the real wine, beer and tea samples by comparing the concentrations determined (1) with log*IE* or log*RF* based method and (2) calibration graph-based method. As seen from the Table 5, Table 6 and Table 7, good correlation could be obtained, when we use measured and predicted concentrations.

The analysis of the predicted concentrations vs concentration measured with calibration graph resulted in R^2 of 0.90 and slope of 0.71 over four beer samples (including both MRM and scan data). Slope value below 1 shows that concentration calculation via prediction slightly underestimate it. Comparing MRM and san modes, the square of correlation coefficient over all beer samples is 0.85 and 0.93 and slopes 0.60 and 0.75 respectively. For the wine samples, R^2 for four wine samples was 0.92 and slope 0.72 (including both MRM and scan data). Slope value below 1 show, that concentration calculation via prediction slightly underestimate them. Comparing MRM and scan, R^2 is 0.89 and 0.98 and slopes 0.55 and 0.65 respectively. For two tea samples R^2 values over 0.8 were observed when comparing log*IE* or log*RF* with calibration graph method.

Two sets are given for wines and beers, with and without proline, since proline has 7800 times higher concentration in some wine and 1700 times higher in some beer (compared to lowest concentration found) and also to show better correlation in lower range of concentrations. For the comparison sample-by-sample R^2 values between 0.95 and 1.00 are observed over both MRM and scan measurement modes with proline and between 0.70 and 1.00 without proline in case of beers. R^2 values between 0.80 and 1.00 are observed over both MRM and scan measurement modes with proline and between 0.50 and 0.99 without proline in case of wines. Overall correlations between log*RF* and calibration graph and log*IE* and calibration graphs for wine, beer and tea, are presented on **Figure 15, Figure 16** and **Figure 17.**

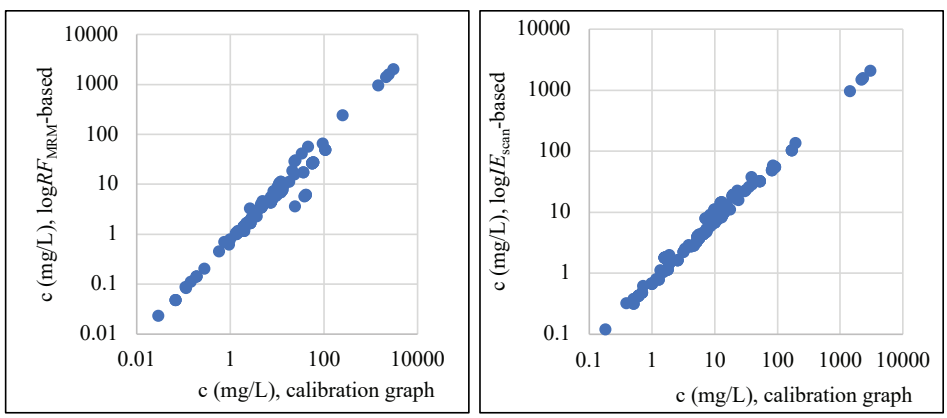

Figure 15. The fit between concentrations of amino acids in **wines** estimated with calibration graph vs estimated with logRF_{MRM} (left) or logIE_{scan} (right) based method.

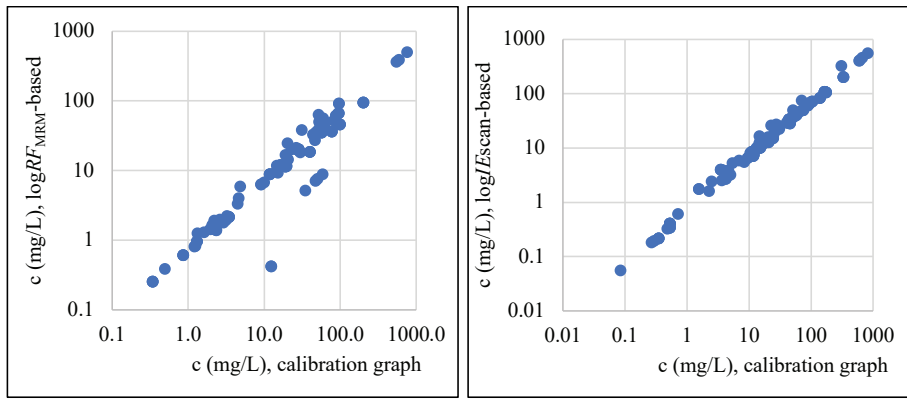

Figure 16. The fit between concentrations of amino acids in **beers** estimated with calibration graph based method vs estimated with logRF_{MRM} (left) or logIE_{scan} (right) based method.

