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Derivatization-targeted LC-MS analysis of compounds containing amino group

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

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II. Marengo, A., **Maciel, L. S.**, Cagliero, C., Rubiolo, P., & Herodes, K. (2023). Free Amino Acids and Biogenic Amines Profiling and Variation in Wild and Sub-Endemic Cardueae Species from Sardinia and Corse. *Plants*, *12*(2). https://doi.org/10.3390/plants12020319

III. **Maciel, L. S.**, Malenica, D., Herodes, K., Kass, M., & Bhat, R. (2024). Detection and quantification of free amino compounds in selected agricultural by-products with derivatization followed by liquid chromatography-tandem mass spectrometry analysis. *Food Chemistry Advances*, *4*(January), 100629. https://doi.org/10.1016/j.focha.2024.100629

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Paper I: One of the main people responsible for planning and performing the experiments, data treatment, visualization and writing the manuscript.

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ABBREVIATIONS

AA	amino acid
ACN	acetonitrile
AP	apple pomace
AQC	6-Aminoquinolyl-N-hydroxysccinimidyl carbamate
BA	biogenic amine
BCAAs	branched-chain amino acids
DDA	data-dependent acquisition
DEEMM	diethyl ethoxymethylenemalonate
DIA	data-independent acquisition
dMRM	dynamic multiple reaction monitoring
DoE	design of experiments
ESI	electrospray ionization
EtOH	ethanol
FLD	fluorescence detector
Fmoc-Cl	fluorenylmethyloxycarbonyl chloride
FTICR	Fourier-transform ion cyclotron resonance
GC-MS	gas chromatography-mass spectrometry
HCl	hydrochloric acid
HILIC	hydrophilic interaction liquid chromatography
HPH	hempseed hull
HPLC	high-pressure liquid chromatography
HRMS	high-resolution mass spectrometry
IEC	ion-exchange chromatography
LC-MS/MS	liquid chromatography-tandem mass spectrometry
LC-MS	liquid chromatography-mass spectrometry
MDPF	2-Methoxy-2,4-diphenyl-3(2H)-furanone
ME	matrix effect
MeOH	methanol
NITC	2-naphthylisothiocyanate
NLS	neutral loss scan mode
NMR	nuclear magnetic resonance spectroscopy
NPAA	non-protein amino acids
OB	oat bran
OPA	o-phthalaldehyde
PAA	proteinogenic amino acids
PCA	principal component analysis
PITC	phenylisothiocyanate
PLS-DA	partial least squares discriminant analysis
PIS	precursor ion scan mode
QqQ	triple quadrupole mass analyzer
QToF	quadrupole time-of-flight
RPLC	reversed-phase liquid chromatography

RSD	relative standard deviation
RSM	response surface methodology
RT	retention time
SBL	sea buckthorn leaves
SBP	sea buckthorn pomace
SPTPP	(5-N-succinimidoxy-5-oxopentyl)triphenylphosphonium bromide
TAHS	p-N,N,N-trimethylammonioanilyl N′-hydroxysuccinimidyl carbamate iodide
TCA	trichloroacetic acid
TFA	trifluoroacetic acid
TMPP	(N-Succinimidyloxycarbonylmethyl)tris(2,4,6-trimethoxyphenyl)phosphonium bromide
UASE	ultrasound-assisted solid-liquid extraction
UV-Vis	ultraviolet–visible

1. INTRODUCTION

Non-targeted analysis encompasses the simultaneous detection of a wide range of chemical compounds, which is usually performed by high-resolution mass spectrometry (HRMS). Metabolomics is a field interested in non-targeted analysis to discover and identify metabolites (molecular weight < 1.5 kDa) and translate that into clinical, biomarker discovery and environmental field. There is a high occurrence of amino-containing metabolites due to their biological importance and comprehend proteinogenic, non-proteinogenic and post-translationally modified amino acids, polyamines, peptides, nucleosides, catecholamines and biogenic amines.

Regarding the analysis of amino compounds, reverse-phase liquid chromatography-tandem mass spectrometry (RPLC-MS/MS) has become widespread since it offers information on the molecular mass of compounds and enables distinction of co-eluting compounds based on their molecular masses. Nevertheless, the challenges by this approach include weak retention in a RP column and low ionization efficiency. Both problems can be tackled by employing derivatization. The derivatives present better retention in a RP column, ionization efficiency and a fragmentation pattern in MS/MS.

Derivatization can be employed to detect a specific group of compounds based on their functional groups because of the constant fragmentation pattern of the derivatives. This can be the regular common loss of a neutral molecule enabling the use of neutral loss scan (NLS) mode and/or the presence of a common charged fragment, which allows for the use of precursor ion scan (PIS) mode. This work introduces the derivatization-targeted analysis concept, in which all compounds with a specific functional group are targeted and initially unknown compounds detected. This allows to narrow down the number of possible compounds detected in a sample, thereby reducing the problem of recording excessive number of compounds as is common in non-targeted analysis.

The aim of this work is to develop and make use of the derivatization-targeted approach for the detection of amino compounds in complex samples, such as plant extracts. The derivatization reagent diethyl ethoxymethylenemalonate (DEEMM) was employed followed by the screening of amino compounds in NLS mode in a triple quadrupole (QqQ) instrument since it is more sensitive, allowing for the detection of compounds at low concentrations. Derivatization reagents other than DEEMM were evaluated with respect to their derivatives' fragmentation pattern as well.

2. LITERATURE REVIEW

2.1. Amino compounds

Amino compounds or amines are a group of compounds with a nitrogen present in its chemical structure and have the general formula R_3N, where R represents hydrogen or an organic substituent. Amines can be primary ($-NH_2$), secondary (-NH-) or tertiary (-N<) and encompass proteinogenic (PAA), non-protein (NPAA) and post-translationally modified amino acids, polyamines, peptides, nucleosides, catecholamines and biogenic amines. The classification is not mutually exclusive, *e.g.* a biogenic amine can also be classified as a catecholamine.

2.1.1. Amino acids

The chemical structure of amino acids (AA) always contains amine and carboxylic acid groups and for PAA, both groups are bonded to the same carbon (α-carbon). Biologically speaking, 20 AAs are the building blocks of proteins, so they are relevant for protein as well as nucleotides and neurotransmitters synthesis (J. Wang et al., 2017), cell growth (Yu, Zhang, Wang, et al., 2022) and are a source of metabolic energy. Although rarely, 2 other AA can be incorporated into proteins by post-translational modification and are referred to as the 21st and 22nd AA, namely selenocysteine and pyrrolysine, respectively (Clark & Pazdernik, 2013).

PAAs can be divided into groups based on their chemical structure or based on their ability to be synthesized in the body. In the latter case, AA are divided into non-essential and essential, which means essential AA need to be obtained from exogenous sources, *i.e.* food or supplements, and includes valine, leucine, isoleucine, lysine, threonine, methionine, histidine, phenylalanine, and tryptophan in the case of humans and farm animals (Galili & Amir, 2013; Litwack, 2018). Each AA needs to be consumed daily and can be obtained mostly from milk, eggs and meat, but also from cereals and legumes (Galili & Amir, 2013). Branched-chain amino acids (BCAAs), which include valine, leucine and isoleucine, are an important AA class in the fitness world due to their role in building muscle, since they are mainly metabolized in the muscle. In addition, they participate in protein synthesis, glucose homeostasis and animals' intestine health (Nie et al., 2018). They represent 40 % of the total required amount of AA by the body (Xiao & Guo, 2022).

Methionine is another essential AA intensively studied to increase its content, which is usually very low in crops, plants, and vegetables. Cysteine is a product of methionine metabolism and is responsible for disulfide bonds or bridges that make up the tertiary structure of proteins, thereby supporting their biological activities and it is also a precursor for the synthesis of an important antioxidant, the tripeptide glutathione (Clemente Plaza et al., 2018; Combs & Denicola, 2019). Taurine and hypotaurine are also two of the important metabolites from cysteine.

Taurine is considered one of the most essential substances in the body, even though it does not participate in protein synthesis, but is present in the brain, retina, muscle tissue and organs in general (Ripps & Shen, 2012), provides a substrate for the formation of bile salts and is important for brain development (Clemente Plaza et al., 2018). Taurine content in the blood decreases with age and directly influences the life and health span of mice and monkeys (Singh et al., 2023). Such compound is found in high quantities in seaweed and animal products, while trace concentrations are found in plants (Kataoka & Ohnishi, 1986). Hypotaurine plays a role as an antioxidant as highlighted in a work with *Caenorhabditis elegans*, as a model organism, since its supplementation in food prolonged the lifespan and health span of such organism and its level decreased as the organism aged (Wan et al., 2020). A potential cytoprotective effect of hypotaurine against the deleterious effects of UVA irradiation has also been determined and supports further investigation of hypotaurine supplementation in skincare products, such as sunscreen (Baseggio Conrado et al., 2021). Hypotaurine can be biosynthesized from cysteamine through a different pathway as well (Shen et al., 2021).

2.1.2. Non-protein amino acids

Non-protein amino acids (NPAAs) are analogs of PAAs but are not incorporated into the proteins of the organisms that synthesize them. These secondary metabolites exist in numbers between 600 to 1000 (Rodrigues-Corrêa & Fett-Neto, 2019). Since they are structurally similar to PAAs, they can be embodied into proteins by replacing the actual PAAs, which interferes with biochemical pathways, leading to toxic effects. For instance, azetidine-2-carboxylic acid can be mistakenly incorporated into proteins instead of proline and other examples include canavanine, mimosine, hypoglycine, β-diaminopropionic acid (β-ODAP) and 3,4-dihydroxyphenylalanine (levodopa) (Yamane et al., 2010). Other not as harmful NPAAs include γ-aminobutyric acid (GABA), β-alanine, δ-aminolevulinic acid and 4-aminobenzoic acid (PABA). GABA derives from glutamic acid and is the main inhibitory neurotransmitter for the central nervous system (Colzato, 2015).

These compounds are mostly found in legumes (genera of Fabaceae) and seeds (Yamane et al., 2010) and are employed as a chemical defense in plants (Rodrigues-Corrêa & Fett-Neto, 2019). Some literature points out the high concentration of these compounds as toxic to human and livestock, including the development of neurodegenerative diseases, such as lathyrism, by the excessive consumption of grass pea (*Lathyrus sativus* L.), which contains β-ODAP. Moreover, an increase in the abortion rate in pregnant goats fed with river tamarind (*Leucaena leucocephala*) has been reported due to the presence of a high content of mimosine (Rodgers, 2014; Rodrigues-Corrêa & Fett-Neto, 2019).

2.1.3. Catecholamines

Catecholamines, amino-containing-catechol structures, are neurotransmitters or hormones involved in immune regulation (Gaskill & Khoshbouei, 2022), regulatory systems (Bergquist et al., 2002) and are derived from the AAs phenylalanine and tyrosine. For instance, levodopa, which is derived from tyrosine and used in the treatment of Parkinson's disease (Ahlskog, 2023), is the precursor compound for dopamine, which is the precursor of norepinephrine (noradrenaline), and epinephrine (adrenaline) synthesis (Gaskill & Khoshbouei, 2022; Meiser et al., 2013).

Dopamine has many functions in the animal's brain, mostly related to behavior, and so is responsible for motivation, reward, mood, learning and sleep (Juárez Olguín et al., 2016). Noradrenaline and adrenaline are released under nerve stimulation, mainly the flight-or-fight response in periods of anxiety or stress. In general, an increase in levels of catecholamines in humans is often associated with changes in blood pressure, heart rate and the emergence of migraines. Plants and animal's physiological functions are similarly disturbed (Płonka et al., 2022).

2.1.4. Biogenic amines

Biogenic amines (BAs) are products of AA enzymatic decarboxylation, reductive amination or transamination, especially by bacteria or have endogenous emergence. For example, putrescine is derived from ornithine and arginine and is associated with the enzyme activity of the bacteria from the family Enterobacteriaceae and the genus *Clostridium*, which include several pathogens (Sánchez-Pérez et al., 2018). The study of BAs in fermented foods, cheese, fish and fish products is of importance because some of them might have toxic effects. Levels of histamine, a product from the decarboxylation of the AA histidine, higher than 500 ppm results in poisoning (Naila et al., 2010). In the USA, the concentration of histamine is restricted to 50 ppm in seafood (Tsiasioti et al., 2023). The BAs putrescine and cadaverine are the most common polyamines found in fermented products (EFSA Panel on Biological Hazards (BIOHAZ), 2011) and their synergetic toxicity is also a relevant topic, *e.g*. they could magnify the toxicity of histamine and tyramine (Jain & Verma, 2018).

BAs also have endogenous origins and are found at different concentrations in various foodstuffs. Polyamines, such as putrescine, spermidine, spermine and agmatine are important for cellular functions (Galgano et al., 2012; Madeo et al., 2018). The presence of putrescine and its by-product spermidine is ubiquitous in various foods, such as grains, vegetables, beans, nuts and mushrooms at different concentration levels (Nishimura et al., 2006). For instance, spermidine has been found in all samples of five tropical fruits, followed by spermine and putrescine (Santiago-silva et al., 2011) and in twelve vegetables typical in the Brazilian cuisine, followed by putrescine, agmatine and spermine (Starling et al., 2021). Agmatine was only found in pineapple, papaya and passion fruit (Santiago-silva

et al., 2011) and cadaverine was found only in bean sprouts (Starling et al., 2021). In general, it is difficult to establish to what degree the presence of BAs in plant-origin products is from endogenous origins or due to bacterial activity (Sánchez-Pérez et al., 2018). However, the concentration of BAs found in BA-rich foods was shown to be toxic to *in vitro* intestinal cell cultures, in the case of cadaverine, at concentration levels higher than 510 mg/kg (del Rio et al., 2019, 2024).

Another group of amino compounds are indolamines or tryptamines and they can also be classified as BAs. Tryptophan is metabolized to 5-hydroxytryptophan by the enzyme *tryptophan hydroxylase*, which is further transformed into serotonin by the enzyme *5-HTP decarboxylase*. Melatonin is also a product of the aforementioned enzymatic reactions, originating from *N*-acetylserotonin (Seithikurippu R, 2015). Both are present in plants and animals and are considered vital signaling compounds since they regulate numerous physiological functions, such as sleep, memory and behavior (Abbasi et al., 2020; Erland et al., 2015; Tittarelli et al., 2015). Tryptamine itself is found at low concentrations in the human brain, but there are more than 1500 tryptamine varieties in nature. Tryptamine-based compounds are also used as recreational drugs from either natural sources, *e.g.* mushrooms, cacti and Ayahuasca, or are synthetic (Daéid, 2005; Tittarelli et al., 2015).

2.1.5. Amino compounds as biomarkers

Considering the vital role of amino compounds in biological processes, they are important metabolites. Metabolites are defined as small endogenous compounds ranging from 80 – 1200 Da (Adamski, 2016) or with a mass lower than 1500 Da (Wishart et al., 2007). The field of metabolites study is called metabolomics.

The role of amino compounds in metabolomics has been significant. For example, the effect of the drug dapagliflozin in diabetic rats was studied to understand the changes in the metabolic pathways by comparing treated and non-treated rats and, among other findings, the AAs metabolism is affected by the drug (Alosaimi et al., 2024). High levels of BCAAs in plasma are linked to an increase in the risk of diagnosis of pancreatic cancer as well as in the initial stage of the disease (Mayers et al., 2014).

In the field of botany, metabolic profiling was carried out for parts of psyllium (*P. ovata* Forsk) and it was concluded the plant is a promising candidate as a functional food, dietary supplement, etc (M. K. Patel et al., 2016). Through network analysis, five amino-containing compounds were found to be present in the various parts of *Fritillaria thunbergii* primary metabolic process (Cui et al., 2018). The content of 16 amino-containing compounds, including AAs, changed for the transgenic wheat line due to its higher tolerance to abiotic stress (Niu et al., 2020).

2.3. Metabolomics

The definition of metabolomics differs across many publications. Metabolomics compels the study of metabolites in healthy and unhealthy states (Khamis et al., 2017), the study of metabolic pathways (Kaddurah-Daouk & Krishnan, 2009), the identification and quantification of small molecules in a sample (K. Wang et al., 2023) and comprehends the analysis of metabolites in biological samples (Delvaux et al., 2022). The metabolome corresponds to the metabolites and their interactions and is the closest representation of the phenotype (Fessenden, 2016). The field of metabolomics has gained attention in the recent years, and the number of publications has rapidly increased with a growth from 23 publications in 2001 to 10480 publications in 2023 (Figure 1), since metabolomics is important in the field of clinical research, disease biomarker discovery, personalized medicine, nutrition, drug discovery and environment.

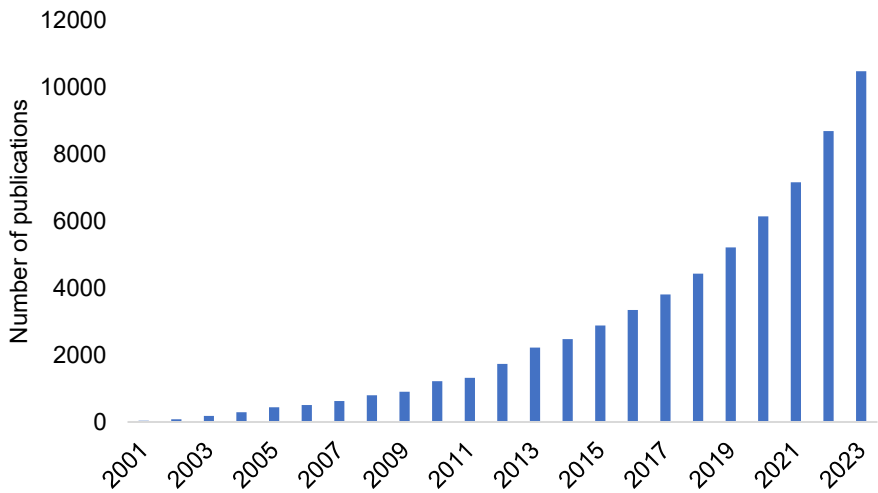


Figure 1. Number of publications between 2001 and 2023 searching for the word "Metabolomics" on ScienceDirect website (ScienceDirect, 2024).

The metabolites are usually in a wide range of chemical and physical properties, such as polarity, which makes the coverage of the whole metabolome a challenge. The metabolomics workflow usually consists of sample preparation, data acquisition by nuclear magnetic resonance spectroscopy (NMR), gas chromatography-mass spectrometry (GC-MS) and/or liquid chromatography-mass spectrometry (LC-MS), followed by data cleaning, database search, statistical analyses, and interpretation of the results. GC-MS and LC-MS have higher sensitivity and specificity, being able to detect a large number of metabolites. However, since more metabolites are not volatile, LC-MS is still preferred over GC-MS.

Metabolomics can be divided into non-targeted and targeted analysis. Targeted metabolomics deals with the quantification of a pre-defined set of metabolites. Non-targeted metabolomics focuses on the simultaneous detection of a wider range of metabolites (from hundreds to thousands) and on their identification (Y. Chen et al., 2022). There are more than 200,000 metabolites in the plant kingdom (M. K. Patel et al., 2016) and currently there are approximately 230,000 metabolites registered in the Human Metabolome Database (Wishart et al., 2007). In that way, the number of possible compounds present in a sample is innumerous in non-targeted analysis.

For non-targeted metabolomics analysis, LC-MS is often used. To characterize and systematize the level of confidence in the compound identification process, a five-level system has been proposed (Schrimpe-Rutledge et al., 2016): at level 5, the molecular mass is determined by MS measurement with accuracy depending on the mass analyzer. The higher the mass accuracy, the fewer the number of possible compounds within a sample. At level 4, molecular formula is defined by determining charge state, adduct ion and isotopic abundance distribution; at level 3, database search and match based on MS1 results; at level 2, MS/MS database search and match based on fragmentation pattern together with other data, such as retention time and from NMR measurements; and level 1, injection of an authentic reference standard and confirmation of the identity (Schrimpe-Rutledge et al., 2016). An example of such classifications is given in Table 1.

Table 1. Levels of identification in non-targeted metabolomics and the respective example. Adapted from (Schrimpe-Rutledge et al., 2016)

Level	Example
5	354 ± 1 Da (*e.g.* QqQ) 354.16 ± 0.03 Da (*e.g.* Orbitrap)
4	$C_{20}H_{22}N_2O_4$
3	Tentative structure based on a metabolite database
2	Putative identification based on fragmentation data in MS/MS library
1	3-[(E)-[(2S)-2-[(E)-(2,3-dihydroxyphenyl)methyleneamino]cyclohexyl]iminomethyl]benzene-1,2-diol

Depending on the desired approach, different instrumentation might be employed. Generally speaking, different mass analyzers can be used in LC-MS, such as single or triple quadrupole (QqQ), ion trap, quadrupole time-of-flight (QToF), orbitrap, and Fourier-transform ion cyclotron resonance (FTICR) with the latter three being considered as HRMS, and commonly employed in non-targeted metabolomics because of their high mass accuracy and resolution. HRMS is usually employed in full scan (Martín-Blázquez et al., 2019; Olkowicz et al., 2021; Rochat et al., 2018), or followed by the more widely employed data-dependent acquisition (DDA) (Barco et al., 2022; B. Li et al., 2023; Torres et al., 2023) or

data-independent acquisition (DIA) (Guo & Huan, 2020; Ledesma-Escobar et al., 2023; Shah et al., 2023). In targeted metabolomics, QqQ is usually the go-to mass analyzer since, in general, it presents a better sensitivity (lower limit of detection – LoD) and dynamic range.

Specifically speaking about the analysis of amino compounds, AA can be either in a free or protein-bound form. The reference method for AA analysis in native form is based on ion-exchange chromatography (IEC), UV detection and post-column derivatization with ninhydrin with a short sample preparation step, but offers lengthy run times and low specificity (Held et al., 2011; Phipps et al., 2020). Ion-pairing chromatography and hydrophilic interaction liquid chromatography (HILIC) are also often employed. Amino compounds can be analyzed by GC and capillary electrophoresis, but it is not as popular as LC methods with detection by ultraviolet (UV), fluorescent (FLD) or MS (Jain & Verma, 2018; Violi et al., 2020a). The latter detector provides information on compounds' molecular mass and allows for the co-elution of compounds with different molecular masses, so LC-MS/MS has become more widespread (K. Patel & Master, 2020). However, analysis of AAs by LC-MS/MS provides its challenges due to their small size and ionic nature (Violi et al., 2020b), such as weak retention in an RP column and low ionization efficiency.

2.2. LC-MS/MS analysis of amino compounds

Amino compounds, especially AAs, in the native form are usually analyzed by HILIC or ion chromatography because of their polar and ionic nature due to the presence of charged amino and, in the case of AAs, charged carboxylic acid groups as well (Violi et al., 2020b). Currently, HILIC is the second most used chromatographic technique after RPLC, since peak asymmetry, splitting and broadening might occur due to the interactions from the protic sample solvent with the polar stationary phase (Morán-Garrido et al., 2022), there is a higher consumption of organic solvent (Kahsay et al., 2014) and a need for gradual column stabilization (Dias et al., 2021). To overcome these potential issues while analyzing amino compounds by HILIC, RPLC is the technique of choice, but the hydrophobicity of the amino compounds needs to be increased to guarantee enough retention in an RP column (*e.g.* C18 column). This can be achieved by the introduction of a chemical tag to the amino compounds, which is called derivatization.

Derivatization is a chemical reaction where the compound of interest is transformed into a derivatized product that has more favorable properties than the underivatized analyte, with respect to the chromatographic separation and or/ detectability. In a nutshell, a chemical moiety, *i.e.* the derivatization reagent, is tagged to the analyte of interest. The labeled compounds have better retention in RPLC, and higher ionization efficiency under electrospray ionization (ESI). The derivatization procedure can be carried out either pre-column or post-column, but the latter requires a more complex instrumental set-up (Violi et al., 2020b). The derivatization reagent must comply with a high reaction yield, have a hydro-

phobic moiety, improved stability and separation, cost reduction and no need for a post-derivatization sample clean-up.

There are several derivatization reagents for amino compounds in the literature, but the ones used in this work are explained in detail and their reaction schemes are presented. O-phthalaldehyde (OPA) is a versatile reagent that can be used with several detectors and presents a straightforward procedure, but it is mostly used for FLD, since only the derivatization products are fluorescent, but the reagent itself is not (Oguri, 2005). A few examples of its use are for the quantification of AAs in fermented beverages by HPLC-UV (de Sousa Fontes et al., 2024), biogenic phosphonates in seawater by HPLC-FLD (S. Wang et al., 2018) and glucosamine in biological samples by LC-MS/MS (Song et al., 2012). However, it does not react with secondary amines and its derivatives have lower stability compared to other derivatives (Munir & Badri, 2020).