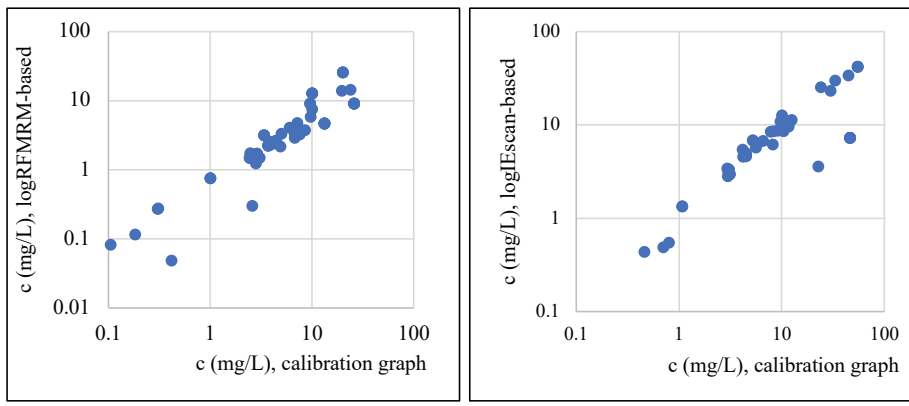

Figure 17. The fit between concentrations of amino acids in **teas** estimated with calibration graph based method vs estimated with logRF_{MRM} (left) or logIE_{scan} (right) based method.

In every sample correlation between measured and predicted concentrations in scan mode is better than in MRM mode. Some compounds, like glycine and alanine, have higher standard deviation for logRF_{MRM} measurement and this influences the accuracy of concentration prediction. At the same time, logIE measurements have better precision. These poorer precisions in logRF_{MRM} measurements also influence the prediction, since in MRM mode, for example, we can see that glycine and alanine which had poor precision, are also the outliers in correlation between measured and predicted concentrations.

Additionally, the trueness was calculated by using the concentration estimated with the calibration graph approach as a reference value.

$$Trueness = average\left(\frac{c_{IE\ based\ method}}{c_{calibration\ graph\ method}}\right) \cdot 100\ \%$$

For the MRM based method, the average trueness value is 70.5 %. For full scan based method the average trueness is 75.5 %. For both modes, most of the values are within 50 and 125 %. Notably, in MRM mode glycine has a very low trueness value. This is due to the less accurate logRF_{MRM} values available for glycine. The numerical trueness values for all analytes for both beer and wine samples are brought in Table S3.

The influence of matrix effect was evaluated based on the trueness values from sample to sample. In case matrix effect would have high contribution to the methods accuracy we would expect to see high variations in the trueness values between different samples. This is due to the fact that in different samples analytes are coeluting with different matrix components and are, therefore, expected to show scatter in trueness values. Especially, this would be expected while comparing wine and beer samples.

For all samples, both wine and beer samples, the concentration prediction accuracy does not depend on the sample, rather on the compound. Therefore, it was concluded that the influence of the matrix effect on the method accuracy is negligible.

It gives a very good opportunity to predict concentration levels of different compounds in wines and evaluate potentially hazardous compounds in wines via only knowing the log*IE* or log*RF* value of derivatized compounds both in scan and in MRM mode.

Comparison of amino acid concentrations in wines, beers and tea

It was observed, that in Estonian apple wine amino acids concentrations are in much lower concentrations than in classical red grapes wines. The same trend was observed independently with the quantification methods (logIE_{scan}, logRF_{MRM} or calibration graph) for all wine samples (**Figure 18**). The patterns observed for wines are the same independent of the quantification method. Calculated and predicted concentrations are average of 100- and 1000-fold diluted samples. Found concentrations in beers and wines are comparable with previous result.[3]

Found concentration of two tea samples were also correlated between classical multi-point calibration graph method and log*IE* or log*RF* estimated values.

It is observed that the beer from Õllenaut has the lowest amino acid concentration. Generally, the patterns observed for beers are the same independent of the quantification method (**Figure 19**). This allows suggesting that logRF_{MRM} based quantification approach can be useful in the characterization of suspected compounds in beverages if the calibration graph cannot be prepared due to lack of standard compounds. In order to further validate this assumption, the method was partially validated for relevant method performance characteristics.

Figure 18. The variation of amino acid concentration between different wines determined by the logRF_{MRM} method (left) and calibration graph-based method(right).

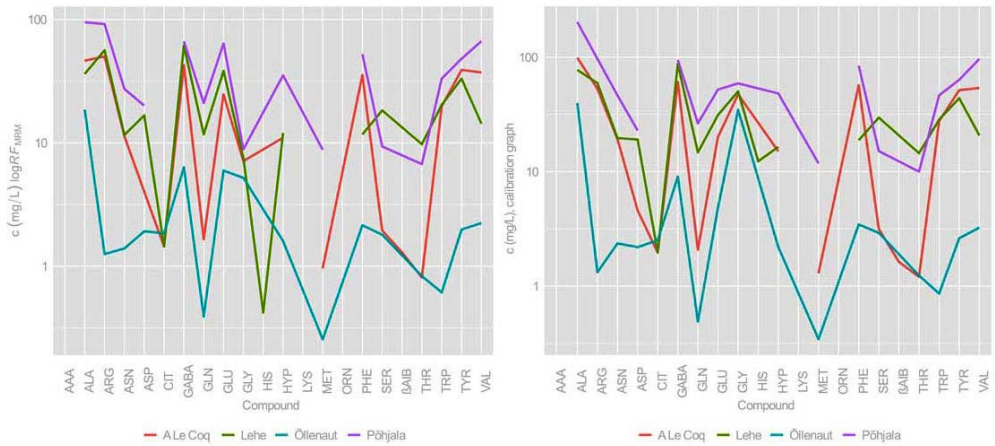

Figure 19. The variation of amino acid concentration between different beers and determined by the logRF_{MRM} method (left) and calibration graph-based method(right).

SUMMARY

Liquid chromatography electrospray ionization (tandem) mass spectrometry LC/ESI/MS(MS) is the most versatile and powerful analytical method for analyzing hundreds of analytes from various different matrices in very low quantities. Until today, majority of studies, methods and application still use standard substances to quantitatively determine analytes in question. The scale of ionization efficiency (log*IE*) in scan modes have been constructed previously to partly solve this problem for screening methods. The aim of this doctoral thesis was to provide solution to eliminate standard substances for targeted analysis of derivatized compound for analysis carried out with LC/ESI/MS/MS.

First, the method of how to measure log*IE* values for derivatized amino acids was needed in order to study how derivatization changes ionization efficiency (IE) in the ESI source. The method had to be different from the originally designed log*IE* measurement method using a well-controlled (solvent composition, analytes in the ESI source) flow-injection method. Log*IE* measurement of derivatized compounds needed a method allowing the use of a chromatographic column and a changing eluent composition. In this work, such a method was developed and this allowed to measure and evaluate the IE of different compounds, mainly for amino acids and biogenic amines, which are important constituents in different foodstuff and beverages. Log*IE* values of both derivatized and underivatized analytes were measured and results showed that in most cases, derivatization enhances IE. In addition, log*IE* values obtained in this work were also linked to previously measured log*IE* scales in order to further broaden these scales.