Several other reagents based on the displacement of a chloride atom have been extensively explored: dansyl chloride, in the quantification of adenosine in tumors by LC-MS/MS (Goodwin et al., 2019); dabsyl chloride, in the quantification of BAs in meat samples by HPLC-UV (De Mey et al., 2012); benzoyl chloride, in the quantification of 70 neurochemicals in biological samples (Wong et al., 2016); and fluorenylmethyloxycarbonyl chloride (Fmoc-Cl), in the determination of fumonisins in feed by HPLC-FLD (Smith et al., 2017). These reagents can be employed with several different detectors. On the other hand, they also react with phenolic compounds (Lacroix & Saussereau, 2012; Pernica et al., 2015; Yu, Zhang, Zhang, et al., 2022), which may raise selectivity problems if amines are the only compounds of interest.

Other reagents include phenyl isothiocyanate (PITC, Figure 2), which has been used for the quantification of free catecholamines and glycine in biological fluids (D. Chen et al., 2021; Wilson et al., 2011; J. Zheng et al., 2018), with no side-products but it presents an extensive and a lengthy sample preparation procedure with a need for sample clean-up (G. Zheng et al., 2015). The reagent 2-naphthylisothiocyanate (NITC), also belonging to the isothiocyanate family, has been explored as well (S.-H. Chen et al., 2006; Neidle et al., 1989; Rizzi et al., 1995). The reagent 6-aminoquinolyl-N-hydroxysccinimidyl carbamate (AQC), developed by Waters® company has been widely studied (Gwatidzo et al., 2013).

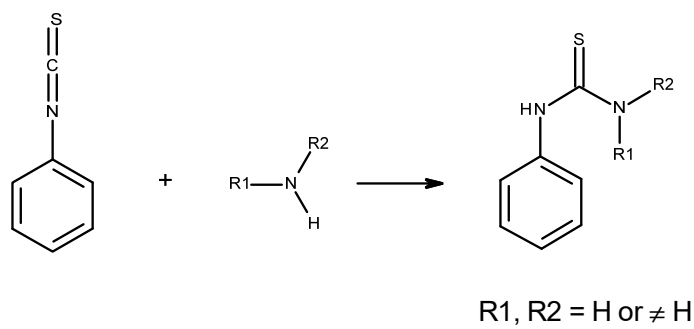


Figure 2. Reaction between amino compounds and PITC.

Diethyl ethoxymethylenemalonate (DEEMM, Figure 3) has been widely employed in the research group, *e.g.* for honey, onion, plant extracts and beverages analyses (Maciel et al., 2021, 2024; Rebane et al., 2014; Rebane & Herodes, 2010; Zapata Flores et al., 2022), has also been employed in the determination of free amino compounds in cheeses (Poveda et al., 2016) and grapes (D'Amato et al., 2023). Overall, the experimental procedure is straightforward, but secondary amines react with DEEMM at a slower rate.

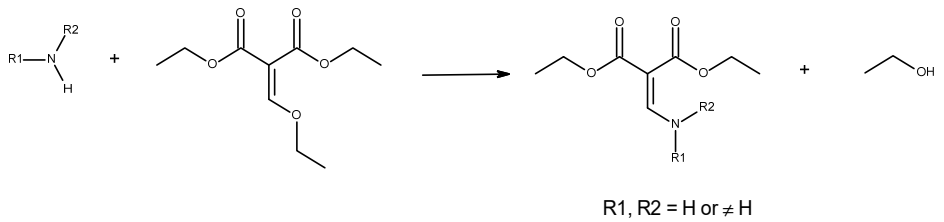


Figure 3. Reaction between amino compounds and DEEMM.

Fluorescamine (Figure 4) was originally developed for HPLC-FLD analysis, *e.g.* in the quantification of cephalosporins in biological samples (Blanchin et al., 1988) and lisinopril in pharmaceutical products (Mohammed et al., 2018), which is a favorable detector since the reagent itself and its hydrolysis product are not fluorescent (Derayea & Samir, 2020). Studies of amino compounds with this reagent by LC-MS/MS have not been undertaken to the same extent, a few examples are the determination of levodopa and carbidopa in rat and monkey plasma (Junnotula & Licea-Perez, 2013) and dimethyl arginine in plasma (Junnotula et al., 2020). The reagent might also react with alcohols, thiols and secondary amines, and if present in excess, they might interfere with its reaction with primary amines (Castell et al., 1979). Moreover, the primary product might undergo dehydration depending on the conditions employed (Junnotula & Licea-Perez, 2013).

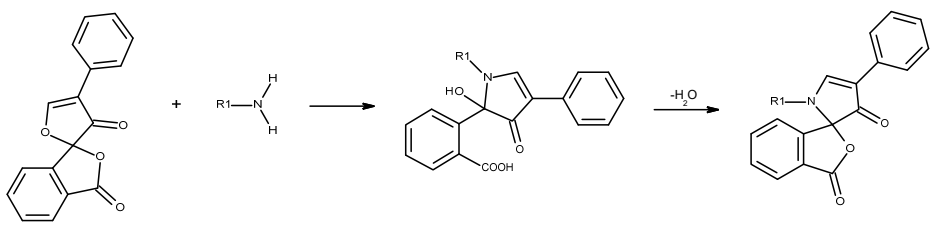


Figure 4. Reaction between primary amino compounds and fluorescamine.

In the case of another fluorescent reagent, 2-methoxy-2,4-diphenyl-3(2H)-furanone (MDPF, Figure 5), the application to amino compounds determination in

real samples is scarce, but it has been employed for protein labeling (Alba et al., 2009; Nawaz et al., 2015; Weigele et al., 1973). The reaction pathways are different if the reaction site is a primary or a secondary amine. Due to the absence of works regarding this reagent, it would be interesting to investigate it for the determination of amino compounds by LC-MS/MS.

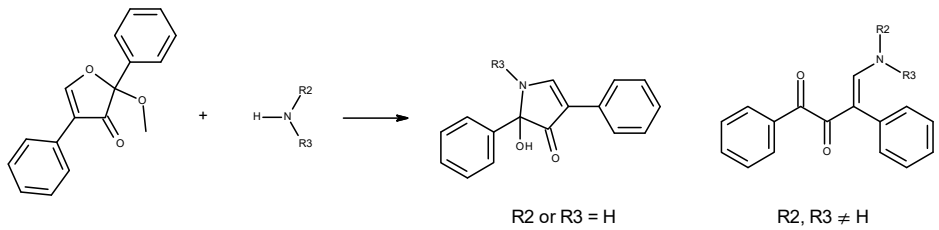


Figure 5. Reaction between amino compounds and MDPF.

Permanently-positively-charged derivatization reagents, mostly based on the presence of triphenylphosphonium moiety (Zhe et al., 2016), improve ionization efficiency due to the positive charge located in the phosphorous present in the structure, resulting in lower LoD and LoQs. Most widely known are (5-N-succinimidoxy-5-oxopentyl)triphenylphosphonium bromide (SPTPP) (Inagaki et al., 2010; McNaney et al., 2014), and *p-N,N,N*-trimethylammonioanilyl *N*′-hydroxysuccinimidyl carbamate iodide (TAHS) (Shimbo et al., 2009), which are not commercially available. Another example is 2,5-dioxopyrrolidin-1-yl N-tri(pyrrolidino)phosphoranylideneamino carbamate (FOSF), synthesized in the research group (Rebane et al., 2012). On the contrary, (N-Succinimidyloxycarbonylmethyl)tris(2,4,6-trimethoxyphenyl)phosphonium bromide (TMPP, Figure 6) is commercially available and contains a trimethoxyphenylphosphonium moiety. It has been employed only for the determination of proteins in bottom-up proteomics (Bagal & Gibson, 2021; Deng et al., 2015) and not for the determination of other amino-containing compounds. It is an interesting candidate to be explored further for that purpose, since charged reagents provide lower LoDs and LoQs.

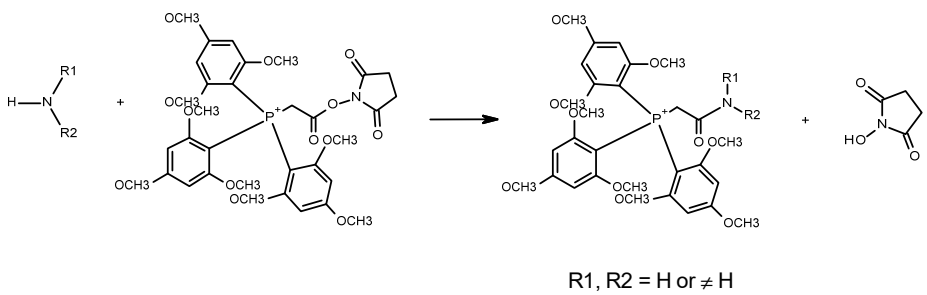


Figure 6. Reaction between amino compounds and TMPP.

Another group of charged derivatization reagents is based on the presence of a pyridinium moiety in a reaction that can be referred to as the Katritzky reaction (Figure 7). Quantification of BAs in fish has been carried out with 2,4,6-triethyl-3,5-dimethyl pyrylium trifluoromethanesulfonate in a simple procedure (Shiono et al., 2021), but the reagent is not commercially available, whereas 2,4,6-triphenylpyrylium (pyry) is and has been employed for labeling of peptides (Waliczek et al., 2016). This reagent is also an interesting candidate for amino compound analysis, since it might provide low LoD and LoQs.

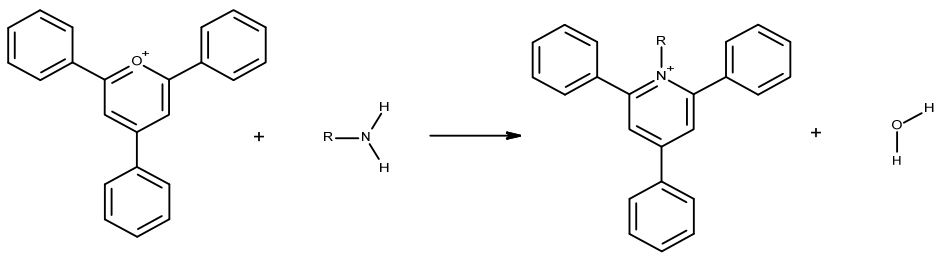


Figure 7. Reaction between primary amino compounds and pyry.

Overall, each derivatization reagent has its advantages and drawbacks, and it is necessary to make a case-by-case assessment. Tertiary amines and amides are not derivatized by any of the aforementioned derivatization reagents.

2.4. Derivatization-targeted analysis

A way to overcome the enormous number of detected compounds in non-targeted analysis is by derivatizing a group of compounds, based on a functional group of interest, and relying on a constant fragmentation pattern of the derivatives. Two types of patterns can be observed: the presence of a common charged fragment, in which case precursor ion scan mode (PIS) can be used; or the loss of a neutral molecule, so constant neutral loss scan mode (NLS) can be employed. In NLS, both MS1 and MS2 scan simultaneously in sync, a peak is registered with a mass spectral signal from the precursor ion (MS1) that yielded the pre-selected neutral loss in the collision cell (Figure 8). In the case of PIS mode, the MS1 is in scanning mode and MS2 is set at the charged product ion of interest (Figure 9).

Multiple neutral loss scanning has been employed to detect and identify 98 triacylglycerols in salmon muscle tissue (Yeo & Parrish, 2020) and 15 new pentacyclic triterpenes in sea buckthorn (Sun et al., 2019) while constant neutral loss scan mode combined with product ion scan has been employed for the detection and the identification of 10 prenylated dihydrostilbenes in *G. uralensis* leaves (Meng et al., 2020). Closely related to this work, AAs have been detected in negative NLS mode (loss of 194) in miso samples after derivatization (Sakamoto et al., 2021).

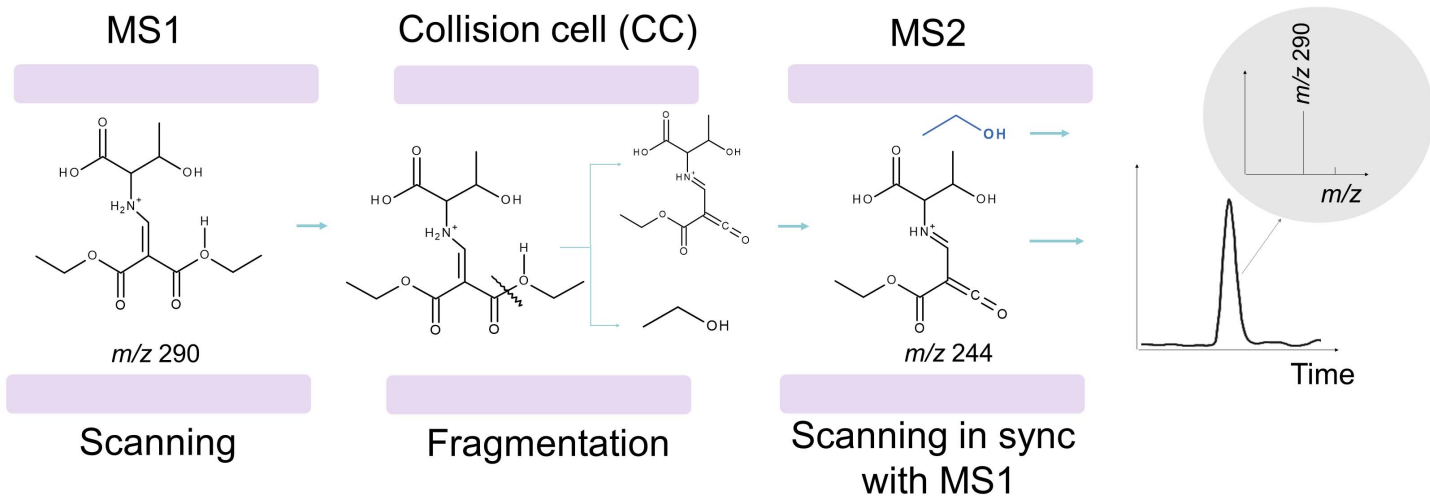


Figure 8. DEEMM-derivative of threonine under neutral loss scan mode in QqQ (2D).

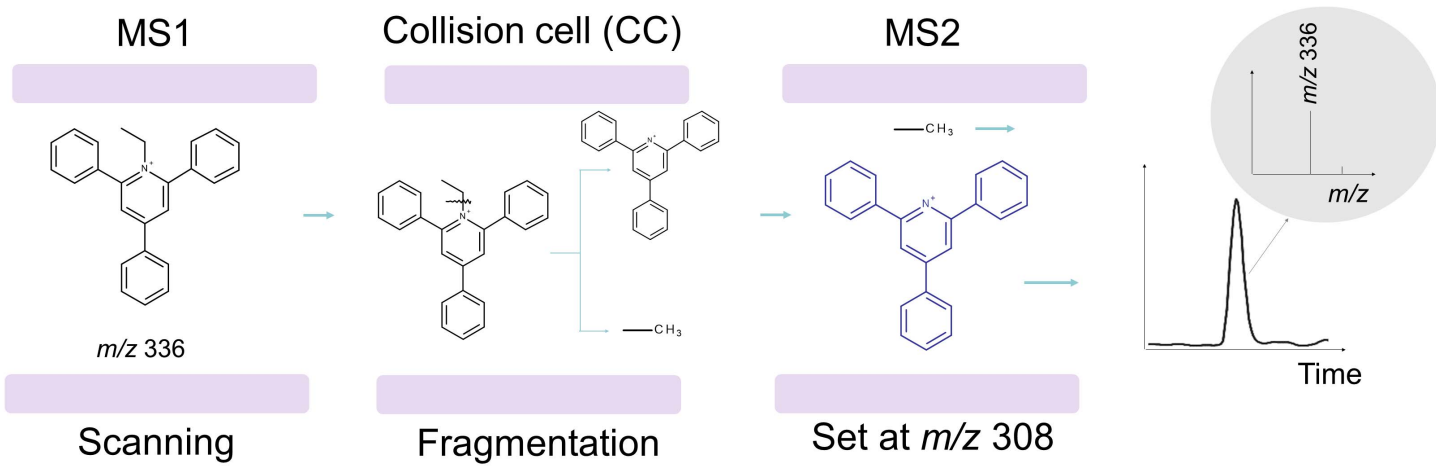


Figure 9. Pyry-derivative of ethylamine under precursor ion scan mode in QqQ (2D).

The mass analyzer chosen for this work is the QqQ, since its sensitivity is usually higher than for HRMS instruments (Rochat, 2016), so it allows for the detection of less abundant compounds. In Table 2, a comparison between QqQ and HRMS from different vendors is presented. The strict comparison of the highest-end instruments from each company was not possible, because some information on the specifications of their instruments is not openly available. Nonetheless, the signal-to-noise ratio (S/N) is significantly higher for QqQ instruments. Some works in literature have compared both instruments. For example, better LoD and better precision for the analysis of 25-hydroxyvitamin D in human serum were achieved in QqQ instrumentation rather than in QToF (Geib et al., 2016). A comparison between Q-Exactive™ (in full scan and MS/MS modes) and QqQ (in MS/MS mode) for the analysis of illicit drugs in wastewater showed that some analytes have better LoQs in HRMS while others have better LoQs in QqQ. HRMS in full scan mode had the lowest score regarding selectivity (Fedorova et al., 2013).

Table 2. Comparison of QqQ and HRMS instrumentation regarding their sensitivity.

Instrument (type)	Sensitivity	Reference
Thermo Scientific™ Orbitrap Exploris™ 480 Mass Spectrometer (HRMS)	50 fg reserpine on-column (MS/MS), S/N 100:1	(Thermo Scientific, 2024)
Agilent Revident LC/Q-TOF (HRMS)	1 pg reserpine on-column (S/N (RMS) > 1000:1)	(Agilent Technologies, 2024)
Agilent 6495D Triple Quadrupole LC/MS (QqQ)	IDL (instrument detection limit) < 0.4 fg of reserpine	(University, 2024)
SCIEX 7600 ZenoTOF (HRMS)	1 pg reserpine on-column, S/N > 2500	(University, 2024)
SCIEX Triple Quad™ 4500 LC-MS/MS System (QqQ)	1 pg reserpine on-column (MRM), S/N > 200,000	(Sciex, 2024)
Shimadzu QToF LCMS-9050 (HRMS)	1 pg reserpine on-column (RSM, MS/MS mode), S/N > 10,000:1	Contact with a Shimadzu representative in Estonia
Shimadzu LCMS-8060NX (QqQ)	1 pg reserpine on-column (RSM), S/N > 1,500,000:1	(Shimadzu, 2024)

Utilizing NLS or PIS modes initially offers an advantage by specifically detecting derivatives of amino compounds, thereby reducing the number of potential compounds detected in a complex sample. Each peak in the chromatogram corresponds to the *m/z* of the protonated molecule or sodium-adduct of derivative, $[M+H]^+$ or $[M+Na]^+$, respectively, allowing for the molecular weight of the amino compound to be back-calculated. This calculated value is then subjected to a molecular weight search in a relevant database, considering the chemical ele-

ments within the compound's structure (CHN, CHON, CHNS, or CHONS), and the resulting list is assessed to determine the compounds likely occurring in the sample. Subsequently, the amino compounds are identified using reference standards and quantified via multiple reaction monitoring (MRM), which offers superior sensitivity in general. This workflow streamlines the process of selecting compounds for quantification compared to the trial-and-error approach by only using MRM (Figure 10).

This approach is not completely non-targeted, because compounds from different classes are not encompassed. At the same time, it is not predetermined which amino compounds will be detected or quantified, therefore, the approach cannot be called targeted analysis. For that reason, this work introduces the derivatization-targeted analysis concept and it has been cited as one of the current approaches to map the polar metabolome (Lioupi et al., 2023).

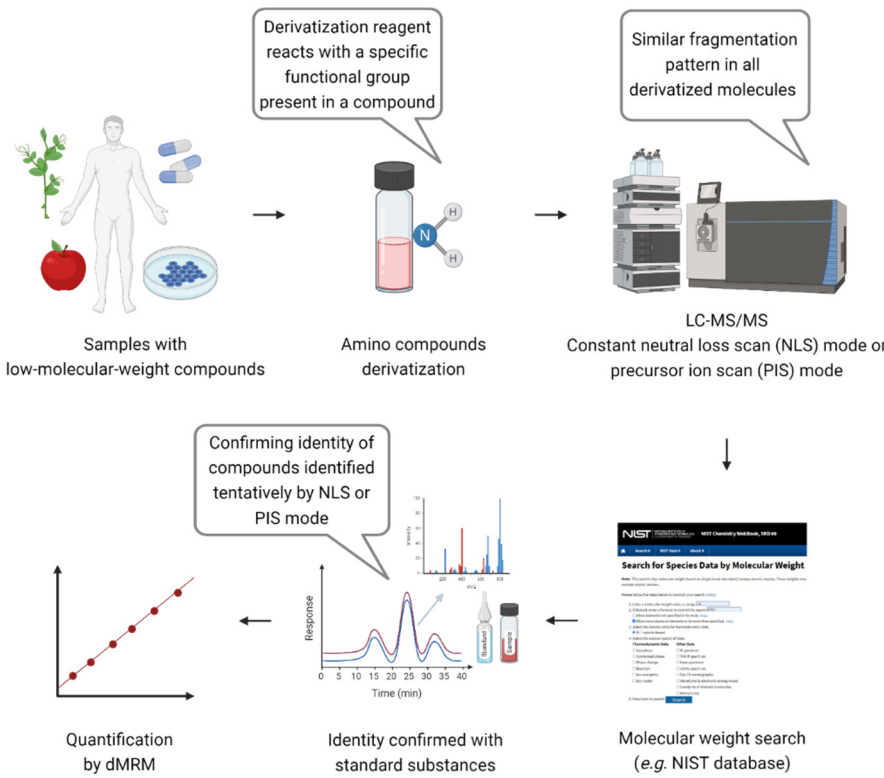


Figure 10. Derivatization-targeted analysis workflow.

The disadvantage of this method is the need to analyze the data manually since there is no available software capable of processing the generated chromatogram and its mass spectra under NLS or PIS modes. Besides, there is no database available for derivatized compounds.

3. EXPERIMENTAL

3.1. Chemicals and equipment

The chemicals and equipment used in this research are detailed in their respective publications, except for fluorescamine, 2-Methoxy-2,4-diphenyl-3(2H)-furanone (98%), 4-dimethylamino-1-naphthyl isothiocyanate (> 98%), sarcosine (≥ 98%), O-phosphoserine (> 98%), from TCI Chemicals, (N-Succinimidyloxycarbonyl-methyl)tris(2,4,6-trimethoxyphenyl)phosphonium bromide (≥ 98.5%), 4-sulfo-phenylisothiocyanate sodium salt monohydrate (technical grade), 2-aminophenol (99%), aniline ≥ (99.5%), diethylamine (≥ 99.5%), isobutylamine (99%), butyl-amine (99.5%) from Sigma-Aldrich, phenyl isothiocyanate (≥ 98 %), 1-naphthy-lisothiocyanate (> 98.5%) from Fluka, 2-(4-morpholinyl)ethylisothiocyanate (97%), kynurenine (> 99%), 2,4,6-triphenylpyrylium tetrafluoroborate (97%), methionine sulfoxide (99.85%) from Alfa Aesar, thiaproline (98%) from Thermo Scientific.

3.2. Stock and standard solutions

Individual stock solutions of the amino compounds were prepared in different concentrations between 1 – 6 mg/g in 30 % methanol in aqueous 0.1 M HCl and stored at –20 °C. Standard solutions were prepared at a pre-defined concentration of amino compounds in the same solvent.

3.3. Sample preparation

An ultrasonic-assisted-solid-liquid extraction was carried out for all the samples at room temperature in an ultrasonic bath (Bandelin Sonorex). In paper I and II, the extracts were submitted to centrifugation for 10 minutes at maximum speed (MTS MPW 340 centrifuge), while in paper III, the extracts were centrifuged for 10 minutes at 10,000 rpm (14,869 × g) (Eppendorf centrifuge 5430 R). The supernatants were filtered through a syringe filter (0.20 µm pore size, 25 mm diameter, hydrophilic regenerated cellulose; Chromafil®Xtra, Macherey-Nagel) and then submitted to derivatization, except in paper III, in which case the super-natants were diluted 6-fold beforehand. Parameters such as the mass of the sample, solvent and solvent volume, extraction time and cycles are presented in Table 3.

Table 3. Details about the ultrasonic-assisted-solid-liquid extraction about all the samples in this work.

Sample	Sample mass (mg)	Solvent	Solvent volume (mL)	Extraction time (min)	Extraction cycles	Paper
Carduus nutans subsp. *macrocephalus* (Desf.) Nyman	100	30:70 mixture of methanol and 0.1 M aqueous HCl	5	20	1	I
Carduus nutans subsp. *macrocephalus* (Desf.) Nyman	200		3	10		II
C. argyroa						
C. cephalanthus						
C. pycnocephalus						
P. casabonae from Corse						
P. casabonae from Sardinia (Gennargentu)						
P. casabonae from Sardinia (Iglesias)						
Hemp (*Cannabis sativa L.*) seed hull	100		2	15	1 and 3	III
Sea buckthorn (*Hippophae rhamnoides*) leaves						
Oat (*Avena sativa*) bran						
Apple (*Malus domestica* Borkh., variety Belorusskoje malinovoje) pomace						
Sea buckthorn (*Hippophae rhamnoides*) pomace		50:50 mixture of acetonitrile and water				

3.4. Derivatization procedure

DEEMM derivatization procedure (Paper I and II): Derivatization was carried out at room temperature in a 1.5 mL glass vial by addition of solutions in the following order and mixing after each addition: 588 µL of the sample extract in 30% methanol in aqueous 0.1 M HCl, 875 µL of borate buffer (0.75 M, pH 9), 7 µL of DEEMM. After 2 h, 30 µL of the quenching reagent (hydroxylamine, 1.5 M) was added.