Together with log*IE* studies of protonated compounds, a sodium-adduct formation and ESI negative ion mode were also studied. In general, it was noted, that sodium-adduct formation and deprotonation in negative ion mode (in conditions targeted to positive ion mode) are very erratic and correlation couldn't be drawn between different modes. But in one case, PrCl-derivatized amino acid, one interesting observation was made, where weak (R^2=0.5) correlation was achieved between positive ion mode and formation of sodium-adducts. However, this shows that for log*IE* application for standard substance free analysis, protonated analytes should be preferred.

During the method development and application of the new log*IE* measurement system using the chromatographic column, it was compared how different ionization sources and eluents would correlate with each other in respect of log*IE*. Essentially, the idea behind it was to show that already published log*IE* values can be used to evaluate the sensitivity when a chromatographical analysis is applied. This was done by measuring sets of compounds with four different setups. It was seen, that in case of atmospheric pressure chemical ionization (APCI) source, correlation between log*IE* and log*Slope* of chromatographic method while using methanol or acetonitrile was not so good (R^2=0,42), and probably high errors would occur, if one wants to predict the sensitivity of

chromatographic method from log*IE* values of the same system. But in case of ESI source, a higher correlation (R^2=0.71 and 0.79) was observed and in this case, log*IE* values measured in flow-injection mode would be highly beneficial to assess sensitivity for chromatographic method with either solvent.

Since most practical applications nowadays use MS/MS analysis, the next aim was to evaluate the correlation of measured log*IE* values with response factors (log*RF*) in MS/MS, which would yield opportunity to use RF for evaluating content of analytes. It was found that in case of amino acids derivatized with AQC, a very high correlation (R^2=0.80) between log*IE* and log*RF* in MS/MS occurred, which means, that log*IE* values indicate log*RF* values with high accuracy. This allowed to move into the last step of the method development.

Since the ultimate goal in this thesis was to test if an analysis without standard substances is possible, the final study was to see if log*RF* values could be used to estimate analyte concentration without standard substances in different matrices. Matrices chosen were beer, wine and tea, where amino acid analysis has been previously used to evaluate their quality, origin, etc. This method showed good correlation between log*IE* and log*RF* values, which were both used for estimating amino acid concentration in beer, wine and tea. Both estimates (log*IE* and log*RF*) showed very high correlation compared to concentrations obtained with a calibration curve method and the method was also validated to show its trueness and other parameters. Therefore, it was concluded that this approach would be useful tool to make a targeted scan very effective for many analytes together to evaluate their concentration, by which it is possible to distinguish counterfeit drinks from the original ones.

As demonstrated, standard free quantitation for targeted MS/MS analysis is possible. It will open lots of possibilities for a wide area of different application to perform such analysis. This application could possibly be expanded to other reagents and analytes which in turn widens even more range of use such approach.

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SUMMARY IN ESTONIAN

Derivatiseeritud ainete ionisatsiooniefektiivsuste uurimine LC/ESI/MS jaoks ja nende rakendamine suunatud analüüsiks

Vedelikkromatograafia elektropihustus-ionisatsiooni massispektromeetria LC/ESI/MS(MS) on kõige mitmekülgsem ja võimsam analüüsimeetod sadade erinevatest maatriksitest pärit analüütide määramiseks väga väikestes kogustes. Kuni tänapäevani kasutatakse enamikus uuringutes, meetodites ja rakendustes vaatlusaluste analüütide kvantitatiivseks määramiseks standardaineid, sest molekulide ioniseerumise efektiivsus võib ionisatsiooniallikas erineda mitmeid miljoneid kordi. Mittesuunatud analüüsi jaoks (ingl *nontargeted*) on varasemalt koostatud ionisatsiooniefektiivsuse skaalasid (log*IE*), et seda probleemi osaliselt skriinimismeetodite jaoks lahendada. Selle doktoritöö eesmärk oli pakkuda lahendus standardainete elimineerimiseks suunatud (ingl *targeted*) analüüsi jaoks derivatiseeritud ühendite analüüsiks MRM mõõtmise režiimis.

Esiteks oli vaja välja töötada log*IE* mõõtmise meetod derivatiseeritud ainete jaoks, sest seni kasutusel olnud meetod, mida peamiselt kasutati puhaste ainete jaoks, ei sobinud. Selleks uuriti, kuidas derivatiseerimine (aine spetsiifiliste omaduste muutmine) muudab ionisatsiooniefektiivsust (IE) ESI allikas ning võrreldi erinevate analüütiliste tingimuste komplekte nagu eluendi koostis ja kromatograafiakolonni lisamine, et hinnata, kuidas need omakorda mõjutavad IEd. Samuti ei saa derivatiseeritud ühendeid segus mõõta ilma kolonnita, nagu seni tehtud puhaste ühendite puhul. Selle tulemusel töötati välja kromatograafiline meetod erinevate ühendite, peamiselt derivatiseeritud aminohapete ja biogeensete amiinide, IE mõõtmiseks ja hindamiseks, mis on olulised koostisosad erinevates toiduainetes ja jookides. Oli selgelt näha, et derivatiseerimine suurendab enamikul juhtudel IE-d. Tulemused seoti ka varem mõõdetud IE skaaladega, et laiendada olemasolevate skaalade kasutusvõimalusi.

Koos log*IE* uuringutega uuriti ka naatrium-addukti moodustumist ja ESI negatiivsete ioonide tekkimist negatiivses režiimis. Üldiselt täheldati, et naatrium-addukti moodustumine positiivses ja deprotoneerimine negatiivsete ioonide režiimis (tingimustes, mis on pigem sobilikud positiivse iooni tekkel) on väga ebakorrapärane ja korrelatsiooni ei olnud võimalik erinevate režiimide vahel tõmmata. Kuid ühel juhul, PrCl-ga derivatiseeritud aminohapetega, tehti üks huvitav tähelepanek, kus positiivse iooni tekkimise ja naatriumi adduktide tekkimise vahel saavutati nõrk ($R^2 = 0.5$) korrelatsioon.

Järgnevalt võrreldi, kuidas erinevad ionisatsiooniallikad ja eluendid log*IE* suhtes korreleeruvad. Selleks tehti nelja erineva seadistusega ühendikomplektide mõõtmine. Oli näha, et atmosfäärirõhu keemilise ionisatsiooni (APCI) allika korral pole log*IE* ja kromatograafilise meetodi log*Slope* vaheline korrelatsioon metanooli või atseetonitriili kasutamisel nii hea ($R^2 = 0.42$) ja tõenäoliselt oleks vead suured, kui soovitakse ennustada kromatograafilise meetodi

tundlikkust sama süsteemi log*IE* väärtuste põhjal. Kuid ESI allika puhul täheldati pisut kõrgemat korrelatsiooni (R^2 = 0.71 ja 0.79) ja sel juhul oleks ilma kromatograafilise kolonnita mõõdetud log*IE* väärtused kromatograafilise meetodi tundlikkuse hindamiseks kummagi lahustiga väga kasulikud.

Kuna enamus tänapäevaseid analüüsirakendusi kasutavad MS/MS süsteemi, siis järgmine eesmärk oli hinnata mõõdetud log*IE* väärtuste korrelatsiooni MS/MS tundlikkusfaktoriga (RF), mis annaks võimaluse kasutada RFi analüütide sisalduse hindamiseks suunatud analüüsil. Leiti, et AQC korral on väga kõrge korrelatsioon (R^2 = 0.80) log*IE* ja log*RF* vahel, mis tähendab, et log*IE* väärtused näitavad suure täpsusega log*RF* väärtusi. Sellest saab tuletada käesoleva doktoritöö lõpliku eesmärgi.