DEEMM derivatization procedure (Paper III): Derivatization was carried out at room temperature in a 2.0 mL Eppendorf tube by addition of 100 µL of the extract, 488 µL of 30% methanol in aqueous 0.1 M HCl, 875 µL of borate buffer (0.75 M, pH 9), 7 µL of DEEMM, with and without addition of 30 µL of the quenching reagent (hydroxylamine, 1.5 M) after 2 hours. If the quenching reagent was not added, 518 µL of 30% methanol in aqueous 0.1 M HCl was used.

Derivatization procedure for other reagents: for isothiocyanate reagents, 200 µL of amino compound standard solution (10 mg/kg) in bicarbonate buffer (0.020 M, pH 8.6), 50 µL of acetonitrile and 150 µL of isothiocyanate reagent solution were mixed together. The reagent 4-SPITC was dissolved in the buffer solution (2 mg/ml); PITC and 2-(4-morpholinyl)ethyl isothiocyanate (MORPHO) were diluted in acetonitrile (1:50); and NITC and 4-dimethylamino-1-naphthyl isothiocyanate (4-AMITIC) were dissolved in acetonitrile (2 mg/ml). In the case of other reagents, 300 µL of borate buffer (0.25 M, pH 8 or 9), 30% acetonitrile aqueous solution, 1 µL of amino compound stock solution and 25 µL of derivatization reagent solution in acetonitrile (2 mg/ml) were employed.

3.5. LC-MS/MS analysis

All the analyses were conducted on an HPLC-MS system with an Agilent 1290 Infinity II quaternary pump, column thermostat, an autosampler and an Agilent 6460 Triple Quadrupole (QqQ) mass spectrometer (MS) with Agilent Jet Stream Technology (heated electrospray) ionization source (AJS-ESI). The column was maintained at 40°C. The mobile phase was composed of 0.1% aqueous formic acid (component A) and acetonitrile (component B). The LC-MS/MS parameters employed in this work are shown in Table 4 as well as the MRM transitions (Table 5) used for the quantification of amino compounds (paper III).

Table 4. LC-MS/MS parameters employed in this work.

Parameter	Paper I	Paper II	Paper III	Exploring existing derivatization reagents for derivatization-targeted analysis
LC parameters				
Column	Zorbax Eclipse Plus C18 (3.0 × 100 mm, 1.8 µm)		Zorbax Eclipse Plus C18 (3.0 × 100 mm, 1.8 µm) and guard column (3.0 × 5 mm, 1.8 µm)	Waters ACQUITY UPLC BEH C18 (2.1 × 50 mm, 1.7 µm)
Flow rate (ml/min)	0.5	0.5	0.4	0.3
Gradient (% B)	Gradient I: 0–2 min, 10% 2–17 min, 10–100% 17–19 min, 100% 19–21 min, 100–10% Gradient II: 0–2 min, 10% 2–27 min, 10–100% 27–29 min, 100% 29–31 min, 100–10%	0–2 min, 10% 2–13 min, 10–25% 13–32 min, 25–75% 32–35 min, 75–100% 35–37 min, 100% 37–38 min, 100–10%	0–2 min, 10% 2–27 min, 10–98% 27–29 min, 98% 29–31 min, 98–10%	0–2 min, 10% 2–11 min, 10–98% 11–13 min, 98% 13–15 min, 98–10%

Parameter	Paper I	Paper II	Paper III	Exploring existing derivatization reagents for derivatization-targeted analysis
Post time (minutes)	4	5	4	
Injection volume (µl)	5		2	
ESI-MS parameters				
Scan mode	Constant neutral loss		Constant neutral loss and dynamic multiple reaction monitoring	Full scan and product ion
m/z range	50 – 500 500 – 1000 1000 – 1500 1500 – 2000	50 – 600		200 – 800 600 – 900
Mode	Positive			Positive and negative
Fragmentor (V)	90			
Collision energy (V)	8			0 – 110 in steps of 10 V
Drying gas temperature (°C)	320			
Drying gas flow (L/min)	9			
Nebulizer gas pressure (PIS)	45			
Sheath gas temperature (°C)	400		380	
Sheath gas flow (L/min)	12			
Capillary voltage (V)	3000			
Nozzle voltage (V)	0			

Table 5. MRM transitions and retention time (minutes) for the quantified DEEMM-derivatives in paper III in alphabetical order. Fragmentor voltage 90 V and collision energy 8 V.

Compound Name	Precursor Ion $[M+H]^+$	Product Ion $[M+H]^+$	Retention time (min)
3-methoxytyramine	338	292	13.57
5-aminovaleric acid	288	242	11.13
α-aminobutyric acid	274	228	11.89
Agmatine	301	255	7.49
α-alanine	260	214	10.33
Aminoadipic acid	332	286	9.56
Arginine	345	299	6.28
Asparagine	303	257	6.96
Aspartic acid	304	258	8.15
β-alanine	260	214	9.36
Cadaverine	443	397	17.23
Citrulline	346	300	8.02
Cysteine	462	416	14.08
Ethanolamine	232	186	8.42
Ethylamine	216	170	12.54
γ-aminobutyric acid	274	228	10.00
Glutamine	317	271	7.30
Glutamic acid	318	272	8.59
Glycine	246	200	8.88
Glycylglycine	303	257	7.77
Histidine	326	280	4.79
Histamine	282	236	6.05
Hypotaurine	280	234	6.63
Isoleucine	302	256	14.40
Isopentylamine	258	212	18.24
Leucine	302	256	14.60
Lysine	441	395	14.90
Methionine	320	274	12.67
Methionine sulfone	352	306	8.66
Methylamine	202	156	10.32
Octopamine	324	278	10.82
Ornithine	473	427	14.09

Compound Name	Precursor Ion $[M+H]^+$	Product Ion $[M+H]^+$	Retention time (min)
Phenylalanine	336	290	14.40
Phenylethylamine	292	246	17.44
Proline	286	240	10.41
Putrescine	383	337	16.20
Serine	276	230	7.60
Serotonin	347	301	12.58
S-methylcysteine	306	260	11.75
Taurine	296	250	6.06
Threonine	290	244	8.76
Tryptophan	375	329	13.57
Tryptamine	331	285	16.63
Tyrosine	352	306	11.03
Tyramine	308	262	13.42
Valine	288	242	12.91

3.6. Software

All the chemical structures were drawn in BIOVIA Draw 2021 (Systemes, 2024). Figure 10 was created in Biorender (BioRender, 2024). The optimization of the fragmentor voltage and collision energy was carried out by the Agilent Mass-Hunter Optimizer software version B.08.02. Agilent Quantitative Analysis Version B.08.00 / Build 8.0.598.0 was employed to obtain peak areas and Agilent Qualitative Analysis navigator version B.08.00 / Build 8.0.8208.0 was used for chromatogram treatment. The correlation plot was created in the R software (R Core Team, 2020; Simko & Wei, 2017) as well as the design of experiments (DoE) results analysis and plots (Lenth, 2009). The heatmap was created by using Morpheus software (Broad Institute, 2024).

4. RESULTS AND DISCUSSION

4.1. Optimization of neutral loss scan mode of DEEMM derivatives (Paper I)

The derivatives of DEEMM present an interesting fragmentation pattern with the loss of an ethanol molecule (-46 Da) from the protonated precursor ion $[M+H]^+$ under CID conditions. The site of protonation of the derivatives can be either on the oxygen or on the nitrogen due to the intramolecular hydrogen bonding between the hydrogen bonded to the nitrogen and the oxygen from the carbonyl group (Figure 11) (Gómez-Alonso et al., 2007).

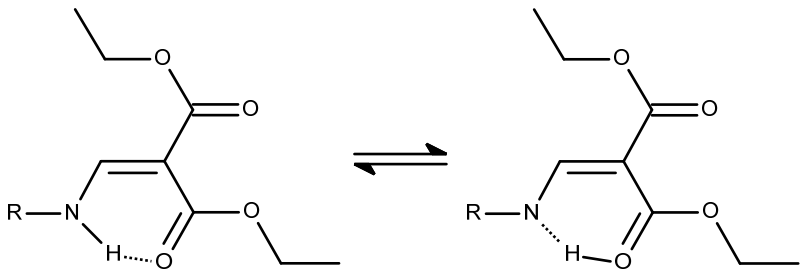


Figure 11. Primary-amine DEEMM-derivative and its hydrogen bonding between nitrogen and oxygen.

Regardless of the protonation site, the charged fragment generated has the same structure and the release of an ethanol molecule takes place (Figure 12).

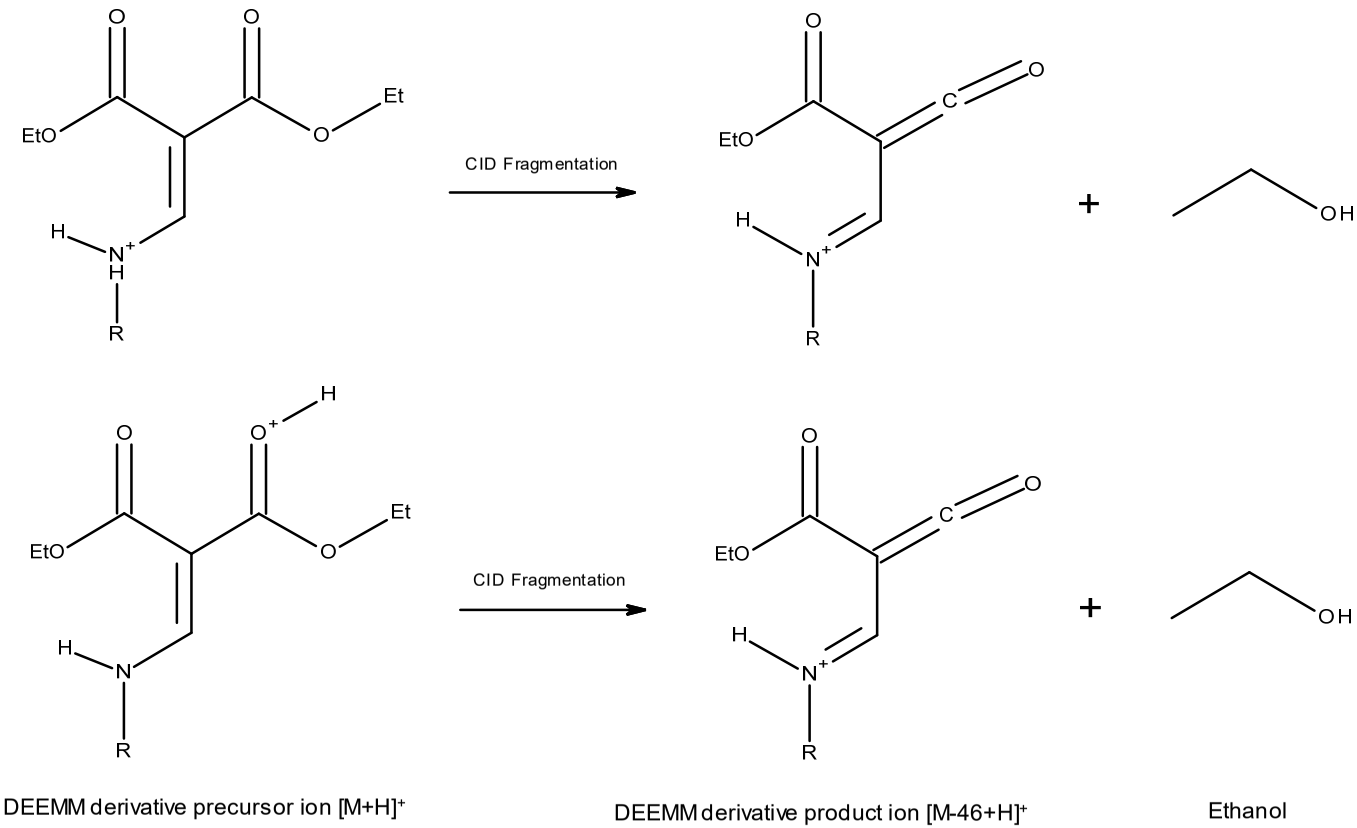


Figure 12. Different protonation sites of the precursor ion $[M+H]^+$ of a DEEMM derivative.

In derivatization-targeted analysis, unlike MRM, the aim is to see clearly as many peaks as possible. When the NLS mode chromatogram was generated from the optimized reaction parameters (Rebane & Herodes, 2010), several peaks from the reaction blank were present, such as DEEMM itself (*m/z* 217), the methanolysis (*m/z* 203) and the hydrolysis (*m/z* 161 and 189) products (Figure 13). The methanolysis peak was confirmed by changing the solvent to isopropanol and detecting the peak of the product between DEEMM and isopropanol (*m/z* 231).

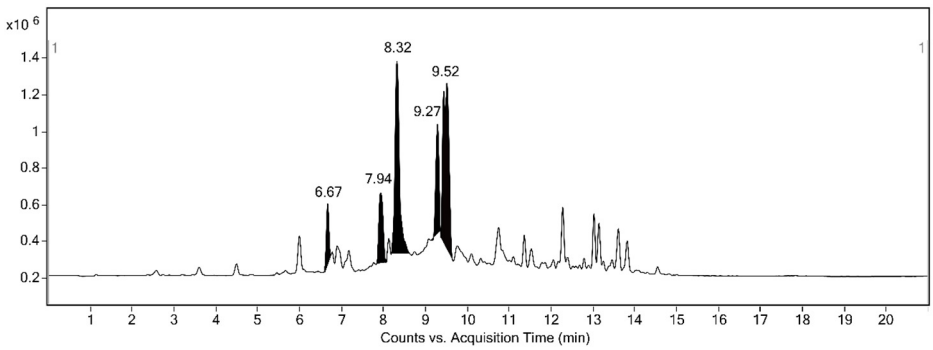


Figure 13. Chromatogram in NLS mode of a solution of DEEMM highlighting the major peaks: 6.67 min (*m/z* 188 – DEEMM-ammonia derivative); 7.94 min (*m/z* 161 – DEEMM hydrolysis product); 8.32 min (*m/z* 203 – DEEMM methanolysis product); 9.27 min (*m/z* 189 – DEEMM hydrolysis product) and 9.52 min (*m/z* 217 – DEEMM).

Since one of the main peaks corresponded to the methanolysis product, the volume of methanol was decreased by employing neat DEEMM instead of a DEEMM solution in methanol (1:50) and the volumes of the other reagents were adjusted accordingly (Figure 14).

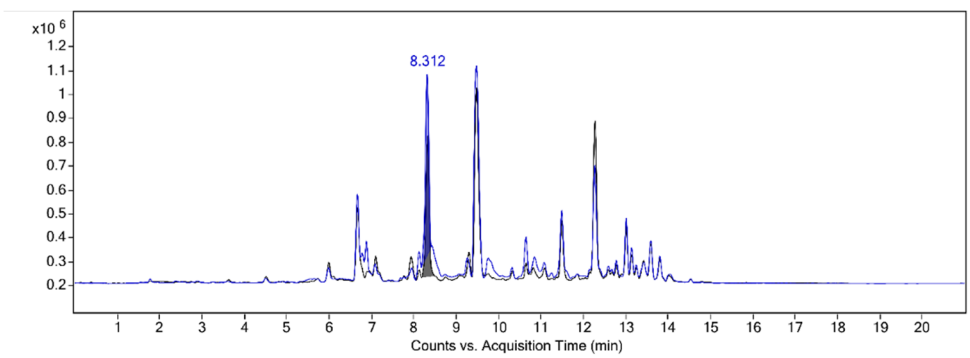


Figure 14. Comparison between chromatograms in NLS mode, employing diluted (blue line) and neat DEEMM (black line). Analysis after 24 h. The peak at RT = 8.31 minutes corresponds to the methanolysis product. Diluted refers to the method in which a solution of DEEMM in methanol (1:50) was employed and neat refers to the method in which DEEMM is added as it is, not diluted in any solvent.

The peak corresponding to the methanolysis product was not fully eliminated by employing neat DEEMM. To overcome this, the reaction was carried out in solvents other than methanol: acetonitrile and ethanol. The methanolysis peak was no longer present at the same intensity (Figure 15) and new peaks seemed to arise, *e.g.* a peak corresponding to *m/z* 216 when ethanol was employed. The next step was to understand how the solvent affects the sensitivity of DEEMM derivatives of amino compounds.

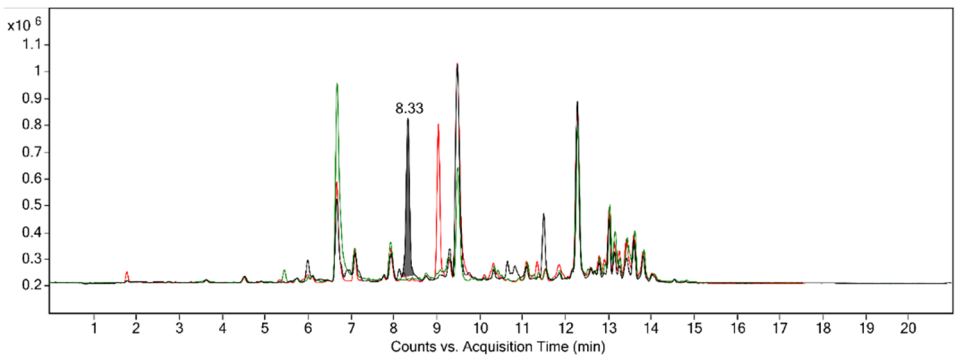


Figure 15. Comparison between chromatograms in NLS mode employing methanol (black line), ethanol (red line) and acetonitrile (green line). Analysis after 24 hours. The peak at RT = 8.33 minutes corresponds to the methanolysis product.

Nine chemically different AAs were chosen for optimization of the reaction conditions in a standard solution, namely arginine, aspartic acid, threonine, proline, tyrosine, tryptophan, phenylalanine and isoleucine (Table 6).

Table 6. LC-MS information on the DEEMM derivatives of AAs concerning the protonated derivative $[M+H]^+$, the retention time in minutes, which corresponds to the method used for method development (gradient I in Table 4).

Amino acid derivative	$[M+H]^+$	Retention time (minutes)
Arginine	345	5.20
Aspartic acid	304	6.35
Threonine	290	6.74
Proline	286	7.81
Tyrosine	352	8.19
Tryptophan	375	9.75
Phenylalanine	336	10.13
Isoleucine	302	10.27

These compounds were submitted to derivatization in different solvents, ethanol and acetonitrile, and their peak areas were recorded in NLS mode (Figure 16).

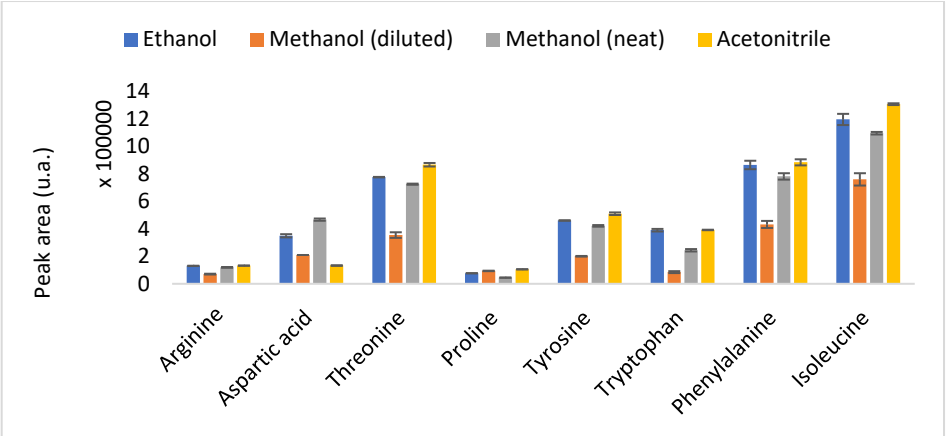


Figure 16. Average peak area comparison for DEEMM-derivatives of 8 amino acids in a standard solution for the solvents ethanol, methanol, acetonitrile (neat) and methanol (neat and diluted) as the reaction medium ($n = 2$).

The one-way ANOVA p-values are statistically significant for all the AAs ($p < 0.001$) mainly because of the lowest peak area given by the diluted methanol medium. Proline is an exception since diluted methanol and acetonitrile present similar peak areas. Additionally, the peak area of aspartic acid in the acetonitrile solution is considerably lower than in the other solvents because peak splitting took place, which might be due to the difference between the strength of the mobile phase and the injected solution. In general, acetonitrile and ethanol as derivatization reaction medium provided higher peak areas followed by methanol (neat). The latter solvent was chosen for further experiments since the derivatization yield for DEEMM was evaluated for it.

Several quenching reagents were tested for it to react with the DEEMM excess and perhaps prevent the methanolysis and the hydrolysis reaction. Hydroxylamine (1.5 M), diethylamine (10 M), butylamine (10 M), 2-amino-2- (hydroxymethyl)-propane-1,3-diol (1.5 M) and glycine (0.5 M) were chosen based on their chemical structure (on the extremes of polarity) and their solubility in 30% methanol in aqueous 0.1 M HCl. Heptylamine was also tested since its derivative was expected to have a long retention time, but its solubility in the water-based solvent was not sufficient for the reaction. The retention time of their derivatives is an important parameter because the peak of the quenching reagent should not overlap with the peaks of the analytes of interest (Table 7). The quenching reagent was added 2 hours after starting the derivatization reaction.

Table 7. Different quenching reagents for DEEMM derivatization and their respective protonated *m/z* and retention time (minutes).

Quenching reagent	$[M+H]^+$	Retention time (minutes)
Hydroxylamine	158	1.77
2-amino-2-(hydroxymethyl)propane-1,3-diol (Tris)	292	5.52
Glycine	246	6.77
Diethylamine	244	10.15
Butylamine	244	11.60

Hydroxylamine is a good candidate for quenching due to the high polarity of its DEEMM derivative and therefore leading to a short retention time (RT = 1.5 minutes). The chromatographic profile became cleaner as a result of the addition of the quenching reagent after 2 hours (Figure 17).

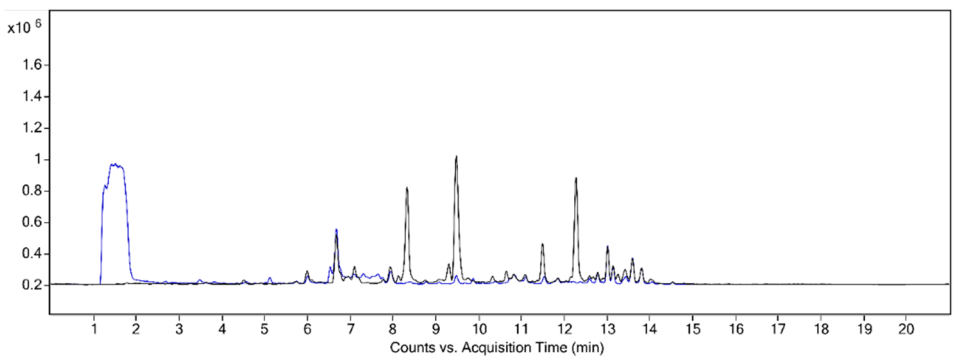
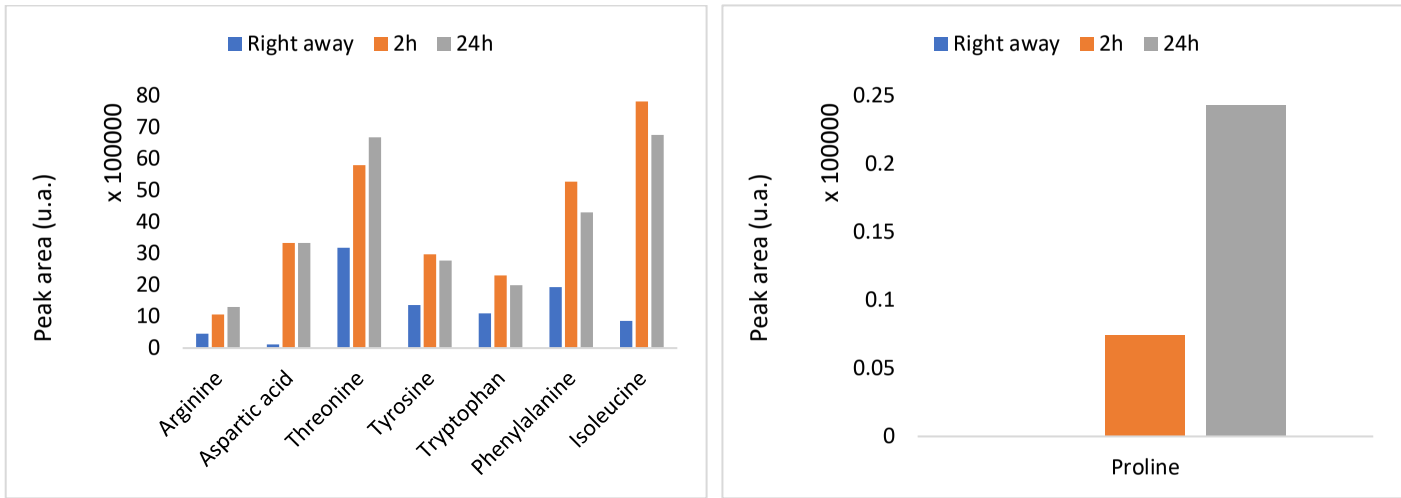


Figure 17. NLS profile of a blank solution derivatized with DEEMM without quenching (black line) and with hydroxylamine as quencher (blue line). Derivatization was carried out in 30% methanol in aqueous 0.1 M HCl and analysis was performed after 24 hours.

The addition time of the quenching reagent was also tested at three different times, right away, after 2 hours and after 24 hours. However, the right-away addition proved to affect the derivatization step significantly (Figure 18), especially in the case of aspartic acid, isoleucine and proline, which is shown by the lower peak areas. The results of 2 and 24 hours are comparable, and, in some cases, derivatives present a higher peak area for a quenching carried out after 2 hours, with the only exception being proline. Therefore, since quenching after 24 hours would make the procedure too long, 2 hours was used as the quenching time.



(a) (b)

Figure 18. Peak areas of the DEEMM derivatives of (a) 7 AAs and (b) proline after the addition of the quenching reagent right away, after 2 and 24 hours and analysis time 24 hours after quenching (n = 1).

After establishing the addition of the quenching reagent after 2 hours, different analysis times were tried out too (Figure 19). At first, there is an interference peak between 6.5 and 8 minutes, which disappears already after 160 minutes. Consequently, analysis time should be at least 3 hours after the quenching is carried out.

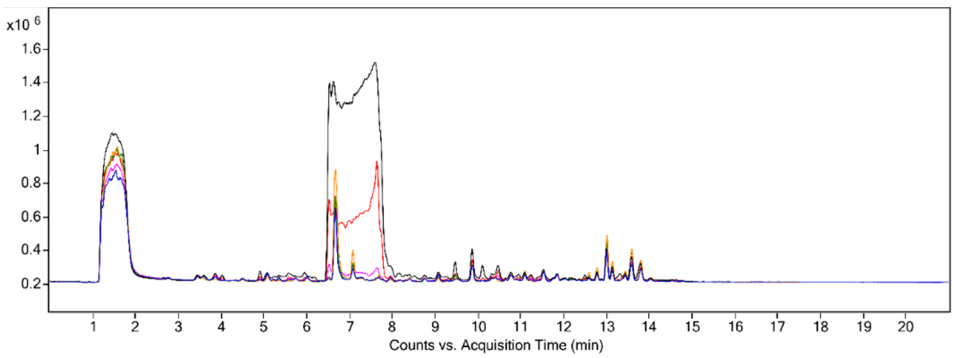


Figure 19. Analysis over time of hydroxylamine addition to DEEMM derivatization mixture with the addition of hydroxylamine in 30% methanol in aqueous 0.1 M HCl solution: immediately (black line), after 40 minutes (red), 80 minutes (pink), 160 minutes (blue), 440 min (green) and 720 minutes (orange) (paper I).

The influence of the quenching reagent on the peak area of the 9 AAs was studied and the *p*-value was used to compare the two treatments (Figure 20). Proline's peak area is affected by the addition of the quenching reagent with a significant decrease ($p < 0.001$), as previously pointed out (Figure 18b), emphasizing how slowly secondary amines react with DEEMM. Other AAs showed higher peak areas with the addition of the quenching reagent. An exception is aspartic acid, which showed comparable peak areas for both treatments. Other than that, tryptophan was the only AA that presented a statistically significant difference ($p < 0.001$) between the two treatments.

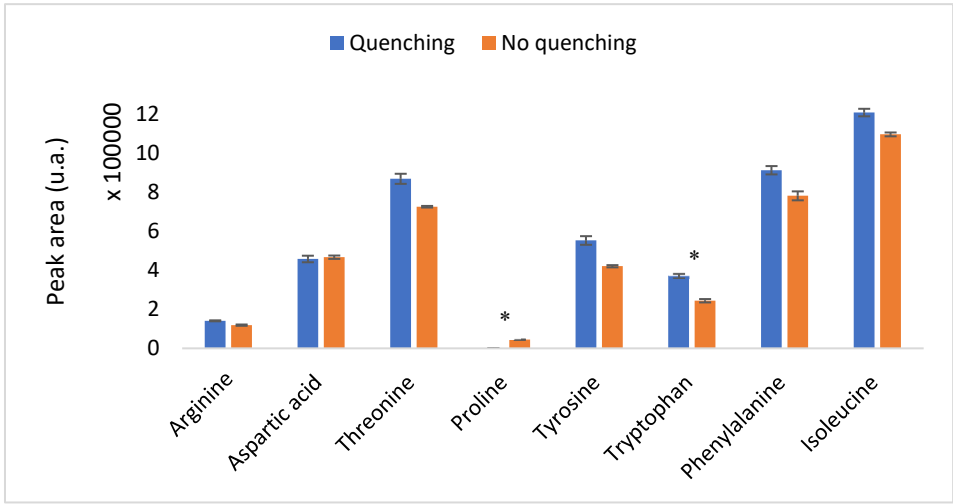


Figure 20. Comparison between peak areas in NLS mode of nine AA DEEMM-derivatives with and without the addition of the quenching reagent ($n = 2$). * $p < 0.001$ for the one-way ANOVA test.

In order to apply the developed method to a real sample, the extract of *Carduus nutans* subsp. *macrocephalus* (Desf.) Nyman, a wild edible species that grows in the Mediterranean territory, was chosen to explore its amino compound content since its metabolic profiling is restricted to polyphenol compounds (Marengo et al., 2017). Polyphenols are secondary or specialized metabolites in plants and are responsible for defense against microorganisms (antimicrobial) and used in signaling pathways, produced under biotic stress situations. Plant primary metabolites participate in the plant's growth and can vary due to abiotic stress and include vitamins, carbohydrates and AAs, among others (Salam et al., 2023).

In total, 21 amino compounds were detected and identified by the injection of their reference standard and 7 remained unidentified, labeled as "unknown" (Table 8). However, the presence of α-aminobutyric acid, β-aminobutyric acid (*m/z* 274 at 9.67 and 10.64 min, respectively) and ethanolamine (*m/z* 232 at 7.283 min) was speculated.

Table 8. List of identified derivatives in the extract of *Carduus nutans* subsp. *Macrocephalus* (Desf.) Nyman. Each compound is described by their retention time (gradient II on Table 4), protonated molecule (*m/z*), molecular weight of the derivative and amino compound (g/mol) and identified compound name (paper I).

RT (min)	$[M+H]^+$	Derivative molecular weight (g/mol)	Molecular weight (g/mol)	Compound name
1.540	159.0	158.0	33.0	Hydroxylamine derivative
3.742	325.9	324.9	154.9	Histidine
5.145	282.0	281.0	111.0	Unknown
5.546	345.0	344.0	174.0	Arginine
5.846	365.0	364.0/342.0[2]	194.0/172.0[2]	Unknown
6.014	302.9	301.9	131.9	Asparagine
6.364	317.0	316.0	146.0	Glutamine
6.632	275.9	274.9	104.9	Serine
6.832	258.9	257.9/235.9[2]	87.9/65.9[2]	Unknown
7.116	303.9	302.9	132.9	Aspartic acid
7.283	232.0	231.0	61.0	Unknown
7.734	290.0	289.0	119.0	Threonine
8.319	259.9	258.9/236.9[2]	88.9/66.9[2]	Unknown
8.970	274.0	273.0	103.0	γ-aminobutyric acid
9.304	260.0	259.0	89.0	Alanine
9.388	286.0	285.0	115.0	Proline
9.668	274.0	273.0	103.0	Unknown
9.956	288.0	287.0/265.0[2]	117.0/95.0[2]	Unknown
10.056	352.0	351.0	181.0	Tyrosine
10.641	274.0	273.0	103.0	Unknown
10.858	324.0	323.0/301.0[2]	153.0/131.0[2]	Unknown
11.459	242.0	241.0/219.0[2]	71.0/49.0[2]	Unknown
11.894	288.0	287.0	117.0	Valine
12.311	308.0	307.0	137.0	Tyramine
12.545	375.0	374.0	204.0	Tryptophan
12.829	427.0[1]	472.0	132.0	Ornithine
13.096	336.0	335.0	165.0	Phenylalanine
13.280	302.0	301.0	131.0	Isoleucine
13.481	302.0	301.0	131.0	Leucine
13.547	441.0[1]/509.0[2]	486.0	146.0	Lysine
14.733	383.0[1]	428.0	88.0	Putrescine
15.903	292.0	291.0	121.0	Phenylethylamine
16.454	353.0	352.0/330.0[2]	182.0/160.0[2]	Unknown

[1] Loss of 46 from the disubstituted derivative, [2] Sodium adduct $[M+Na]^+$

4.2. Discrimination of plant samples in neutral loss scan mode (Paper II)

The method developed in Paper I was further applied to six individuals of five different species of *Carduus* genus, all collected on the island of Sardinia, Italy, as well as the species *Ptilostemon casabonae* from different locations: *C. argyroa* (CA); *C. cephalanthus* (CC); *C. nutans* subsp. *Macrocephalus* (CN); *C. pycnocephalus* (CP); *P. casabonae* from Corse (PCC); *P. casabonae* from Sardinia (Gennargentu, PCG); *P. casabonae* from Sardinia (Iglesias, PCI). The amino compound profiles were investigated and compared among these samples to understand if the discrimination of the plant species and geographical origins was possible as it has been found and determined for the polyphenol profiles of *Carduus* species (Marengo et al., 2017). The statistical analyses principal component analysis (PCA), partial least squares discriminant analysis (PLS-DA) and hierarchical clustering were employed for such purpose.

In order to optimize the ultrasound-assisted solid-liquid extraction (UASE) procedure, a design of experiment (DoE) was used. Central composite design (CCD) was employed to identify any statistically significant parameters among the volume of solvent (3 – 10 mL), the amount of solid sample (50 – 200 mg) and the extraction time (10 – 40 min). In CCD, star or axial points are included in the design in order to estimate the curvature of the model as well as for the estimation of first and second-order terms (Bhattacharya, 2021).

The results of the preliminary DoE showed that the only parameter that was not statistically significant in the sum of the peak areas of all the detected compounds was the extraction time, which was set at 10 minutes in the following experiments for optimization. Another CCD was employed for the optimization of the extraction procedure with regard to the volume of the solvent and the amount of plant matrix (Table 9).

Table 9. DoE for the optimization of the UASE extraction with the variables volume of solvent (mL) and plant weight (mg).

Volume of solvent (mL)	Plant material (mg)
3	140
3	140
5	140
5	140
3	200
3	200
5	200
5	200
4	170
4	170
4	170
4	170
4	170
2.6	170
5.4	170
4	128
4	212

The generated data, based on the sum of the peak areas in NLS mode, was analyzed by response surface methodology (RSM) (Hanrahan et al., 2005; Sarabia & Ortiz, 2009). The model was evaluated based on its *p*-value as well as the lack of fit, the parameters volume's and mass' and their interaction's *p*-value (Table 10). The most statistically significant variable was the solvent volume followed by the plant mass ($p < 0.05$).

Table 10. *p*-values of the variables volume (V), mass (m), volume and mass (V:m) interaction, model, and lack of fit and adjusted R^2.

Factor	*p*-value	
Intercept	1.26E-08 ***	7.46E-10 ***
V	0.018 *	0.011 *
m	0.067 *	0.049 *
V:m	0.72	-
Model	0.043 *	0.013 *
Lack of fit	0.146	0.159
Adjusted R^2	0.58	0.63

*** $p \approx 0$; ** $p < 0.001$; * $p \approx 0.05$

Although the response surface plot (Figure 21) suggests that the reduction of the solvent volume and the increase of the plant mass will enhance extraction efficiency, the variables were set at 3 mL and 200 mg, respectively, due to experimental limitations.

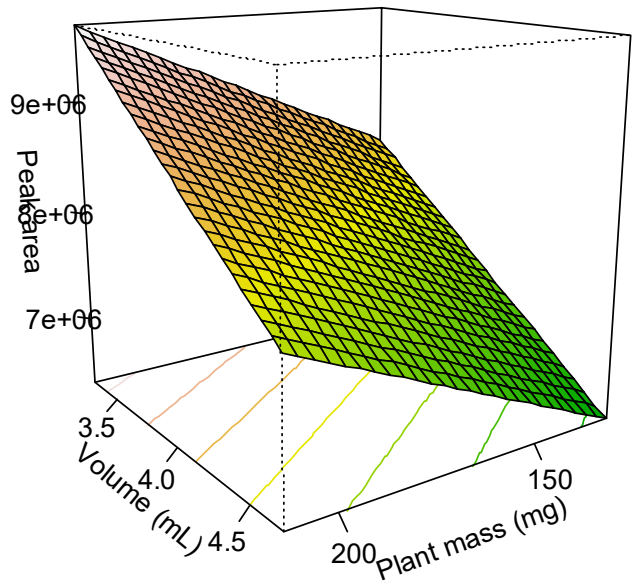


Figure 21. Response surface plot of the dependence of the sum of peak areas on the variables volume (mL) and plant mass (mg).

Compared to the previous work, 9 additional compounds were detected, namely taurine, histamine, glycine, glutamic acid, methylamine, β-aminobutyric acid (BABA), α-aminobutyric acid (AABA), ethylamine, dopamine, pyrrolidine and isopentylamine since the extraction procedure was optimized and a slower gradient was employed, thus resulting in improved peak separation and enabling the detection of more amino compounds.

The NLS mode was also employed for the semi-quantification of the compounds based on a single-point external calibration. The amino compounds present at high concentrations are proline, glutamine, asparagine and glutamic acid (approximately 1 g/kg). The high concentration of proline is due to its accumulation in the plant, since it might play a role in stress tolerance (Trovato et al., 2021). The results were also used to build a heatmap with hierarchical clustering based on Pearson distance (Figure 22).

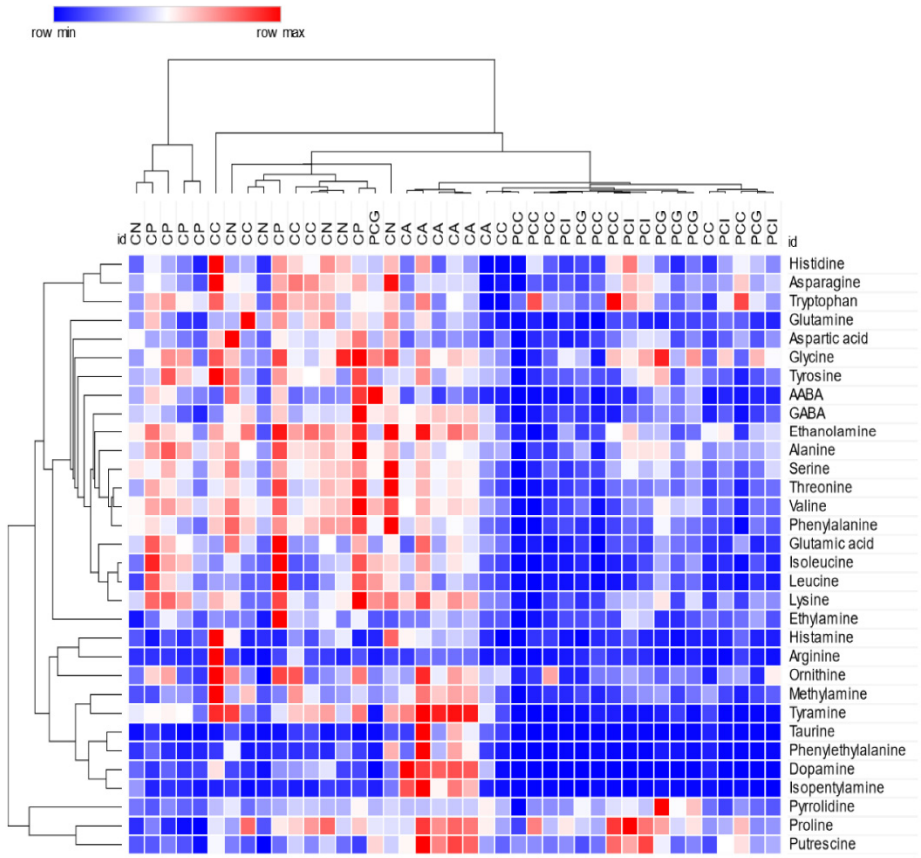


Figure 22. Hierarchical clustering (Pearson distance) and heat-map visualization for six individuals of 5 different *Carduus* species and *Ptilostemon casabonae* from different locations based on the semi-quantification of 32 compounds in NLS mode. Blue color represents lower concentration and red represents higher concentration values (paper II). CA: *C. argyroa*; CC: *C. cephalanthus*; CN: C. nutans subsp. Macrocephalus; CP: *C. pycnocephalus* CP; PCC: *P. casabonae* from Corse; PCG: *P. casabonae* from Sardinia (Gennargentu); PCI: *P. casabonae* from Sardinia (Iglesias)

The separation between the two different genus *Carduus* and *Ptilostemon* comes from the higher content of amino compounds, in general, in *Carduus* species especially in the case of the species *C. pycnocephalus* and *C. argyroa*. Moreover, *P. casabonae* from different locations did not show any significant differences in terms of their amino compound content. These results show these plants can be distinguished based on their genera and this is a straightforward approach to get a primary amino-containing metabolites profiles of plants.

4.3. Detection of amino compounds in agricultural by-products and their quantification by multiple reaction monitoring (Paper III)

Agricultural by-products consist of peels, leaves, seeds, pomace, bagasse and stems and their valorization comes down to recovering their bioactive compounds which can be applied in livestock feed supplements. The agricultural by-products hempseed (*Cannabis sativa L.*) hull (HPH), sea buckthorn (*Hippophae rhamnoides*) pomace (SBP) and leaves (SBL), apple (*Malus domestica* Borkh., variety Belorusskoje malinovoje) pomace (AP) and oat (*Avena sativa*) bran (OB) were analyzed for their free amino compounds content and to evaluate HPH, SBP and SBL as potential feed supplement while AP and OB were also analyzed because they have been employed as livestock feed (Ralla et al., 2018; Zhang et al., 2021) and, therefore, are used to compare the samples.

Protein-bound AAs need to be digested while free AAs are readily absorbed in the stomach (Eugenio et al., 2022). The higher the AAs bioavailability, the bigger the increase in the concentration of AA in plasma samples as observed in young adults and pigs (Eugenio et al., 2023; Weijzen et al., 2022). Free AA and peptides trigger microbial protein synthesis in the rumen (Schwab & Broderick, 2017) and the fast release of AAs in the bloodstream might affect the first-pass metabolism (Eugenio et al., 2023). However, since there is an increase in the trend to replace intact proteins with hydrolysates and free AAs, there is a need for long-term studies about the correct proportion of intact protein and free AAs to enhance animal performance and health (Eugenio et al., 2022).

The first step was to detect amino compounds by combining DEEMM derivatization and NLS mode. Considering all the samples, 78 peaks were detected (Figure 23), of which 46 were identified by the injection of a reference standard and quantified in dMRM mode. On the other hand, 32 compounds remained unidentified (Table 11).

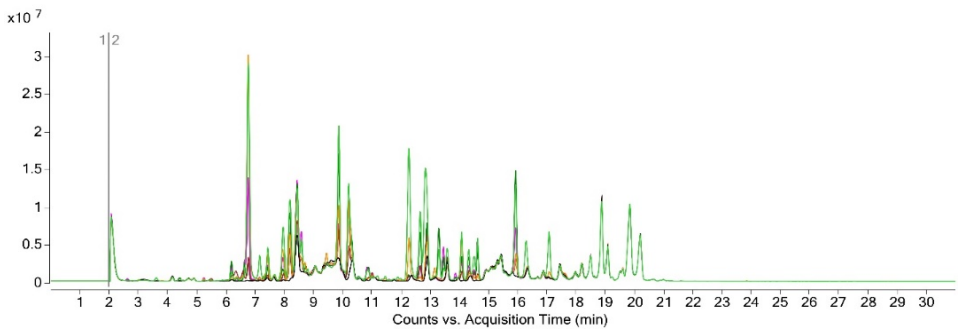


Figure 23. NLS chromatograms comparing the profiles among blank (black line) hempseed hull (dark green line), oat bran (pink line), apple pomace (brown line), sea buckthorn pomace (orange line) and leaves (light green line) (paper III).

Table 11. Peaks of compounds, which were detected but not identified in the samples. HPH: hempseed hull, SBP: sea buckthorn pomace, OB: oat bran, AP: apple pomace and SBL: sea buckthorn leaves. The sign "+" indicates detected and "–" indicates not detected.

Retention time (min)	$[M+H]^+$ DEEMM derivative	Molar mass amino compound* (g/mol)	HPH	SBP	OB	AP	SBL
5.11	340	169	+	-	+	+	-
5.21	334	163	-	-	-	-	+
5.48	386	215	+	+	+	+	-
5.48	340	169	+	-	+	+	-
5.59	373	202	-	-	-	+	+
6.32	350	179	+	+	+	+	+
6.55	345	174	+	-	+	-	-
6.63	350	179	-	+	-	+	+
6.81	356	185	+	-	+	+	+
6.91	422	251	+	+	+	+	+
7.60	303	132	-	+	-	-	-
7.49	346	175	-	-	-	-	+
7.48	336	165	-	-	-	+	+
7.73	276	105	-	-	-	+	+
7.81	226	55	+	-	+	-	-
7.81	259	88	+	-	+	-	+
7.81	375	204	+	-	+	+	-
8.99	285	114	-	+	-	+	-
9.07	379	208	-	-	-	-	+
9.14	317	146	-	+	-	-	-
9.47	416	245	+	-	+	-	-
9.69	287	116	-	-	-	-	+
9.69	317	146	-	+	-	-	-
10.01	301	130	-	-	-	+	+
11.05	338	167	+	-	-	-	-
11.74	447	276	-	-	-	-	+
11.90	379	208	-	-	-	-	+
12.05	324	153	+	+	-	-	-
14.08	521	350	-	-	-	-	+
15.11	369	198	-	-	-	-	+
15.11	415	244	-	-	-	-	+
16.21	429	258	-	-	-	+	+
16.68	331	160	-	-	-	-	+

* assuming it is a monosubstituted derivative

The DEEMM derivatives of amino compounds with different functional groups were submitted to fragmentation at different collision energy values (0 – 40 V) in both positive and negative modes to unravel the chemical structure of the "unknown" compounds. In that way, it is possible to correlate the presence of a fragment or a neutral loss to a functional group. For instance, amino compounds with a primary amide (*e.g.* asparagine), a carbamide (*e.g.* citrulline) or a guanidine (*e.g.* agmatine) group undergo a neutral loss of 17 Da (NH_3) in positive ionization mode. However, in negative mode, a neutral loss of 18 Da is detected for primary amides, while a neutral loss of 43 and 42 Da are detected for carbamide and guanidine-containing compounds, respectively. As for secondary amides, no specific fragments are detected in positive mode, but in negative mode, a neutral loss of 57 Da is present for glycylglycine and 18 Da for theanine.

One example is the peak corresponding to *m/z* 385 (RT = 5.48 min), present in all the samples, except SBL. The results indicate the presence of a carboxylic acid moiety or a hydroxyl group due to the neutral loss of 18 (H_2O) in both positive and negative modes as one of the first fragmentation pathways.

4.3.1 Extraction optimization

Following the work with plant samples, a UASE of the agricultural by-products was carried out, firstly by optimizing the extraction solvent at room temperature and extraction time of 10 minutes. Different aqueous solutions, 80% ethanol, 5% trichloroacetic (TCA) and trifluoroacetic acids (TFA), 0.1 and 0.01 M HCl, water, 50% aqueous acetonitrile and 30% methanol in aqueous 0.1 M HCl were tested for the samples HPH, SBP, AP and OB. In total, 36 amino compounds were evaluated in terms of their peak area under NLS mode (Figure 24). This enables the addition of newly confirmed compounds to the dataset later, which is not possible in MRM mode.