Kuna suunatud analüüsi käigus saavutatakse ühendite määramisel madalad määramispiirid, oleks väga kasulik kasutada log*RF* väärtusi analüüdi kontsentratsiooni määramiseks ilma standardaineteta erinevates maatriksites. See meetod näitas head korrelatsiooni log*IE* ja log*RF* väärtuste vahel, mida kasutati õlle, veini ja tee aminohapete kontsentratsiooni hindamiseks. Mõlemad hinnangud (log*IE* ja log*RF*) näitasid väga kõrget korrelatsiooni võrreldes kalibreerimisgrafiku meetodiga ning see valideeriti ka selle tõesuse ja muude parameetrit osas. Väljatöötatud meetod oleks kasulik vahend, et muuta suunatud analüüs väga tõhusaks paljude analüütide jaoks korraga, ja hinnata nende kontsentratsiooni, mille abil on võimalik võltsitud jooke eristada õigetest jookidest.

Nagu käesoleva doktoritöö raames välja töötatud meetod näitas, on standardainete vaba analüüdisisalduse määramine võimalik ka suunatud MS/MS analüüsi korral. See avab paljusid võimalusi erinevate rakenduste jaoks, mida on võimalik laiendada ka teistele derivatiseerivatele reagentidele ja analüütidele.

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APPENDIX

Table S1. log*IE* values of measured DEEMM-derivatized compounds.

Compound name	*m/z* [M+H]+ underivatized	*m/z* [M+H]+ derivatized	log*IE* underivatized	log*IE* derivatized	Absolute log*IE* change	MW, g/mol
ET4N+	130 (M+)	-	-	-	-	130
AMPA	112	282	0.22	2.66	2.44	111
Asparagine	133	303	0.95	2.21	1.26	132
Aspartic acid	134	304	0.98	2.73	1.75	133
Histamine	112	282	1.80	3.23	1.43	111
Cysteine	122	292	2.16	1.85	-0.31	121
Serine	106	276	2.17	2.87	0.70	105
Histidine	156	326	2.22	3.41	3.22	155
Proline	116	286	2.24	2.16	-0.08	115
Selenocysteine	169	306	2.28	3.41	1.13	168
Spermidine	146	316	2.31	2.68	0.37	145
Threonine	120	290	2.47	3.08	0.60	119
Glutamine	147	317	2.51	3.02	0.51	146
Arginine	175	345	2.57	3.18	0.62	174
Lysine	147	317	2.58	3.55	0.97	146
Selenomethionine	197	367	2.60	3.03	0.43	196
Tryptophan	205	375	2.61	3.45	0.83	204
Glutamic acid	148	318	2.69	3.16	0.47	147
Valine	118	288	2.75	3.23	0.48	117
Methionine	150	320	3.10	3.39	0.29	149
Normetanephrine	184	354	3.11	2.76	-0.35	183
Isoleucine	132	302	3.22	3.31	0.09	131
Leucine	132	302	3.27	3.42	0.15	131
Metanephrine	198	368	3.60	2.87	-0.74	197
Phenyl-alanine	166	336	3.61	3.28	-0.33	165

Table S2. MRM transitions for PrCl reagent

Compound name	Precursor *m/z*	Product ion, *m/z*
Cystine	497	248
		437
		306
Tyrosine	396	136
		308
		336
Histidine	370	196
		110
		284
Lysine	361	301
		170
Ornithine	347	287
		156
		227
Tryptophan	333	245
		273
		230
α-Aminoadipic acid	332	244
		272
Glutamic acid	318	172
		258
		230
Citrulline	304	156
		113
		244
		287
Aspartic acid	304	216
		130
		244
Arginine	303	70
		156
		286
1-Methyl-histidine	298	96
		196
3-Methyl-histidine	298	210
Phenylalanine	294	206
		120
Methionine	278	190
		218
Glutamine	275	172
		84
		215

Compound name	Precursor *m/z*	Product ion, *m/z*
Isoleucine	260	172
		130
		74
4-Hydroxyproline	260	157
Threonine	248	160
		188
		230
Valine	246	158
		116
		186
Proline	244	156
		114
		184
Asparagine	243	157
		115
		201
Serine	234	146
		174
		216
4-Aminobutyric acid	232	172
		130
β-Aminoisobutyric acid	232	172
		130
α-Aminobutyric acid	232	172
		144
Alanine	218	130
		88
Sarcosine	218	88
		130
Glycine	204	144
		118
		162

Table S3. The trueness values for amino acids and amines determined with logRF_{MRM} and logIE_{scan} method.