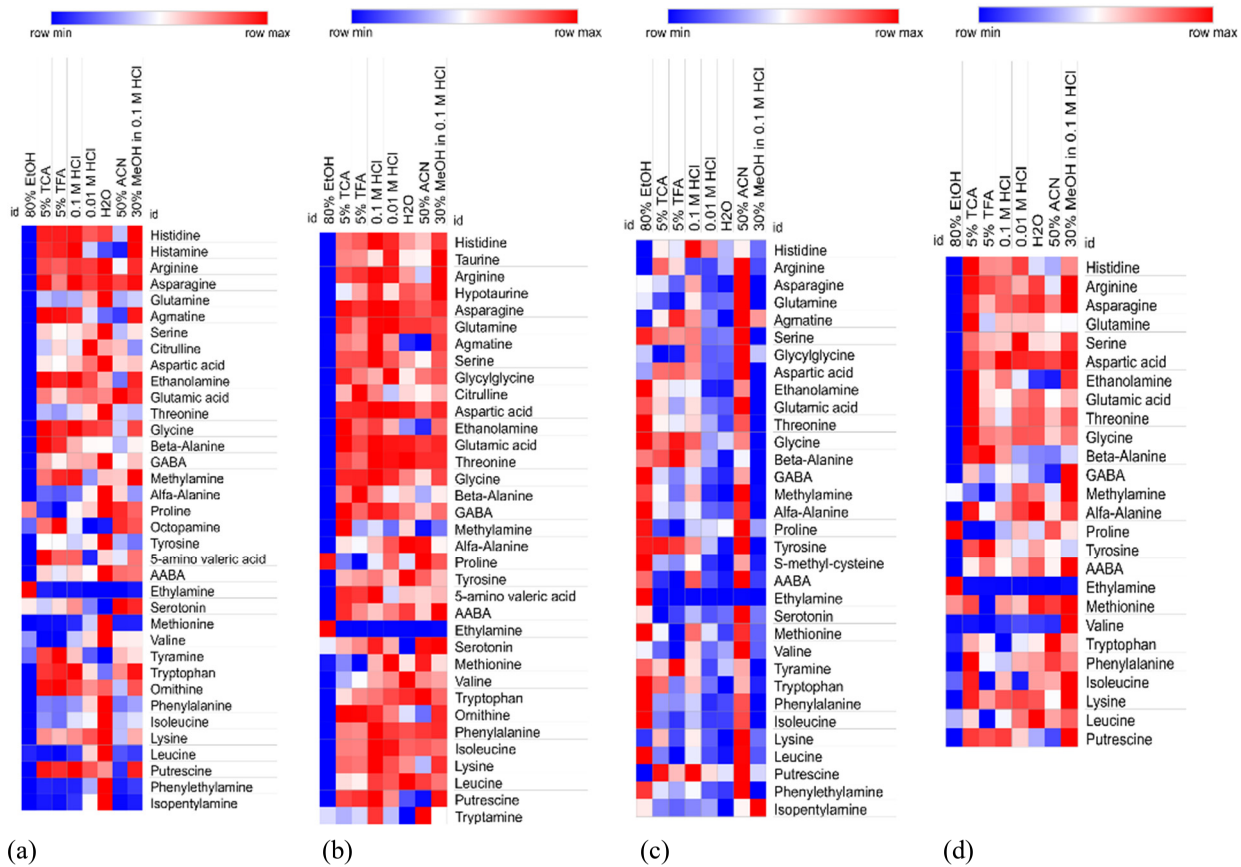


Figure 24. Heatmap for the 8 different solvents employed in the solid-liquid extraction, in terms of analyte peak area in NLS mode, of (A) hempseed hull, (B) oat bran, (C) sea buckthorn pomace and (D) apple pomace. Blue color represents lower peak areas and red represents higher peak areas (paper III).

In general, 80% aqueous ethanol provides lower recoveries due to its relatively lower polarity, but for example, proline has one of the highest extraction rates with this solvent. Serotonin is better extracted by the solvents 50% aqueous acetonitrile and 30% methanol in aqueous 0.1 M HCl. The solvent 30% methanol in aqueous 0.1 M HCl provided a good compromise for most compounds considering extraction efficiency and repeatability (RSD < 20%).

Other equally important extraction parameters are temperature, volume of the solvent, time of the extraction, sample mass, etc. After solvent optimization, the temperature (25 – 60 °C), the volume (2 – 6 mL) and the time (15 – 45 minutes) of the extraction were optimized in terms of the design of experiment (DoE), a full-factorial design with central points was employed (Table 12), and the generated data was analyzed by RSM.

Table 12. Full-factorial design with central points for the study of extraction efficiency by ultrasonic-assisted solid-liquid extraction for temperature (°C), extraction time (minutes) and solvent volume (mL).

Temperature (°C)	Extraction time (min)	Volume (mL)
25	15	2
60	15	2
25	45	2
60	45	2
25	15	6
60	15	6
25	45	6
60	45	6
42.5	30	4
42.5	30	4
42.5	30	4

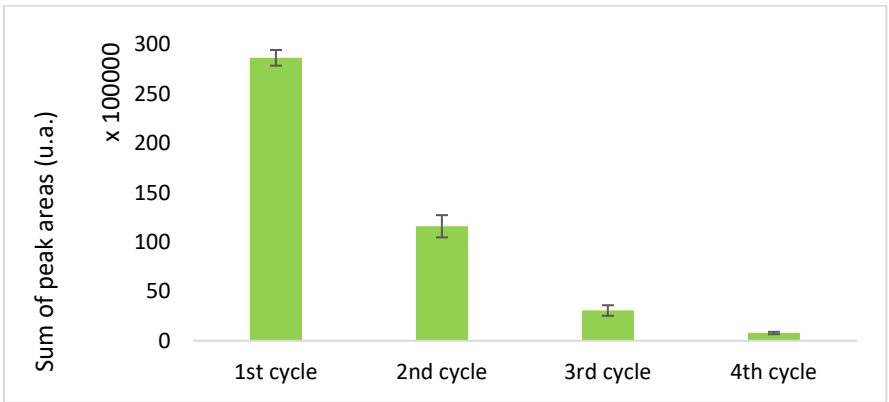
The DoE was carried out for the samples HPH and SBP, since their extraction was done in different solvents. The model was evaluated based on its *p*-value as well as the lack of fit, the parameters temperature's, time's and volume's and their interactions' *p*-value (Table 13). The only statistically significant ($p < 0.05$) variable was the solvent volume for both samples. The lack of fit was not statistically significant ($p > 0.05$) for the sample HPH in the three models built, while the lack of fit significance decreased as statistically non-significant variables were removed from the model for the sample SBP.

Table 13. *p*-values of the extraction variables temperature (T), time (t), volume (V), their interactions, model, and lack of fit and adjusted R^2 for the samples HPH and SBP.

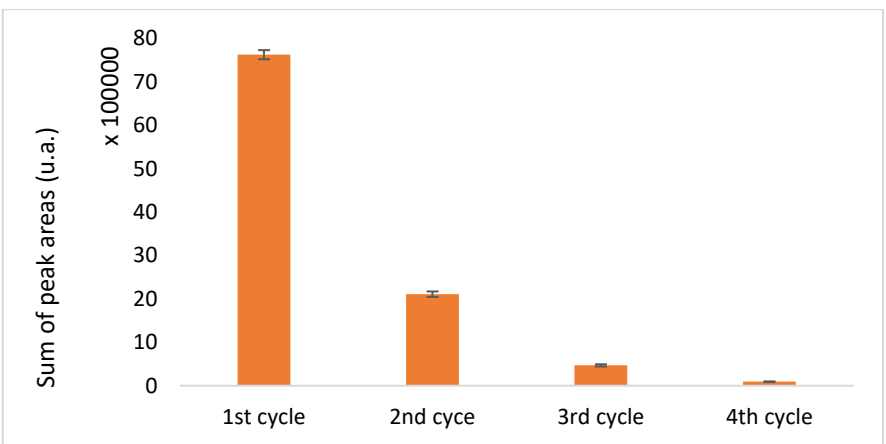
Factor	*p*-value					
	HPH			SBP		
Intercept	7.78E-06 ***	3.02E-08 ***	5.28E-09 ***	2.91E-05 **	2.37E-07 ***	2.11E-08 ***
T	0.515	0.417	0.443	0.640	0.565	0.532
t	0.432	0.328	0.353	0.967	0.959	0.956
V	7.13E-04 **	2.64E-05 **	1.36E-05 **	1.63E-03 *	9.83E-05 **	2.38E-05 **
T:t	0.307	0.203	-	0.988	0.985	-
t:V	0.887	-	-	0.930	-	-
T:V	0.933	-	-	0.733	-	-
Model	0.0100 *	2.95E-04 **	9.95E-05 **	0.0231 *	1.15E-03 *	1.78E-04 **
Adjusted R^2	0.895	0.929	0.919	0.838	0.888	0.904
Lack of fit	0.117	0.219	0.203	7.90E-03	0.0152	0.0190

*** $p \approx 0$; ** $p < 0.001$; * $p \approx 0.05$

After understanding that the extraction time and the temperature were not statistically significant in the studied range, the next step was to evaluate how many extraction cycles were needed for the complete extraction of components with less favorable partition coefficients. The samples HPH and SBP were submitted to a solid-liquid extraction for four rounds or cycles. The graph in Figure 25 shows that the content of free amino compounds is relatively low in the 4th extraction cycle compared to the content in the 1st cycle. On average, less than 3% of the content of amino compounds was present at the 4th cycle, so a three-cycle extraction was chosen for compounds present at higher concentrations. Some analytes present at low concentrations were extracted with only one cycle, especially for the samples SBP and AP, since they presented a lower content of free amino compounds overall.



(a)



(b)

Figure 25. Sum of peak areas in NLS mode of (a) 37 amino compounds in HPH, and (b) 31 amino compounds in SBP after four extraction cycles in NLS mode (n = 2).

4.3.2 Cysteine quantification

The presence of cysteine was expected in all of the samples, but it was not detected. Cysteine is a PAA with a thiol group present in its chemical structure. Thiol groups are highly reactive and, therefore, cysteine can dimerize to form cystine through a disulfide bond. Consequently, the addition of a reducing agent was necessary in order to stop this oxidation process. Moreover, it was found that DEEMM also reacts with the thiol group of cysteine, therefore the disubstituted derivative was detected and used for quantification purposes (Figure 26). Thiol groups have been derivatized to prevent oxidation from taking place in biological samples (Russo et al., 2020).

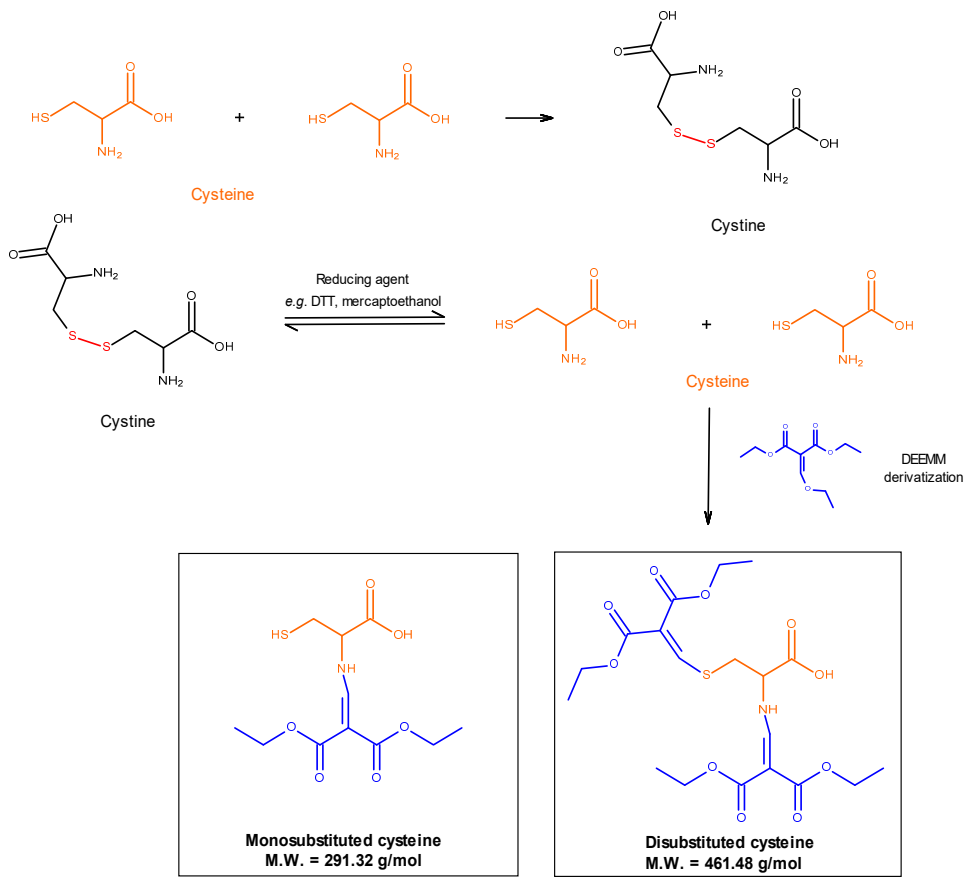
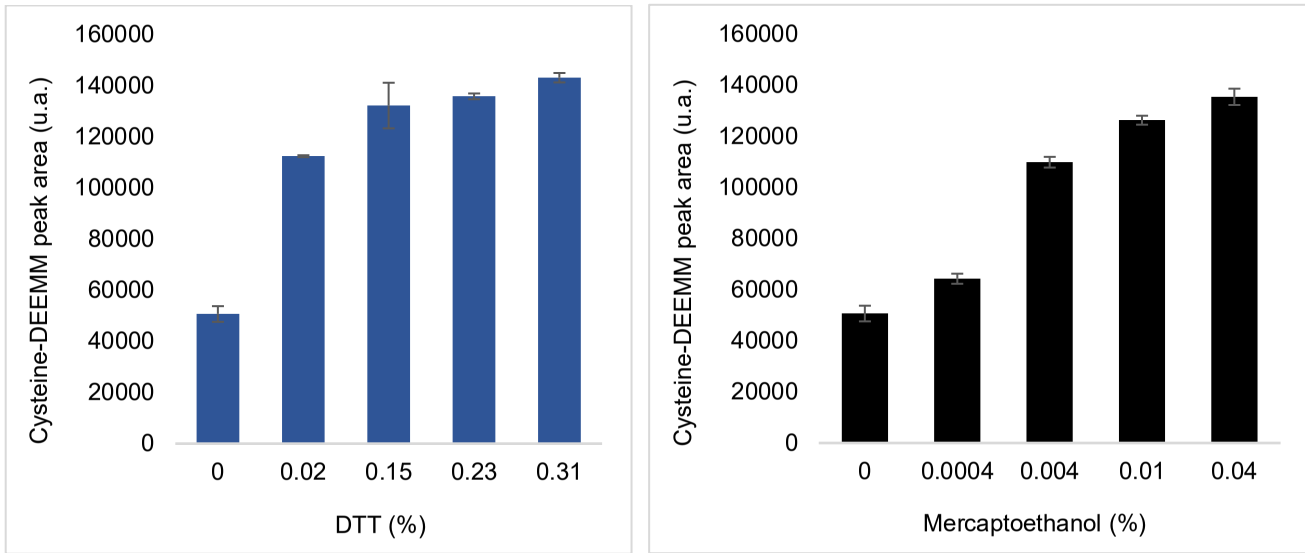


Figure 26. Formation of cystine via oxidation of cysteine, followed by the reduction reaction of cystine, triggered by a reducing agent, leading to the formation of two equivalents of cysteine, and its subsequent reaction with two equivalents of DEEMM.

At first, dithiothreitol (DTT) and 2-mercaptoethanol (MER) were tested, since DTT has been employed for quantification of D-cysteine in cell culture samples and bacteria proteins (Pucciarini et al., 2020; Soriano et al., 2012) and it is usually used in bottom-up proteomics. The MER reagent has been employed for seleno-amino acids quantification in onions (Rebane et al., 2014). The latter was shown to be more effective as a reducing agent since it gives similar results to DTT but with a lower concentration of the reagent needed (Figure 27a and b, respectively).

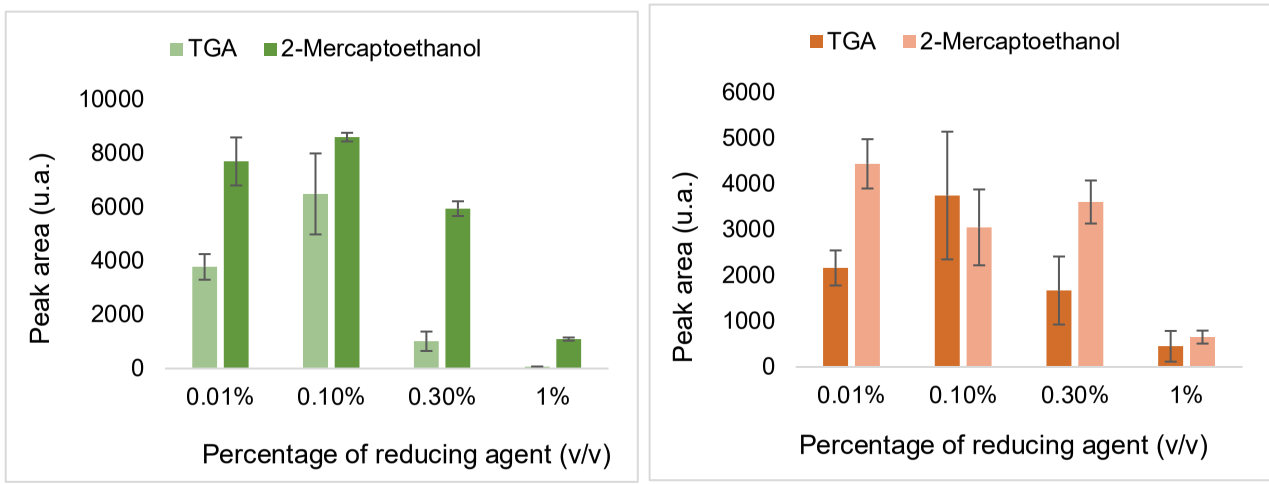
A third reagent, thioglycolic acid (TGA), was also introduced since it has been shown that it is an effective reagent for the protection of cysteine under acidic hydrolysis conditions (Yokote et al., 1986). The effectiveness of TGA and MER were studied for real samples, HPH and SBP (Figure 28a and b, respectively).

The reducing agent TGA was shown to be less effective in preventing the formation of cystine given its lower peak area in all the proportions tested and higher standard deviation in some cases, compared to MER. Therefore, 0.01% MER was chosen as the reducing reagent for the quantification of cysteine in all the samples.



(a) (b)

Figure 27. Peak area of DEEMM-cysteine derivative in a standard solution at different proportions of (a) DTT and (b) 2-mercaptoethanol.



(a) (b)

Figure 28. Comparison between two different reducing agents TGA and 2-mercaptoethanol in different proportions (v/v): 0.01, 0.10, 0.30 and 1% for (A) HPH and (B) SBP (paper III).

4.3.3 Matrix effect

Matrix effect (ME) is a phenomenon that causes a shift in the ionization efficiency of the analyte due to co-eluting compounds present in the matrix (Zhou et al., 2017). Since this influences the signal intensity of the analyte, and, therefore, directly affects quantification accuracy and sensitivity, ME is one of the most important parameters to be evaluated in LC-MS/MS analysis. A serial dilution approach (Oldekop et al., 2014) was employed for the evaluation of ME of 45 amino compounds for the samples HPH, SBP, OB and AP, in which different extract dilution ratios were employed for the quantification, the RSD% was evaluated for the back-calculated concentration values and when it was higher than 20%, then ME was assumed to be present for the analyte in question (Oldekop et al., 2014).

For most of the analytes, the RSD% was lower than 20% in the four studied samples (Table 14). The only exceptions were histidine and ornithine in the SBP sample and proline in the HPH and OB samples. The RSD% values are different across the four samples because the analytes are present in different concentrations and the analyses were carried out on different days. Considering these results, it was assumed that SBL does not present a ME either.

Table 14. RSD% (n = 6, 8 or 10) of concentration values determined at different dilution ratios: 50-, 25-, 12-, 6- and 3-fold. HPH: hempseed hull, SBP: sea buckthorn pomace, OB: oat bran and AP: apple pomace. The sign "-" marks the compound not present or not evaluated in a particular sample.

Amino compound	HPH	SBP	OB	AP
Histidine	10%	23%	9%	19%
Taurine	4%	8%	8%	-
Histamine	6%	17%	-	6%
Arginine	7%	10%	13%	9%
Hypotaurine	-	20%	14%	-
Glutamine	7%	13%	7%	13%
Asparagine	11%	14%	5%	5%
Glycylglycine	14%	-	12%	-
Serine	4%	10%	7%	10%
Agmatine	10%	4%	6%	9%
Citrulline	2%	-	6%	-
Glutamic acid	11%	4%	7%	13%
Aspartic acid	12%	14%	12%	15%
Ethanolamine	12%	12%	15%	9%
Methionine sulfone	-	3%	-	8%
Threonine	17%	2%	5%	13%

Amino compound	HPH	SBP	OB	AP
Glycine	16%	4%	11%	13%
GABA	13%	19%	11%	13%
α-Aminoadipic acid	-	7%	9%	6%
α-Alanine	11%	8%	19%	9%
β-alanine	10%	8%	10%	6%
Methylamine	7%	14%	17%	8%
Octopamine	8%	-	-	-
Proline	22%	19%	21%	17%
5-aminovaleric acid	8%	11%	4%	5%
Tyrosine	12%	5%	11%	11%
AABA	6%	4%	5%	5%
S-methylcysteine	3%	5%	3%	9%
Methionine	10%	14%	9%	5%
Serotonin	7%	13%	9%	-
Ethylamine	3%	6%	8%	7%
Valine	8%	12%	10%	11%
Tryptophan	7%	7%	5%	3%
3-methoxytyramine	4%	-	-	-
Ornithine	9%	21%	15%	2%
Tyramine	10%	14%	-	18%
Isoleucine	9%	7%	5%	8%
Leucine	4%	3%	12%	16%
Phenylalanine	5%	8%	5%	3%
Lysine	20%	10%	19%	16%
Putrescine	14%	10%	11%	9%
Tryptamine	-	10%	12%	-
Cadavarine	6%	-	-	-
2-Phenylethylamine	15%	6%	-	9%
Isopentylamine	3%	5%	10%	12%

4.3.4 Quantification of amino compounds by dMRM

After the detection and the confirmation of the identity of 46 amino compounds, the quantification was carried out in dMRM mode from an external calibration curve prepared from a stock solution with all the amino compounds (Table 15). The samples richest in free amino compounds are SBP and SBL, mainly because of their high content of asparagine (ca. 4 g/kg), however, in terms of the number of amino compounds, OB and HPH are the richest. On the other hand, AP is the poorest in terms of concentration and number of free amino compounds.

The amino compounds GABA, proline and glutamic acid are also quantified at the highest concentration values (ca. 1 g/kg). Hemp seed is rich in compounds with octopamine and tyramine moieties, such as hydroxycinnamic acid amides and phenolamides (Benkirane et al., 2022; Crescente et al., 2018), which explains the presence of octopamine only in HPH and the highest concentration of tyramine in said sample. Moreover, sea buckthorn samples present a higher concentration of serotonin and tryptamine.

The AA lysine, methionine, threonine, and tryptophan are in demand for feed since they are typically supplemented in their free form to feedstuff and are referred to as limiting AAs (Eugenio et al., 2022; Zhao et al., 2022). Among the samples SBL, HPH and SBP, the latter presents the lowest concentration of the AA previously mentioned whereas SBL and HPH have similar concentration values, except for threonine and tryptophan. In terms of these limiting AA, SBL and HPH are preferred feed supplements.

Table 15. Mean concentration of amino compounds per kg of sample ± standard deviation (mg/kg) at repeatability conditions (n = 3). HPH: hempseed hull, SBP: sea buckthorn pomace, OB: oat bran, AP: apple pomace and SBL: sea buckthorn leaves (paper III).

Compound	Concentration (mg/kg)				
	HPH	SBP	OB	AP	SBL
Histidine	53.6 ± 1.7	7.69 ± 0.18	48.6 ± 1.0	4.29 ± 0.26	34.5 ± 0.9
Histamine	0.287 ± 0.035	0.017 ± 0.001	0.074 ± 1.503E-05	0.042 ± 0.004	0.206 ± 0.004
Taurine	0.675 ± 0.036	0.1005 ± 0.0003	0.565 ± 0.006	0.011 ± 4.997E-04	1.18 ± 0.02
Arginine	274.4 ± 7.6	17.3 ± 0.8	181.9 ± 0.8	24.4 ± 1.0	86.1 ± 1.0
Hypotaurine	0.261 ± 0.009	0.0523 ± 0.0005	0.511 ± 0.001	0.111 ± 0.003	0.142 ± 0.003
Asparagine	102.3 ± 3.0	3652 ± 39	856.6 ± 0.4	126.5 ± 7.1	4194 ± 363
Glutamine	32.8 ± 1.9	62.0 ± 2.1	59.0 ± 0.4	14.6 ± 1.9	420.6 ± 42.6
Agmatine	4.77 ± 0.37	0.201 ± 0.002	0.176 ± 0.004	0.047 ± 0.003	0.888 ± 0.018
Serine	39.4 ± 1.6	1.37 ± 0.05	38.0 ± 0.4	11.4 ± 1.4	110.4 ± 6.8
Glycylglycine	0.769 ± 0.070	Below the LoD	0.133 ± 0.005	Below the LoD	Below the LoD
Citrulline	1.41 ± 0.13	Below the LoD	4.10 ± 0.42	Below the LoD	2.88 ± 0.06
Aspartic acid	100.7 ± 1.3	300.1 ± 9.7	190.0 ± 2.9	61.3 ± 2.9	429.9 ± 19.1
Ethanolamine	29.5 ± 1.0	16.2 ± 0.2	8.18 ± 0.05	7.694 ± 0.004	100.0 ± 4.6
Methionine sulfone	0.268 ± 0.011	0.245 ± 0.002	0.554 ± 0.006	0.087 ± 0.002	0.614 ± 0.013
Glutamic acid	555.1 ± 6.6	197.6 ± 4.1	701.6 ± 11.3	89.5 ± 4.8	795.2 ± 1.1
Threonine	29.8 ± 0.6	16.5 ± 0.2	29.2 ± 0.6	8.66 ± 0.87	63.8 ± 1.3
Glycine	39.2 ± 1.7	5.39 ± 0.05	38.6 ± 0.7	15.6 ± 0.8	19.2 ± 0.2
Beta-Alanine	5.02 ± 0.10	5.99 ± 0.09	0.171 ± 0.002	0.087 ± 0.003	0.118 ± 0.002
α-Aminoadipic acid	0.523 ± 0.015	0.459 ± 0.030	1.66 ± 0.05	0.070 ± 0.008	1.17 ± 0.08
GABA	446.9 ± 5.7	224.9 ± 1.7	101.5 ± 0.3	118.2 ± 1.8	953.8 ± 27.4
Methylamine	0.130 ± 0.010	0.759 ± 0.041	0.078 ± 0.007	0.122 ± 0.006	0.459 ± 0.009
Alfa-Alanine	105.6 ± 0.8	141.00 ± 0.03	73.8 ± 0.6	34.4 ± 1.3	174.3 ± 0.5
Proline	239.8 ± 2.9	113.2 ± 3.1	233.4 ± 22.2	23.4 ± 2.1	825.4 ± 18.8
Octopamine	3.16 ± 0.30	Below the LoD	Below the LoD	Below the LoD	Below the LoD

Compound	Concentration (mg/kg)				
	HPH	SBP	OB	AP	SBL
Tyrosine	43.2 ± 2.4	6.453 ± 0.005	29.6 ± 1.4	9.51 ± 0.45	26.8 ± 0.1
5-amino valeric acid	0.862 ± 0.037	0.193 ± 0.006	0.189 ± 0.006	0.045 ± 0.004	4.15 ± 0.26
S-methyl-cysteine	0.1064 ± 0.0008	9.52 ± 0.03	0.083 ± 0.008	0.059 ± 0.006	3.69 ± 0.43
AABA	1.31 ± 0.01	0.682 ± 0.006	0.069 ± 0.003	0.184 ± 0.012	0.553 ± 0.011
Ethylamine	0.707 ± 0.005	0.426 ± 0.004	0.089 ± 0.002	0.514 ± 0.004	0.298 ± 0.005
Serotonin	1.03 ± 0.04	39.1 ± 2.3	2.03 ± 0.15	Below the LoD	473.9 ± 74.1
Methionine	1.38 ± 0.08	0.041 ± 0.002	4.68 ± 0.16	1.50 ± 0.09	1.42 ± 0.04
Valine	108.2 ± 3.0	30.2 ± 0.5	65.2 ± 2.7	46.7 ± 4.6	107.0 ± 11.0
Tyramine	58.3 ± 5.1	0.802 ± 0.040	0.014 ± 0.003	0.0060 ± 0.0005	15.3 ± 1.0
Tryptophan	87.2 ± 2.9	3.06 ± 0.08	101.3 ± 4.4	4.89 ± 0.12	45.4 ± 1.3
3-methoxytyramine	0.245 ± 0.022	Below the LoD	Below the LoD	Below the LoD	Below the LoD
Ornithine	5.17 ± 0.39	0.171 ± 0.008	9.20 ± 1.28	0.029 ± 0.002	2.61 ± 0.14
Phenylalanine	32.5 ± 0.9	81.7 ± 0.2	20.8 ± 0.3	12.01 ± 0.03	73.1 ± 1.6
Isoleucine	32.3 ± 0.7	5.96 ± 0.03	13.3 ± 0.3	10.1 ± 0.1	39.9 ± 0.2
Cysteine	3.68 ± 0.07	2.83 ± 0.13	6.56 ± 0.40	0.63 ± 0.05	13.79 ± 0.16
Lysine	70.9 ± 1.3	9.89 ± 0.05	70.2 ± 1.3	10.2 ± 0.1	76.3 ± 4.4
Leucine	172.5 ± 4.2	6.30 ± 0.02	16.3 ± 0.3	15.60 ± 0.03	50.5 ± 1.35
Putrescine	28.4 ± 0.8	3.54 ± 0.04	7.07 ± 0.22	2.91 ± 0.02	18.4 ± 0.6
Tryptamine	Below the LoD	1.85 ± 0.04	0.107 ± 0.011	0.0087 ± 0.0009	32.9 ± 0.7
Cadaverine	0.724 ± 0.063	Below the LoD	Below the LoQ	Below the LoD	Below the LoD
2-phenylethylamine	0.327 ± 0.021	0.987 ± 0.009	0.022 ± 6.154E-05	0.022 ± 0.001	11.2 ± 0.2
Isopentylamine	0.532 ± 0.021	0.0705 ± 0.0003	0.019 ± 0.001	0.033 ± 6.919E-05	0.145 ± 0.03

A correlation matrix, based on Pearson distance and the concentration of 46 amino compounds shown in Table 15, was built to evaluate the similarity and the difference among the 5 samples (Figure 29), especially between HPH, SBP and SBL against OB and AP, since the two latter have already been employed as livestock feed. Compounds that were not quantified because they were below the LoD had a value of 0 in the matrix.

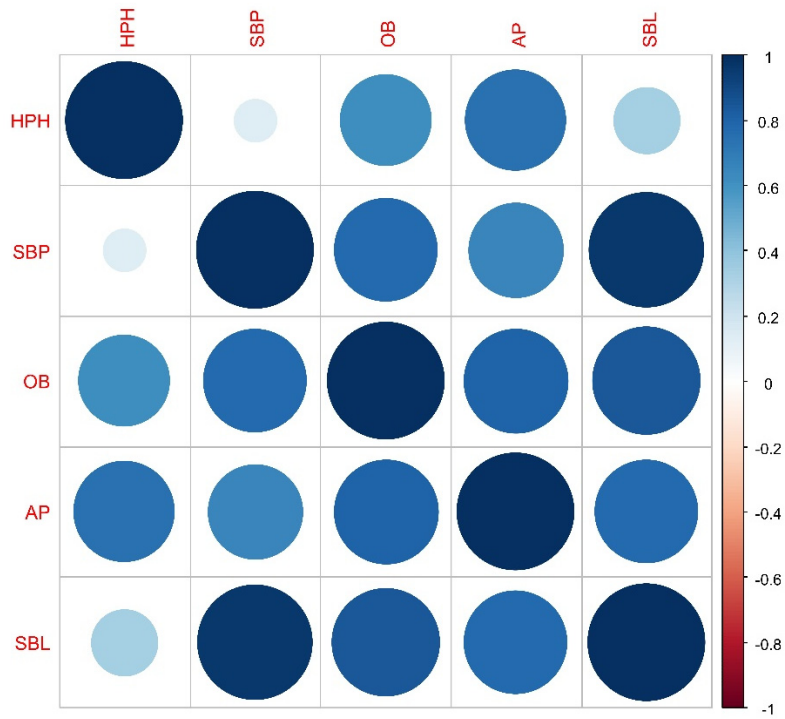


Figure 29. Similarity matrix (based on Pearson distance) among the five samples. HPH: hempseed hull, SBP: sea buckthorn pomace, OB: oat bran, AP: apple pomace and SBL: sea buckthorn leaves. Blue represents positive correlation and red color represents negative correlation. The size of the circle represents how big the correlation is (paper III).

Although different parts, namely the leaves and pomace, of sea buckthorn were analyzed, they are closely related ($r = 0.96$) because of their similar concentration of asparagine, aspartic acid, methylamine, α-alanine, S-methylcysteine, AABA, ethylamine, serotonin, phenylalanine and tryptamine. OB and AP are moderately correlated with SBP ($r = 0.78$ and 0.66, respectively) and HPH ($r = 0.61$ and 0.74, respectively) and more strongly correlated with SBL ($r = 0.85$ and 0.77, respectively). This high correlation between the already employed samples as feed supplements indicates that SBL is more appropriate for livestock feed supplements and the compounds responsible for that similarity between OB and SBL

are methionine sulfone, glutamic acid, β-alanine, α-aminoadipic acid, tyrosine, and lysine. Moreover, the combination of the samples HPH and SBL in different proportions could address any shortcomings observed in one another, *e.g*. the tryptophan content in SBL is lower while the threonine content is higher.

The content of free amino compounds may be affected by the season of the plant collection and the sample pre-treatment, such as the drying process. Moreover, further research is needed to explore the synergistic interactions among the identified amino compounds and how they affect the animal's health and performance. For instance, the presence of putrescine and cadaverine could augment the histamine and tyramine's toxicity (Jain & Verma, 2018).

4.4. Comparison of sensitivities of NLS and MRM modes

The limit of detection (LoD) and quantification (LoQ) were evaluated in dMRM and NLS modes corresponding to the loss of 46 and with the same fragmentor voltage and collision energy values. NLS is a scanning mode by its nature and scanning modes of quadrupole mass analyzers are known to provide lower sensitivity as compared to MRM. The sensitivity of dMRM is expected to be higher, but the magnitude is not known. A calibration curve (5 – 150 µg/kg) was built and analyzed on the same day for both modes and its slope and standard deviation of the response were used to calculate LoD and LoQ (Equations 1 and 2, respectively)

$$LoD = \frac{3.3 * \sigma}{S} \quad (1)$$

$$LoQ = \frac{10 * \sigma}{S} \quad (2)$$

where
σ – standard deviation of the response and
S – slope of the calibration curve

The comparison of LoD and LoQ values between MRM and NLS modes (Table 16), presented as LoQ_{NL}/LoQ_{MRM}, shows that the results are quite close for most analytes, with a maximum of 14 times higher sensitivity of MRM mode in the case of histidine. On average, MRM mode provided 4 times higher sensitivity than NLS mode.

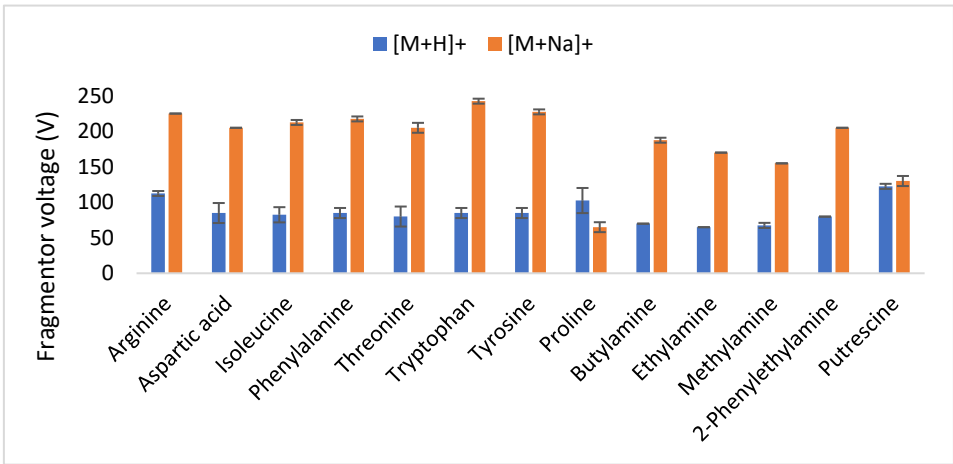
Table 16. Comparison of LoD and LoQ in MRM and NLS modes for standard solution, fragmentor voltage 90 V and collision energy 8 V.

Amino compound	LoD		LoQ		LoQ_{NL}/LoQ_{MRM}
	MRM	NLS	MRM	NLS	
3-methoxytyramine	3.1	5.6	9.3	17.0	2
5-aminovaleric acid	2.0	6.5	6.0	19.7	3
AABA	2.6	7.0	7.8	21.1	3
Agmatine	3.8	4.5	11.6	13.6	1
Arginine	1.8	18.5	5.5	56.1	10
Asparagine	2.4	9.3	7.2	28.3	4
Aspartic acid	2.5	12.8	7.7	38.7	5
Cadaverine	3.1	2.3	9.5	7.0	1
Citrulline	3.0	16.8	9.1	50.9	6
Cysteine *	26.0	52.2	78.7	158	2
Cysteine	338	297	1023	899	1
Ethanolamine	5.7	4.5	17.3	13.7	1
Ethylamine	3.4	3.7	10.4	11.3	1
GABA	1.1	4.0	3.4	12.1	4
Glutamic acid	1.8	23.8	5.4	72.1	13
Glutamine	1.7	18.3	5.0	55.5	11
Glycine	3.3	3.4	10.0	10.2	1
Glycylglycine	2.5	21.5	7.4	65.2	9
Histamine	5.1	4.3	15.5	13.0	1
Histidine	2.0	29.0	6.2	87.9	14
Isoleucine	2.3	3.7	7.1	11.1	2
Isopentylamine	3.2	7.0	9.7	21.2	2
Leucine	2.0	4.5	5.9	13.6	2
Lysine	4.5	7.8	13.7	23.8	2

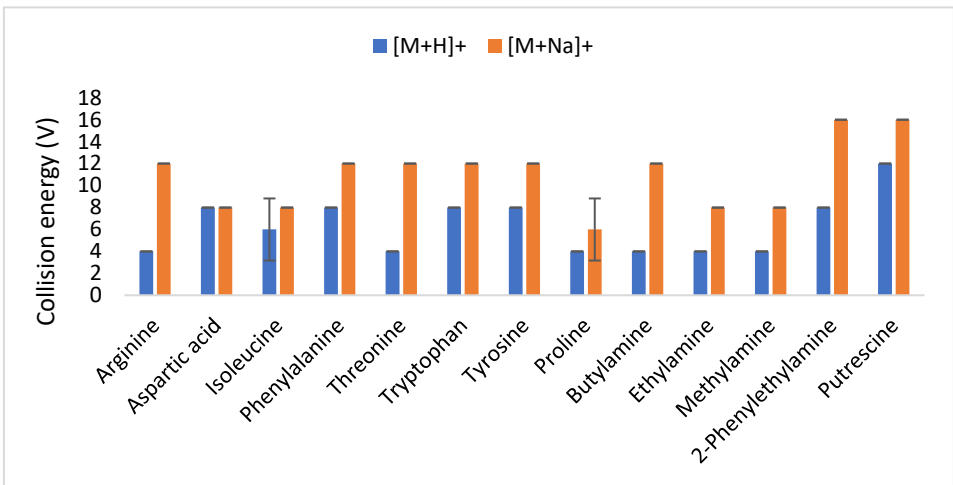
Amino compound	LoD		LoQ		LoQ_{NL}/LoQ_{MRM}
	MRM	NLS	MRM	NLS	
Methionine	1.6	12.2	4.9	37.1	8
Methionine sulfone	2.7	17.8	8.2	54.0	7
Methylamine	4.4	9.8	13.4	29.8	2
Octopamine	1.3	8.2	4.0	24.8	6
Ornithine	4.0	15.8	12.2	47.8	4
Phenylalanine	2.0	4.2	6.0	12.7	2
Phenylethylamine	3.1	4.3	9.5	13.1	1
Proline	6.8	55.5	20.5	168	8
Putrescine	4.3	4.8	13.0	14.6	1
Serine	5.6	21.2	17.0	64.3	4
Serotonin	2.0	4.5	6.0	13.7	2
S-methylcysteine	1.3	5.2	4.1	15.7	4
Taurine	3.2	16.6	9.8	50.2	5
Threonine	1.6	10.4	4.9	31.4	6
Tryptophan	6.4	5.9	19.3	17.8	1
Tyramine	1.4	5.9	4.3	18.0	4
Tyrosine	2.7	12.8	8.1	38.8	5
Valine	1.8	10.1	5.4	30.6	6
α-Alanine	2.4	3.4	7.4	10.2	1
β-Alanine	5.3	6.5	16.1	19.7	1

* with the addition of 2-mercaptoethanol

The LoD and LoQ can be fine-tuned by changing the fragmentor and the collision energy values for each analyte in the case of MRM while it is possible to have only four values of fragmentor voltage and collision energy, in the case of NLS mode, per method. However, these values for the protonated $[M+H]^+$ and sodium adduct $[M+Na]^+$ of DEEMM derivatives are similar to one another as shown by the graph in Figure 30. The only exceptions, in the case of the fragmentor voltage values for the protonated molecule, are the simplest amines methylamine and ethylamine (ca. 65 V) and diamino-compound putrescine (ca. 120 V), otherwise, the range is 70–113 V. As for the collision energy, the range stays between 4–12 V.



(a)



(b)

Figure 30. Optimization of the (a) fragmentor voltage and (b) the collision energy values for the MRM transition corresponding to the loss of 46 on two different days (n = 2).

In case ME is present, extracts should be diluted to reduce it. Therefore, MRM is preferable for accurate quantification. Besides, some compounds were present at low concentrations, *e.g*. histamine and 3-methoxytyramine, so employing dMRM was unavoidable. However, the results of LoD in NLS mode are still adequate for the unraveling of amino compounds in complex samples.

4.5. Exploring existing derivatization reagents for derivatization-targeted analysis

When derivatization reagents are explored for derivatization-targeted analysis, they need evaluation based on different parameters. It is important to know if the reagent reacts with a wide variety of amino compounds. To study the reactivity of derivatization reagents, a group of amino compounds, such as sulfonated, BAs, AAs, secondary amines, diamines, triamines etc. (Figure 31) was selected and their derivatives' fragmentation patterns studied under different collision energy values. The aim was to identify common charged fragments for PIS mode or neutral losses for NLS mode.