Compound	Sample type	Trueness for logRF_{MRM} method	Trueness for logIE_{scan} method
ABA	Wine	73.7	NaN
ALA	Wine	46.8	59.8
ARG	Wine	94.8	69.9
ASN	Wine	59.0	74.0
ASP	Wine	87.5	112.5
ÃŸAIB	Wine	79.5	82.4
C-C	Wine	NaN	69.5
CIT	Wine	73.8	67.6
GABA	Wine	70.0	68.4
GLN	Wine	79.5	NaN
GLU	Wine	122.4	97.1
GLY	Wine	15.0	72.8
HYP	Wine	73.1	75.6
LYS	Wine	NaN	63.3
MET	Wine	74.4	63.3
ORN	Wine	NaN	105.5
PHE	Wine	62.2	66.2
SER	Wine	61.8	84.7
THR	Wine	67.2	65.5
TRP	Wine	71.3	61.3
TYR	Wine	75.6	70.8
VAL	Wine	69.1	69.1

Compound	Sample type	Trueness for logRF_{MRM} method	Trueness for logIE_{scan} method
AAA	Beer	NaN	97.4
ALA	Beer	46.8	59.8
ARG	Beer	94.8	69.9
ASN	Beer	59.0	74.0
ASP	Beer	87.5	112.5
ÃŸAIB	Beer	79.5	82.4
CIT	Beer	73.8	67.6
GABA	Beer	70.0	68.4
GLN	Beer	79.5	77.6
GLU	Beer	122.4	97.1
GLY	Beer	15.0	72.8
HIS	Beer	3.4	NaN
HYP	Beer	73.1	75.6
LYS	Beer	NaN	63.3
MET	Beer	74.4	63.3
ORN	Beer	NaN	105.5
PHE	Beer	62.2	66.2
SER	Beer	61.8	84.7
THR	Beer	67.2	65.5
TRP	Beer	71.3	61.3
TYR	Beer	75.6	70.8
VAL	Beer	69.1	69.1

PUBLICATIONS

CURRICULUM VITAE

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Education

2009–…	University of Tartu, PhD (Chemistry)
2007–2009	University of Tartu, MSc (Chemistry)
2003–2007	University of Tartu, BSc (Chemistry)

Work experience

2011–…	Estonian Environmental Research Centre, Fuel Quality and Customs Services Department
2008–2011	State Agency of Medicines, laboratory

Scientific publications

1. **Gornischeff, Artur**; Kruve, Anneli; Rebane, Riin (2020). Characterization of wines with liquid chromatography electrospray ionization mass spectrometry: quantification of amino acids via ionization efficiency values. Journal of Chromatography A, 1620:461012, 1–7.
2. Rebane, Riin; Kruve, Anneli; Liigand, Jaanus; Liigand, Piia; **Gornischeff, Artur**; Leito, Ivo (2019). Ionization efficiency ladders as tools for choosing ionization mode and solvent in liquid chromatography/mass spectrometry. Rapid Communications in Mass Spectrometry, 33 (23), 1834–1843.
3. **Gornischeff, Artur**; Liigand, Jaanus; Rebane, Riin (2018). A systematic approach toward comparing electrospray ionization efficiencies of derivatized and non-derivatized amino acids and biogenic amines. Journal of mass spectrometry, 53 (10), 997−1004.
4. **Gornischeff, Artur**; Rinken, Toonika (2011). Calculating the output signal parameters of a lactose bienzymatic biosensing system from the transient phase response. Proceedings of the Estonian Academy of Science, 60 (2), 136–140.

ELULOOKIRJELDUS

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Haridus

2009–…	Tartu Ülikool, doktoriõpe (Keemia)
2007–2009	Tartu Ülikool, MSc (Keemia)
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Erialane töökogemus

2011–…	Eesti Keskkonnauuringute Keskus OÜ, Kütusekvaliteedi ja tolliteenuste osakond
2008–2011	Ravimiamet, labor

Publikatsioonide loetelu

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