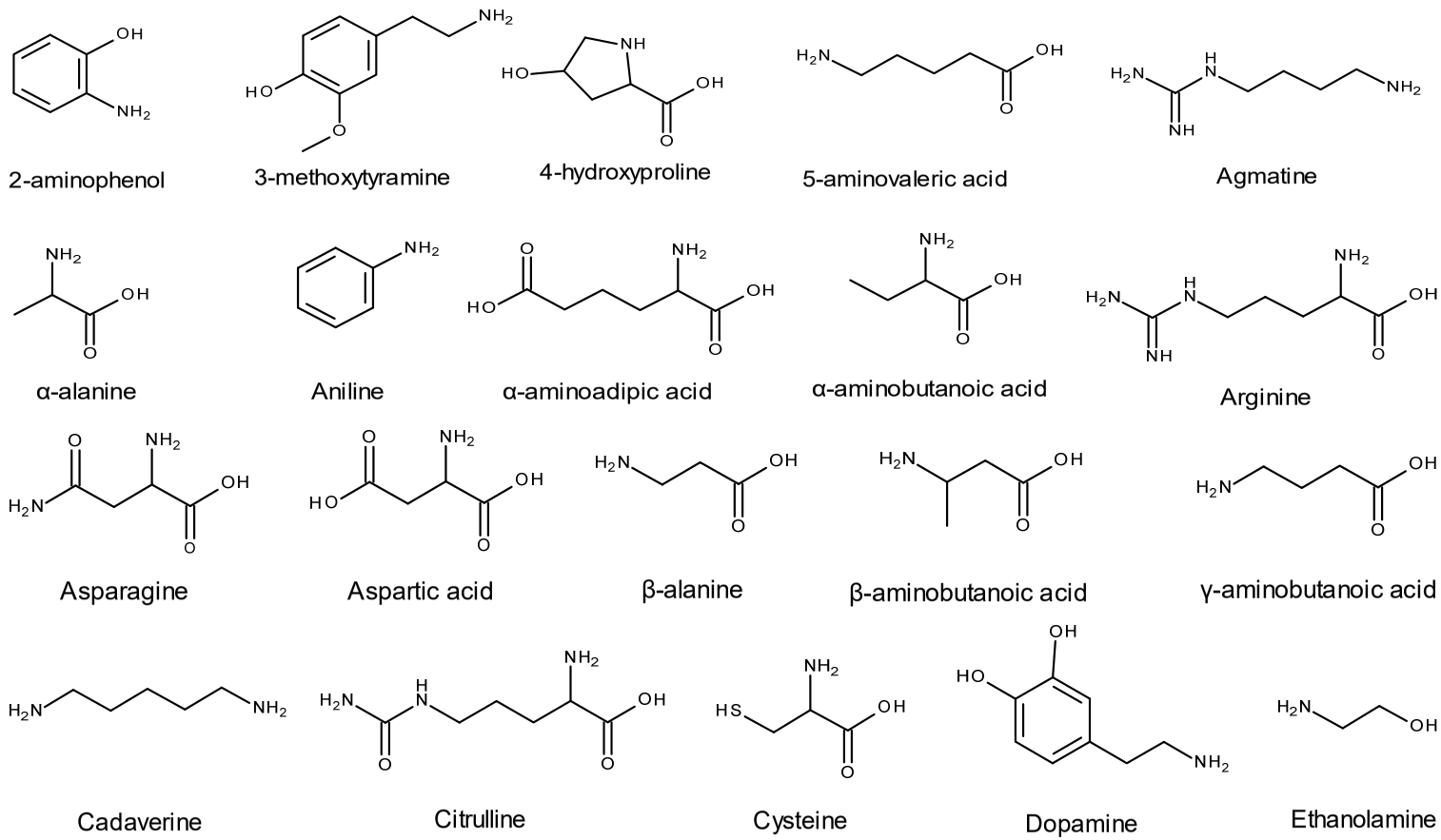


Figure 31. Continued

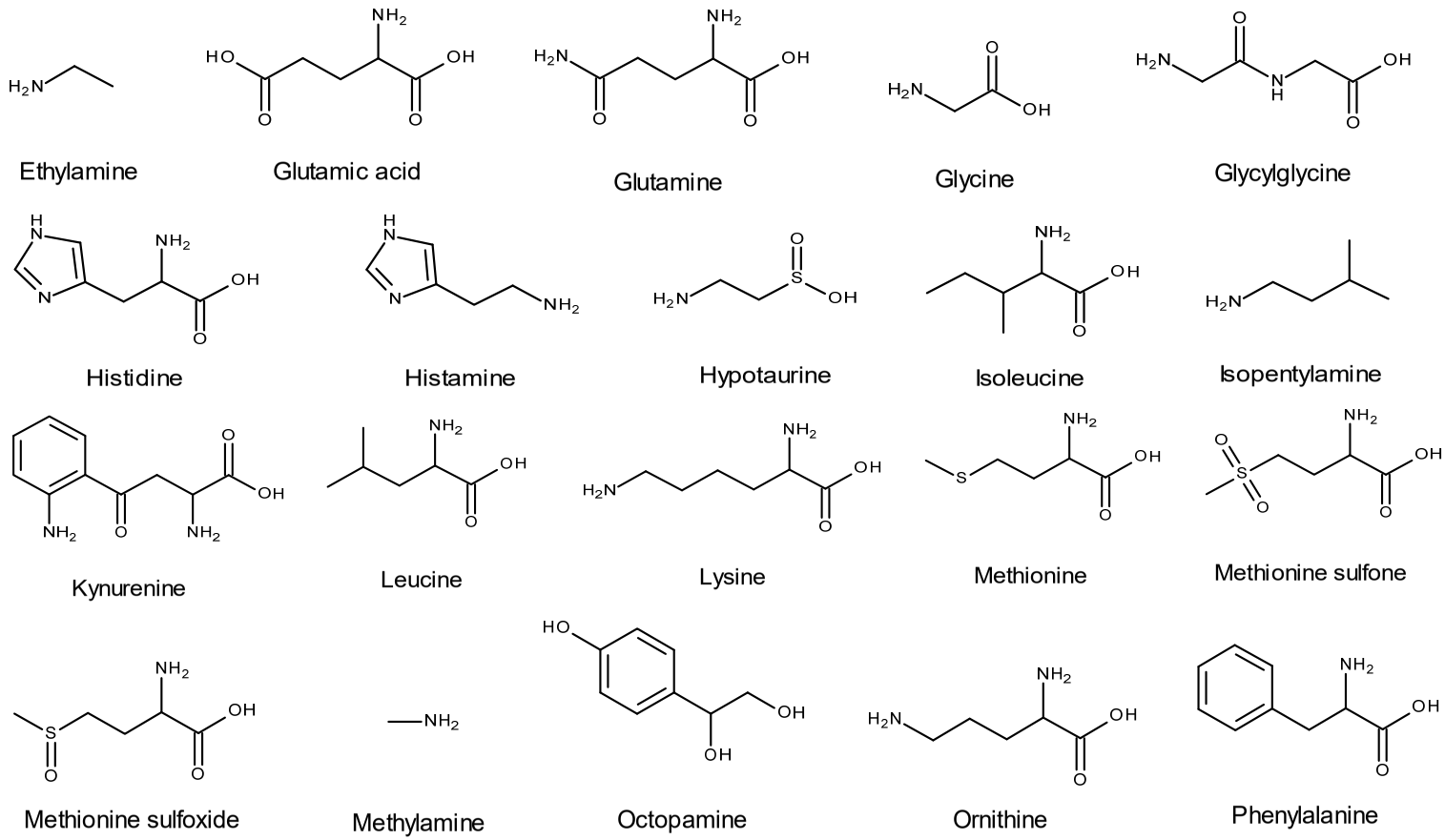


Figure 31. Continued

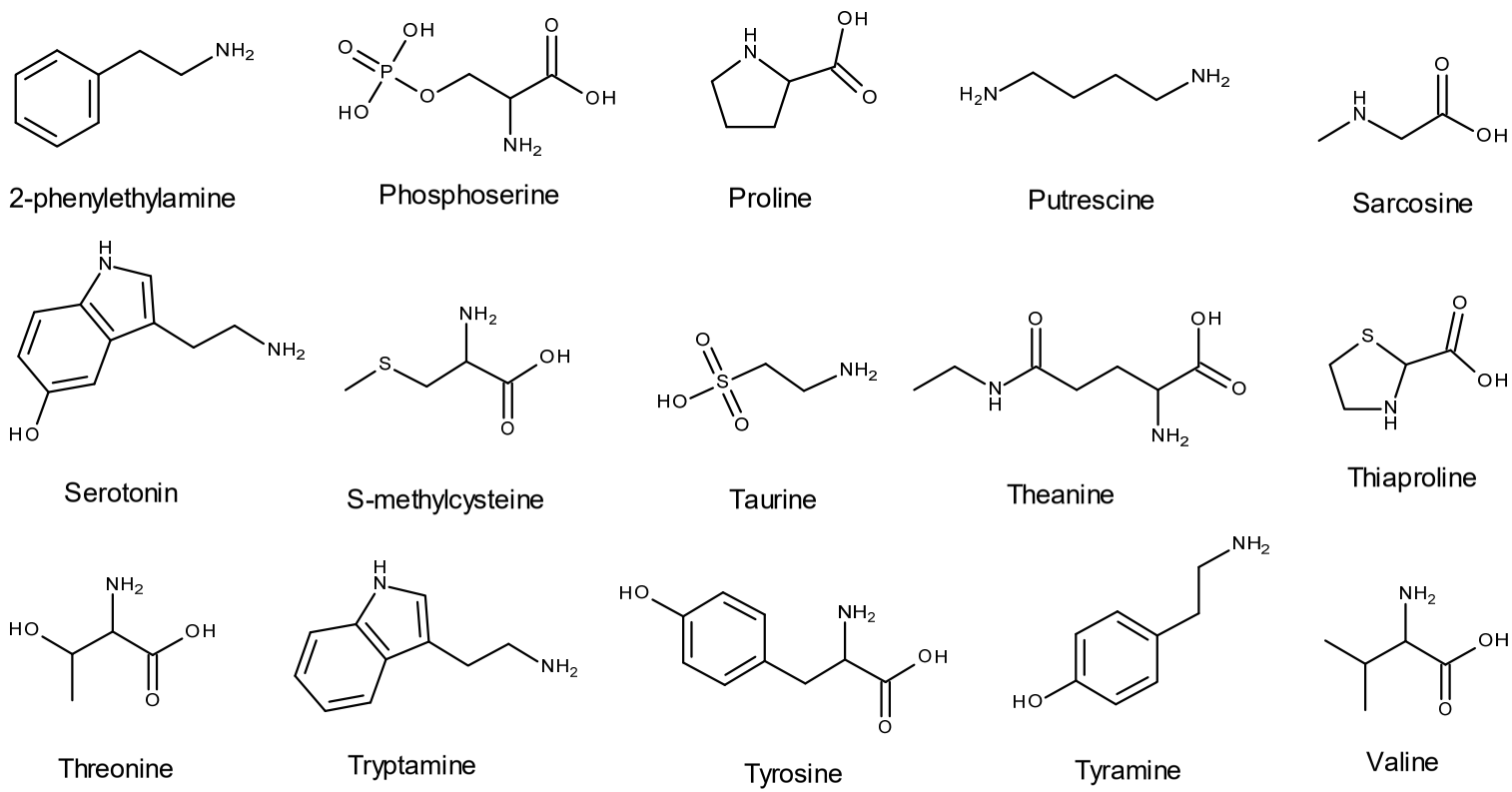


Figure 31. Chemical structure of 54 amino compounds tested for derivatization reagents.

Another important factor is the reactivity between the derivatization reagent towards alcohols and phenols since they are also nucleophiles depending on the reaction conditions. Therefore, 10 alcohols or phenolic compounds (Figure 32) were tested.

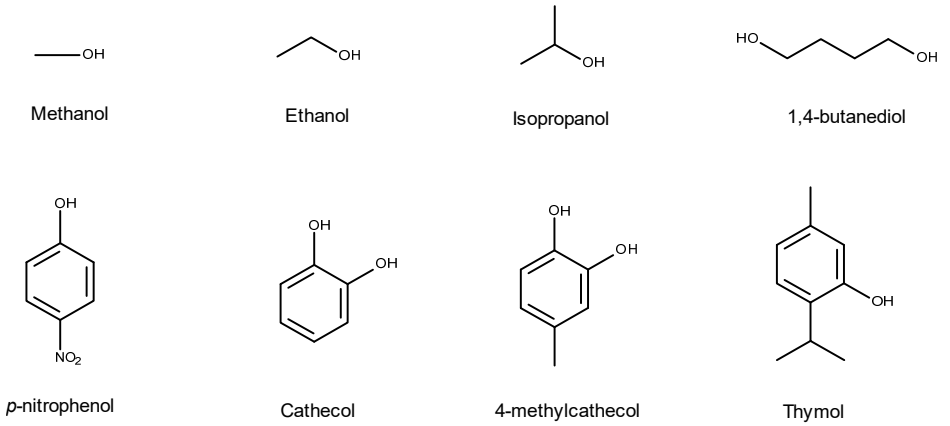


Figure 32. Alcohols and phenolic compounds tested with the derivatization reagents in this work.

The derivatization reagents studied were divided into groups of isothiocyanates, charged and fluorescent reagents. Reagents other than DEEMM are sought because DEEMM has a slow reaction rate with secondary amines and due to the pursuit of possible lower quantification limits in NLS, PSI and MRM modes. None of the reagents used in this part of the work have been employed in derivatization-targeted analysis before and their reaction and LC-MS parameters were not optimized.

4.5.1. Isothiocyanate derivatization reagents

A family of isothiocyanate reagents (Figure 33) was explored based on the fact that PITC and NITC have been widely employed in the derivatization of AAs. Besides, the derivatives of 4-SPITC (Figure 33a) showed the neutral loss of H_2S (34 Da) in negative mode, which sparked an interest in other reagents from the same family. 4-SPITC has been employed in bottom-up proteomics to enhance structural elucidation (J. Franck et al., 2009; Julien Franck et al., 2010).

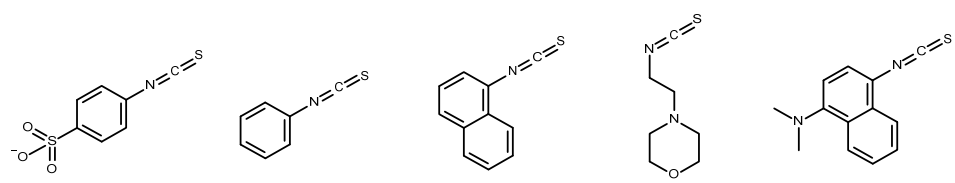


Figure 33. Isothiocyanate derivatization reagents tested in this work (a) 4-SPITC, (b) PITC, (c) NITC, (d) MORPHO and (e) 4-AMITIC.

The loss of 34 Da, corresponding to the loss of H_2S, was not observed for derivatives of secondary amines, such as proline and diethylamine since the hydrogen bonded to the nitrogen participates in the release of this fragment (Figure 34). The loss of 173 Da (Figure 35), corresponding to the loss of 4-aminobenzenesulfonic acid, in both negative and positive modes, was observed for disubstituted amino compounds, such as putrescine and lysine. The presence of *m/z* 216, $[M+H]^+$ in positive mode, and *m/z* 214 $[M]^-$ in negative mode, corresponding to the derivatization reagent, were observed. The *m/z* of the derivatives' dimer was also detected which indicates the presence of hydrogen bonding in the sulfur atom.

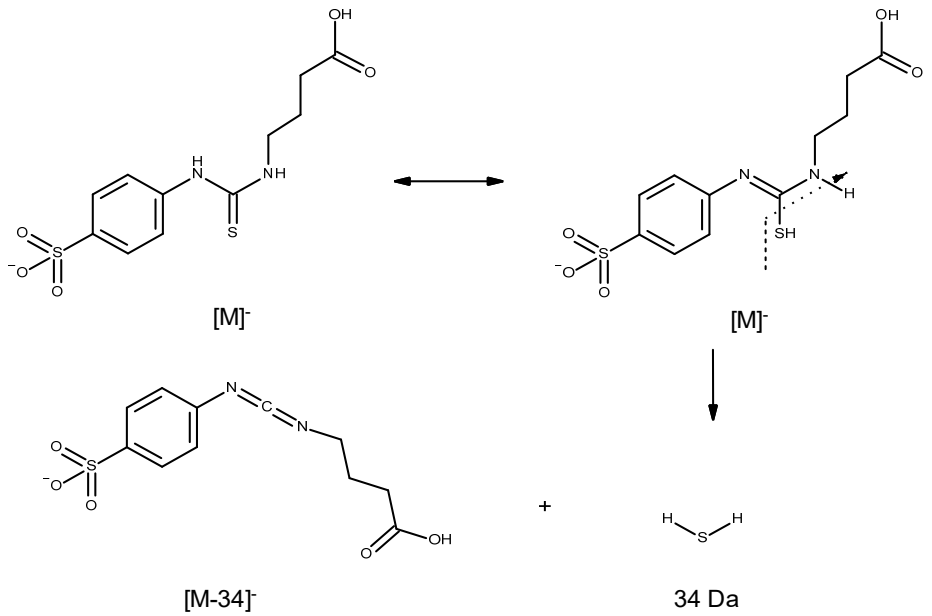


Figure 34. Fragmentation pathway of GABA-4-SPITC derivative with the release of a hydrogen sulfide molecule in negative mode.

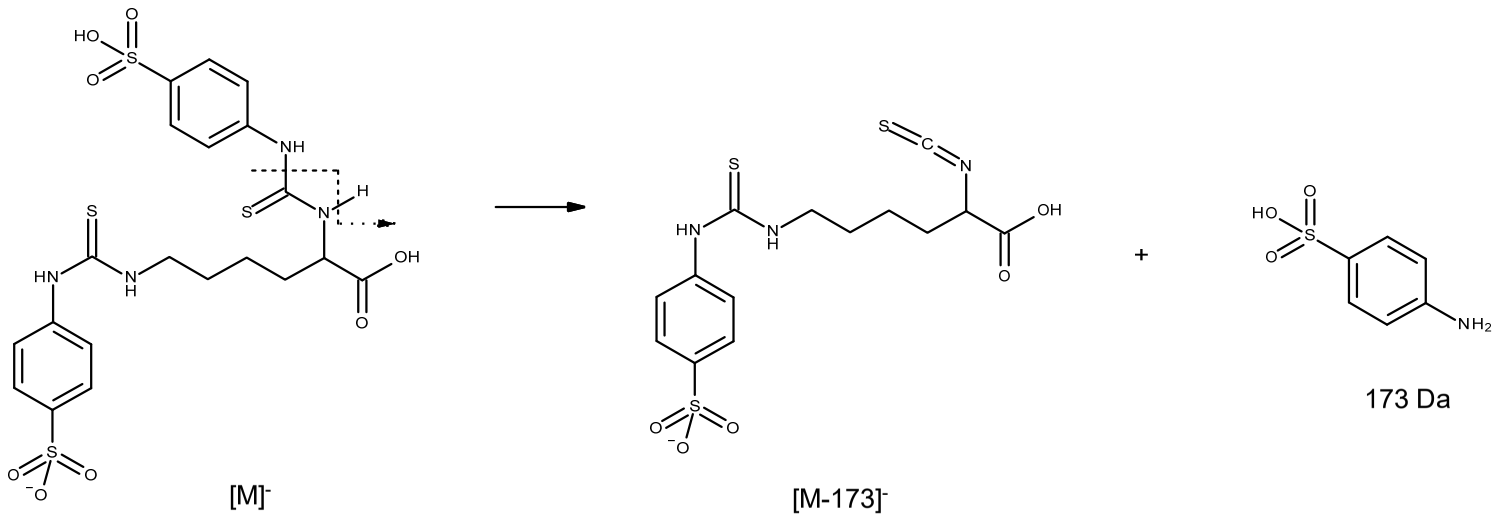


Figure 35. Fragmentation pathway of disubstituted lysine-SPITC derivative with the release of a 4-aminobenzenesulfonic acid molecule in negative mode.

The chromatogram of 4-SPITC derivatives in NLS negative mode is presented in Figure 36 for a solution of a few BAs and another of AAs. The derivatives of methylamine, ethylamine, isopentylamine and phenylethylamine were detected due to the presence of a permanent negative charge in the derivatization tag, unlike the case of putrescine and diethylamine, which was expected based on what was discussed above.

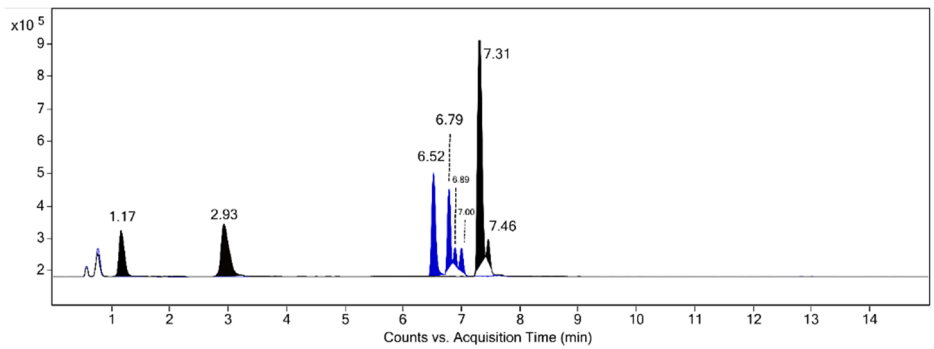


Figure 36. Chromatogram in NLS mode (-34 Da), in negative mode, of 4-SPITC derivatives of methylamine (RT = 1.17 min), ethylamine (RT = 2.93 min), tyramine (RT = 6.52 min), leucine (RT = 6.79 min), phenylalanine (RT = 6.89 min), tryptophan (RT = 7.00 min), isopentylamine (RT = 7.31 min) and phenylethylamine (RT = 7.46 min), *m/z* range 100 – 500. Fragmentor voltage 90 V and collision energy 15 V.

PITC, NITC and 4-AMITIC showed a neutral loss of 34 Da in negative mode as well, but similarly to 4-SPITC, secondary amines did not. Additionally, simple aliphatic amines (*e.g.* methylamine, isobutylamine) do not present this fragmentation pattern either since these compounds do not have a moiety that ionizes in negative mode, such as the group $-SO_3^-$ present in the reagent 4-SPITC. Similarly to 4-SPITC, the loss of aniline (-93 Da) and 1-naphthylamine (-143 Da) in positive mode is observed for disubstituted compounds, in the case of PITC and NITC, respectively. Moreover, the presence of *m/z* 94 (+), 92 (-) and 144 (+), 142 (-) for both PITC and NITC was detected, respectively. The same was observed for 4-AMITIC, *m/z* 229 in positive mode together with *m/z* 214 and 204. The neutral loss corresponding to the loss of the derivatization reagent was also detected.

MORPHO did not show good retention time values, since the derivatized amino compounds were not detected under the chromatographic conditions used (0.1 % aqueous formic acid and acetonitrile as the mobile phases). Although the prices of the reagents are affordable, they were not further explored due to an inconsistent fragmentation pattern and/or poor sensitivity in the modes used. Besides, 4-SPITC is only available as a technical grade reagent.

4.5.2. Charged reagents

Two charged derivatization reagents, TMPP and pyry, were studied due to the lack of studies with them and the fact that charged reagents can provide high ionization efficiencies because of the presence of the permanent positive charge.

In the case of TMPP, there is evidence for its reaction with hydroxyl groups, which can be present in some amino compounds, *e.g.* tyramine, otherwise, the expected phenolic-TMPP derivatives were not detected. Moreover, a product with an additional +14 Da is present as well, which is more prominent for AAs. A disubstituted derivative of cysteine was also detected without the addition of a reducing reagent, giving evidence that TMPP is also reactive towards the thiol group.

The MS characteristics of the derivatives consist of the presence of $[M]^+$, $[M]^{2+}$ and even $[M+H]^{2+}$ in some cases. The TMPP-derivatives are bulky molecules, so no fragments are detected up to collision cell energy of 30 V, but at 30 – 40 V, the loss of 46 Da is detected, correspondent to the loss of dimethyl ether and to the formation of a six-membered ring among the phosphorus, oxygen and two benzene rings (Figure 37) (Breci, 2001). A loss of 46 was not observed for the charged derivatization reagent SPTPP (Inagaki et al., 2010), another phosphonium-based reagent, which corroborates with the fragmentation pathway presented in Figure 37. The fragments *m/z* 181, 151, 136 and121 are also present.

The same fragmentation pattern is observed for the "+14 product", contrary to the TMPP derivatives of agmatine, arginine, hypotaurine and theanine. For disubstituted TMPP derivatives, such as lysine and putrescine, the loss of 23 Da is observed.

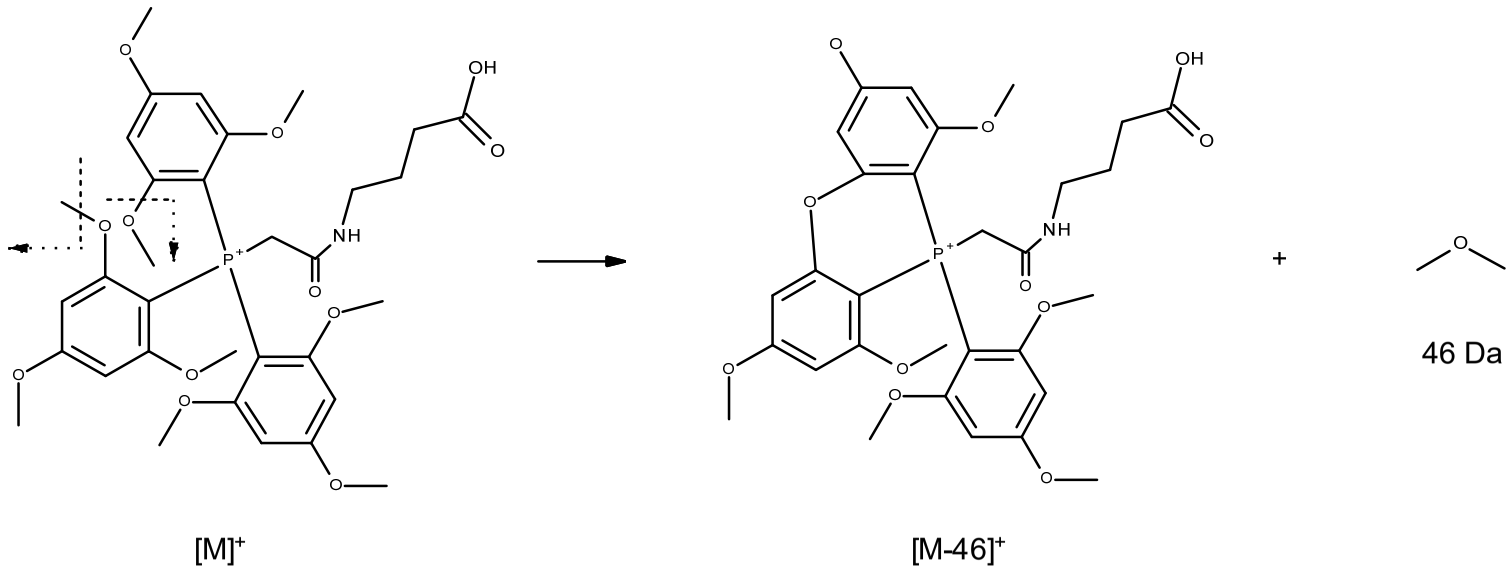


Figure 37. Fragmentation pathway of GABA-TMPP derivative with the release of a dimethyl ether molecule.

The NLS chromatogram of a standard solution of taurine is presented in Figure 38 in an *m/z* range of 610 – 1000, since the peak of the hydrolysis product (*m/z* 591) and the "+14 product" of the hydrolysis product (*m/z* 605) are prominent. In that way, these peaks do not appear in the chromatogram, which makes it cleaner, but then it is not possible to detect the TMPP-methylamine derivative (*m/z* 604) and the $[M]^{2+}$ ion of some derivatives.

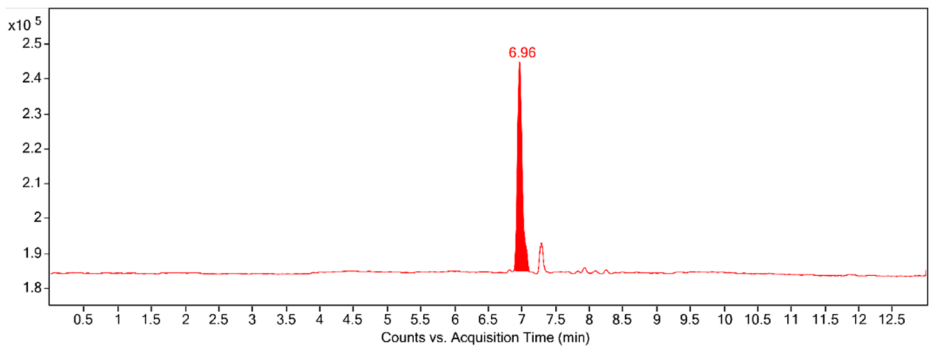


Figure 38. Chromatogram in NLS mode (-46 Da) of a taurine standard solution (RT = 6.96 min), *m/z* range 610 – 1000. Fragmentor voltage 90 V and collision energy 40 V.

The employment of NLS (-46 Da) and/or PIS (*m/z* 181) is a promising approach to detect amino compounds, however, there is a lack of understanding of the formation of the +14 product and consequently, figuring out a way to cease it. Plus, disubstituted products of amino-containing compounds with hydroxyl group in their structure will be also detected by this approach.

The pyrylium-based derivatization reagent reacts with neither secondary amines nor phenolic compounds which was predicted by the reaction pathway. Some publications suggest a water-free environment for the reaction to take place (Bąchor et al., 2020; Katritzky et al., 1980; Y. Li et al., 2021; Waliczek et al., 2016), but when purely acetonitrile conditions were tested, the peak area of pyry-tryptamine derivative was lower when compared to peak area of the reaction which took place in an aqueous solution. The reagent's reactivity towards AAs is lower compared to biogenic amines', for instance, a high temperature (ca. 60 °C) was needed for the reaction of the reagent with valine and isoleucine.

The MS behaviour of pyry-derivatives is based on the formation of a tri-phenylpyridinium ion (*m/z* 308) and the release of a neutral 1,2-alkene compound (Figure 39) at a range of collision energy 0 – 20 V, depending on the amino compound. Unlike other derivatization reagents shown in this work, the fragmentation of pyry-derivatives is limited to the prominent presence of the aforementioned fragment and the fragments *m/z* 203 and 230.

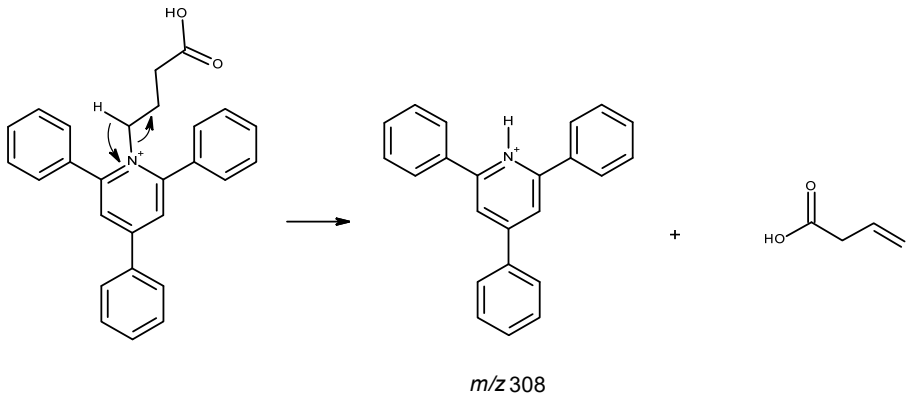


Figure 39. Fragmentation pathway of GABA-pyry derivative leading to the formation of a triphenylpyridinium ion and 3-butenoic acid.

For aromatic amines and methylamine, the *m/z* pattern observed is 306/308/304 (in the order of decreasing intensity) (Figure 40), because the usual fragmentation pathway presented in Figure 39 is not possible.

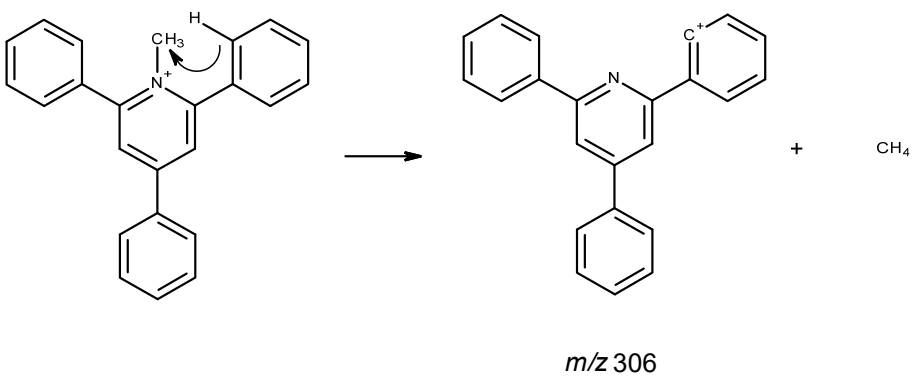


Figure 40. Fragmentation pathway of methylamine-pyry derivative.

The *m/z* of the derivatization reagent (*m/z* 309) is different from the common fragment among the amino compounds (*m/z* 308), so the peak of excess of the reagent does not interfere in the chromatogram when run in PIS scan mode at *m/z* range 310 – 700 (Figure 41). Therefore, primary amino compounds can be detected as pyry-derivatives by employing PIS scan mode set at *m/z* 308, but methylamine and aromatic amines might not be easily detectable due to their distinct fragmentation pattern, so in that case, PIS mode run at *m/z* 306 is necessary too. If the aim is to detect secondary amino compounds as well, then this approach is not applicable.

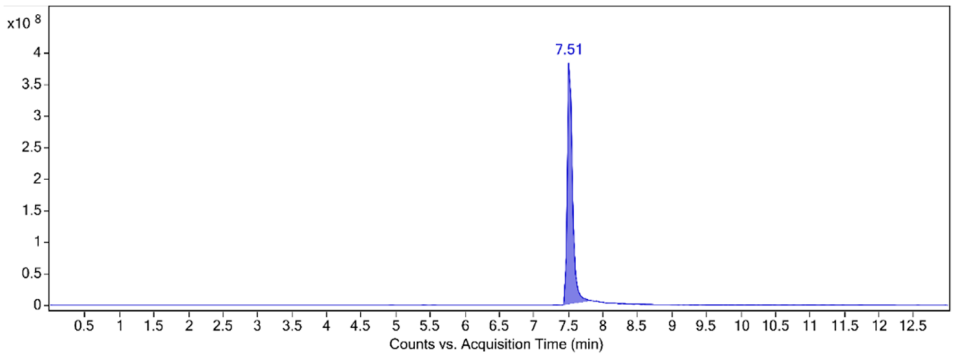


Figure 41. Chromatogram in PIS mode (*m/z* 308) of an isopentylamine standard solution (RT = 7.51 min), *m/z* range 310 – 700. Fragmentor voltage 90 V and collision energy 10 V.

4.5.3. Fluorescent reagents

As mentioned previously, the fluorescent reagent MDPF has not been widely explored in MS analysis while there are few works with fluorescamine (Junnotula et al., 2020; Junnotula & Licea-Perez, 2013). Neither of the reagents reacts with alcohols and phenolic compounds in the tested conditions. The interesting feature of the MDPF reagent is the different products originated from primary and secondary amines, so a distinct fragmentation pattern was expected for both types of amines. The reagent is also readily hydrolyzed, given the presence of a peak with *m/z* 253.

MDPF derivatives under ESI are protonated on the oxygen atom of the hydroxyl group, which is readily released as a neutral compound, H_2O (-18 Da), upon fragmentation in CID (Figure 42) and the presence of the fragment *m/z* 105 at lower collision energy values between 0 – 10 V. The recurrent loss of two H_2O molecules (-36 Da) was observed for some compounds, *e.g.* GABA and asparagine, but there is no pattern in the type of amino compounds for which this loss occurred.

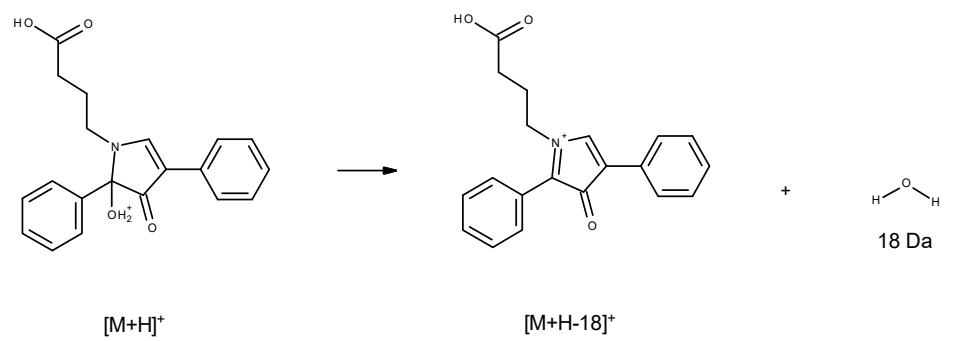


Figure 42. Fragmentation pathway of GABA-MDPF derivative leading to the loss of 18 Da (H_2O).

All the MDPF derivatives of amines can be detected in PIS mode (*m/z* 105) and primary amines can be detected in NLS mode as shown in Figure 43 for the derivative of 3-methoxytyramine together with the presence of two peaks with *m/z* 253, the hydrolysis product as mentioned previously. Secondary amines can be detected by their derivatives' presence in PIS mode and their lack of in NLS mode.

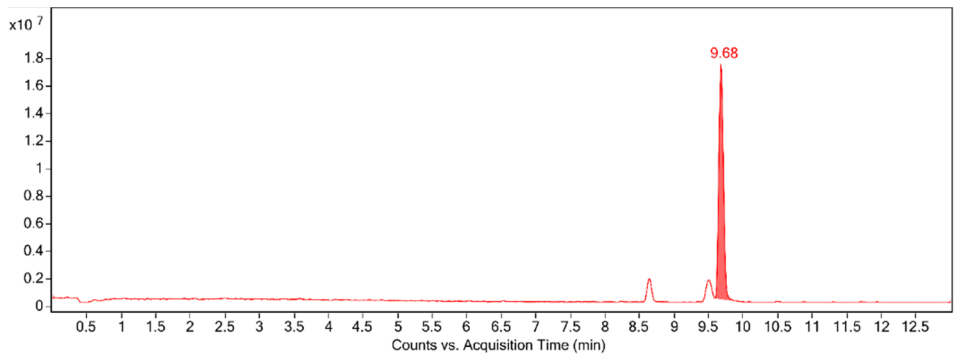


Figure 43. Chromatogram in NLS mode (-18 Da) of a 3-methoxytyramine standard solution (RT = 9.68 min), *m/z* range 100 – 700. Fragmentor voltage 90 V and collision energy 5 V.

The loss of 28 Da (CO) was noticed for secondary amines (Figure 44) and BAs, such as tryptamine and serotonin. The presence of *m/z* 77 ($C_6H_5^+$) was also detected but it is not a good diagnose fragment for MDPF derivatives, since it is a common fragment for aromatic compounds in general.

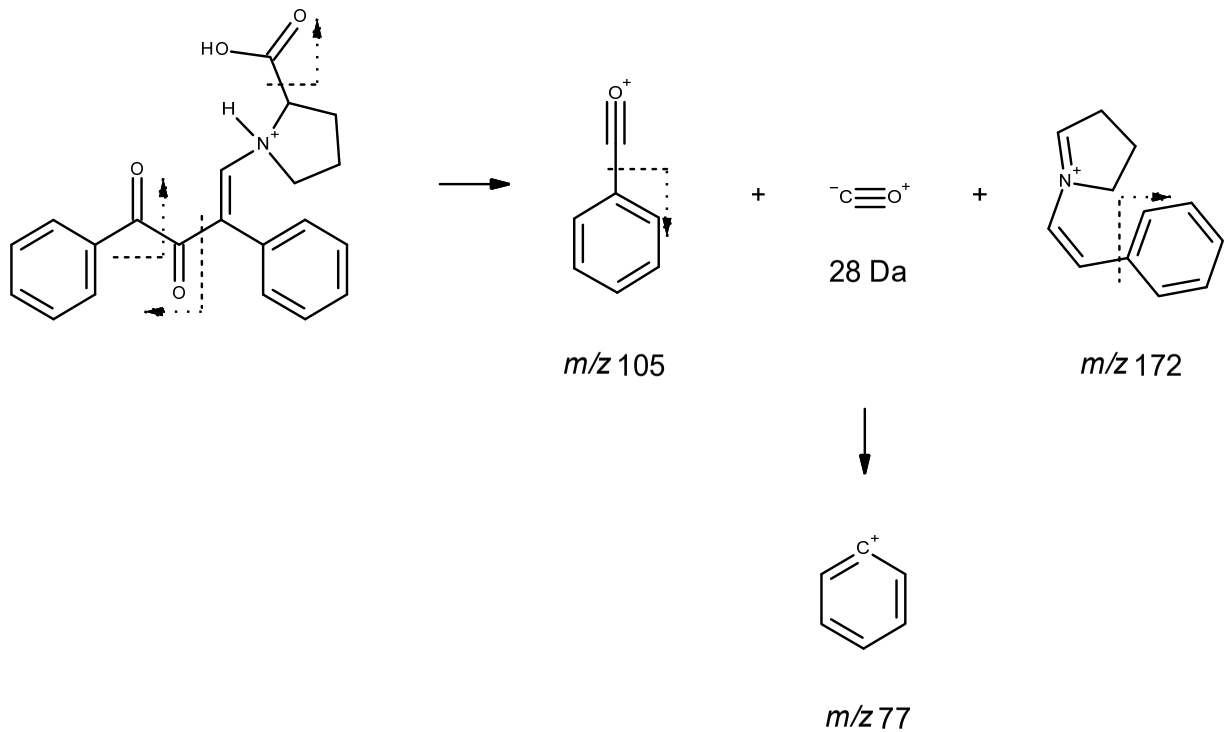


Figure 44. Fragmentation pathway of proline-MDPF derivative leading to the loss of 28 Da (CO), the presence of *m/z* 105, 172 and 77.

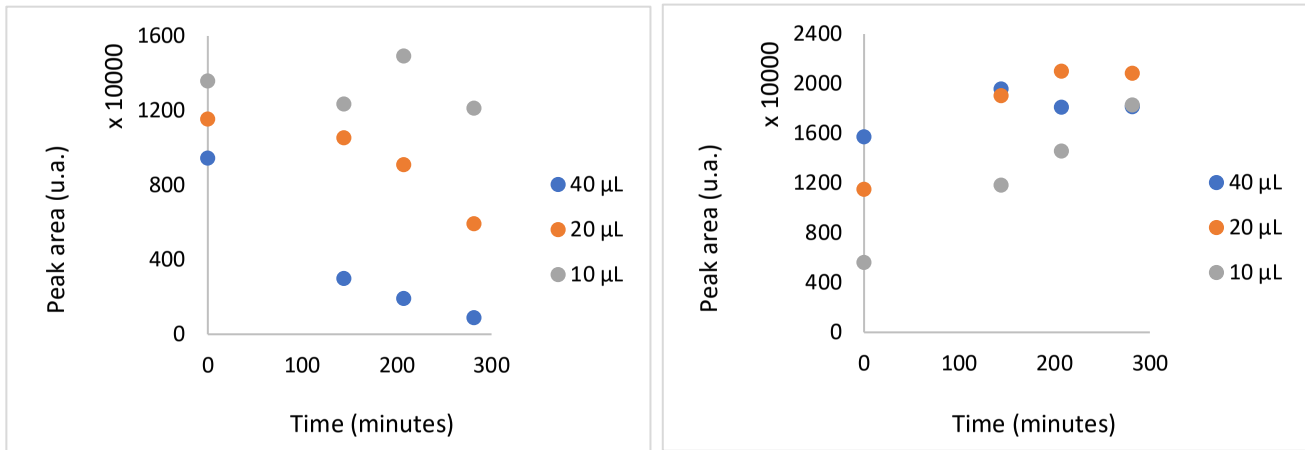
In the case of fluorescamine, two products were expected, one derived from the dehydration of the initial product. The dehydration reaction is affected by the acidity of the reaction medium, hence different volumes of 6 M HCl were added to the reaction solution of tyramine-fluorescamine after 10 minutes and the peak area of both products was recorded (Figure 45). The dehydrated product is favored over time with the least amount of acid (Figure 45a), considering the stable peak area across the four-time points. On the other hand, the dehydrated product is degraded over time when 20 and 40 µL of acid is added with approximately a decrease of 90% in the peak area for the latter. Regarding the derivative as it is (Figure 45b), the profile is stable over time when 40 µL of acid is present, while for 10 and 20 µL, its peak area increases over time. Therefore, a more acidic environment for the reaction is a good start to understanding how to favor one of the products, but considering these preliminary results, a more acidic condition favors the degradation of the dehydrated product while the derivative remains intact.

Fluorescamine derivatives have the same fragmentation pattern as MDPF's, except for the derivatives of secondary amines, which were not studied, since fluorescamine does not react with secondary amines at the tested conditions. The fragmentation pattern of the dehydrated product was also investigated for a few amino compounds: cadaverine, putrescine, theanine, isopentylamine, kynurenine and cysteine and the loss of 18 Da also takes place.

In a nutshell, fluorescamine can be employed for the detection of primary amino compounds by using NLS mode (-18 Da). MDPF can be employed for the detection of primary amino compounds by using NLS mode (-18 Da) too and for the detection of primary and secondary amino compounds using PIS scan mode (*m/z* 105).

A summary of all the derivatization reagents discussed is shown in Table 17, in terms of reactivity towards alcohols and phenolic compounds, fragmentation pattern, possible scan modes and general reaction comments. Overall, the most promising reagents, *i.e.* with fewer issues, are MDPF and triphenylpyrylium.

Now that the reaction features and the fragmentation pattern have been carefully studied, other parameters should also be evaluated, such as reaction yield, ionization efficiency, LoD, LoQ, ME, and LC-MS/MS parameters optimization. In any case, DEEMM showed to have a good performance in many aspects, such as good reactivity towards a large number of amino compounds and good LoD and LoQ in NLS and MRM modes.



(a) (b)

Figure 45. Peak areas profile in different time points for (a) the dehydrated product of tyramine-fluorescamine derivative and (b) the tyramine-fluorescamine derivative with the addition of different volumes of 6 M HCl.

Table 17. Summary of the tested derivatization reagents for derivatization-targeted analysis.

Derivatization reagent	Reaction with alcohols and phenols	Fragmentation pattern	Scan mode	Comments
DEEMM	When in excess	- 46 Da	Neutral loss	Reacts with solvent in excess
MDPF	No	- 18 Da and *m/z* 105	Neutral loss and precursor ion	-
Fluorescamine	No	- 18 Da and *m/z* 105	Neutral loss and precursor ion	Presence of the dehydrated product
Triphenylpyrylium	No	*m/z* 308	Precursor ion	Lower sensitivity towards AAs
TMPP	Yes	- 46 Da and *m/z* 181	Neutral loss and precursor ion	The presence of a +14 product and reacts with phenolic group

5. SUMMARY

The approach named derivatization-targeted analysis is introduced, which makes use of the common MS/MS fragmentation pattern of derivatized amines and amino acids. It employs precursor ion scanning (PIS) mode, when a common charged fragment is present, or neutral loss scanning (NLS) mode, when the loss of a neutral fragment takes place. In that way, the approach targets all compounds with a specific functional group. This allows to narrow down the number of possible compounds detected in a sample, thus reducing the problem of recording excessive number of compounds as in the case of non-targeted analysis.

The combined approach of DEEMM derivatization and NLS mode was applied to 11 plant species for the detection of amino compounds. In the first part of the work, the derivatization reaction was optimized in terms of the reaction conditions and application of a quenching reagent. This allowed to reduce the number of chromatographic peaks due to the derivatization reaction by-products. As a result, more peaks could be detected in NLS mode without interference.

The developed approach was applied to 42 plant samples of 5 plant species, mostly grown in the Mediterranean territory. Hierarchical clustering, based on the semi-quantification of the amino compounds by NLS mode, showed that their content can be used to separate the plants based on their genera, *Ptilostemon* and *Carduus*, mainly due to the presence of a higher content of amino compounds in the latter. In addition, the method was applied to 5 agricultural by-products to evaluate their use as a feed supplement regarding their free amino compound content. In total, 50 amino compounds were detected and identified and out of these, 33 were quantified by NLS mode and 46 were quantified by MRM mode.

Derivatization reagents belonging to the groups isothiocyanate, charged or fluorescent reagents, were studied for their use in derivatization-targeted analysis with respect to their fragmentation pattern. The group of isothiocyanate reagents showed a fragmentation pathway with the loss of 34 Da (H_2S) in negative ionization mode. The charged reagents presented, as their main fragmentation pathway, the loss of 46 Da (C_2H_6O) and the presence of the fragment *m/z* 308 for TMPP and pyrylium ion, respectively. Both fluorescent reagents have the same fragmentation pattern, the loss of 18 Da (H_2O) and the common fragment *m/z* 105. A more in-depth investigation of the reagents in terms of their fragmentation pattern, matrix effects, LoD and LoQ should be carried out.

The derivatization-targeted approach was developed, for the first time, based on DEEMM-derivatization of amino compounds followed by the analysis of the derivatives in NLS mode. The method was successfully applied to several plant samples, proving useful in detecting amino compounds in a significantly higher number than anticipated. Apart from DEEMM, other derivatization reagents were examined, and some demonstrated promising results in terms of their derivatives' fragmentation patterns. The future perspectives include streamlining the proposed approach by automatizing the derivatization and data analysis stages for more efficient elucidation of detected compounds. In general, the derivatization-targeted approach can be further extended to any functional group which can be derivatized.

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SUMMARY IN ESTONIAN

Aminorühma sisaldavate ühendite derivatiseerimine-suunatud LC-MS analüüs

Töötati välja derivatiseeritud amiinide ja aminohapete sarnasel MS/MS fragmentatsioonimustril baseeruv analüüsimeetod, mis nimetati derivatiseerimine-sihitud analüüsiks. Meetod kasutab eellasioonide skaneerimise (PIS) režiimi, kui fragmentatsioonil tekib ühine laetud fragment, ja neutraali kao skaneerimise (NLS) režiimi, kui ühine fragment on laenguta. Seega võimaldab see meetod tuvastada kõiki ühendeid, millel on konkreetne funktsionaalne rühm. See võimaldab vähendada keerulistes proovides tuvastatavate ühendite arvu võrreldes tavapärase avastusanalüüsiga (*non-targeted analysis*).

DEEMM derivatiseerimise ja NLS režiimi kombineeritud lähenemisviisi kasutati aminoühendite tuvastamiseks 11 taimeliigi proovides. Töö esimeses osas optimeeriti derivatiseerimisreaktsiooni tingimused ja reaktsiooni lõpetava reagendi kasutamine. See võimaldas vähendada derivatiseerimisreaktsiooni kõrvalproduktidest tingitud kromatograafiliste piikide arvu, mis lubas NLS režiimis tuvastada rohkem tippe ilma segavate mõjudeta.

Arendatud meetodit rakendati 42 taimeproovile viiest taimeliigist, mis kasvavad enamasti Vahemere piirkonnas. NLS režiimis teostatud poolkvantitatiivsetele analüüsitulemustele rakendati hierarhilist klasteranalüüsi, mis näitas, et aminoühendite sisaldust saab kasutada taimede klassifitseerimiseks perekondadesse, *Ptilostemon* ja *Carduus*, peamiselt tänu viimase kõrgemale aminoühendite sisaldusele. Lisaks rakendati meetodit viiele põllumajandusliku tootmise kõrvalsaadusele, et hinnata nende kasutatavust söödalisandina vabade aminoühendite sisalduse seisukohalt. Kokku tuvastati ja identifitseeriti 50 aminoühendit, millest 33 kvantifitseeriti NLS režiimis ja 46 kvantifitseeriti MRM režiimis.

Uuriti ka teiste derivatiseerivate reagentide sobivust kasutamiseks derivatiseerimine-sihitud analüüsil. Need reagendid grupeeriti kui isotiotsüanaadid, püsiva laenguga ja fluorestseeruvad reagendid ning uuriti vastavate derivaatide fragmentatsioonimustreid. Isotiotsüanaatide rühma kuuluvate reagentide fragmenteerumise ühiseks jooneks oli neutraalse 34 Da fragmendi (H_2S) kadu negatiivses ionisatsiooni režiimis. Laetud reagentidest esines peamise fragmentatsiooni rajana TMPP puhul 46 Da (C_2H_6O) kadu ja pürüüliumiooni puhul laetud *m/z* 308 fragmendi olemasolu. Mõlemal fluorestseeruval reagendil on sama fragmentatsioonimuster, 18 Da kadu (H_2O) ja ühine laetud fragment *m/z* 105. Derivatiseeritud ühendite fragmentatsioon, maatriksefektid, LoD ja LoQ vajavad veel põhjalikumat uurimist.

Töötati välja derivatiseerimine-sihitud analüüsi metoodika, mis põhineb aminoühendite DEEMM-derivatiseerimisel ja järgneval derivaatide analüüsil NLS režiimis. Meetodit rakendati edukalt mitmete taimproovide ekstraktide analüüsil ja see võimaldas tuvastada oluliselt rohkem aminoühendeid, kui oleks olnud võimalik sihitud analüüsi (*targeted analysis*) korral. Lisaks DEEMM-ile uuriti ka teisi derivatiseerimisreagente ning fragmentatsioonimustrite põhjal

võiks mõned neist samuti sobida derivatiseerimine-sihitud analüüsiks. Tulevikus tuleks rõhku panna derivatiseerimise ja andmeanalüüsi etappide automatiseerimisele. Üldiselt on derivatiseerimine-sihitud analüüsi põhimõte kasutatav ka mis tahes funktsionaalsete rühmadega analüütide tuvastamiseks, kui on olemas sobiv derivatiseeriv reagent.

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PUBLICATIONS

CURRICULUM VITAE

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2020–…	PhD program in Analytical Chemistry, University of Tartu – Tartu, Estonia
2018–2020	Master's program in Excellence in Analytical Chemistry, Master of Science University of Tartu – Tartu, Estonia, Uppsala University – Uppsala, Sweden
2012–2017	Bachelor's program in chemistry, Bachelor of Chemistry Federal University of Itajubá, Itajubá, Brazil

Professional employment

Apr/18–Jun/18	Temporary lecturer on general chemistry Federal University of Itajubá, Itajubá, Brazil
Jan/17–May/17	Volunteer research trainee in the biorefining chemistry team Technical Research Centre of Finland Ltd (VTT), Espoo, Finland
Jan/16–Jul/16	Intern at the structural characterization laboratory Solvay Group, Paulinia, Brazil

Scientific publications

1. Malenica, D., **Maciel, L. S.**, Herodes, K., Kass, M., & Bhat, R. (2024). Optimization of Ultrasonic-Assisted Extraction of Antioxidants in Apple Pomace (var. Belorusskoje malinovoje) Using Response Surface Methodology: Scope and Opportunity to Develop as a Potential Feed Supplement or Feed Ingredient. *Sustainability*, *16*(7), 2765. https://doi.org/10.3390/su16072765
2. **Maciel, L. S.**, Malenica, D., Herodes, K., Kass, M., & Bhat, R. (2024). Detection and quantification of free amino compounds in selected agricultural by-products with derivatization followed by liquid chromatography-tandem mass spectrometry analysis. *Food Chemistry Advances*, *4*(January), 100629. https://doi.org/10.1016/j.focha.2024.100629
3. Ben-Othman, S., Bleive, U., Kaldmäe, H., Aluvee, A., Rätsep, R., Karp, K., **Maciel, L. S.**, Herodes, K., & Rinken, T. (2023). Phytochemical characterization of oil and protein fractions isolated from Japanese quince (Chaenomeles japonica) wine by-product. *Lwt*, *178*(December 2022). https://doi.org/10.1016/j.lwt.2023.114632
4. Marengo, A., **Maciel, L. S.**, Cagliero, C., Rubiolo, P., & Herodes, K. (2023). Free Amino Acids and Biogenic Amines Profiling and Variation in Wild and

Sub-Endemic Cardueae Species from Sardinia and Corse. *Plants*, *12*(2). https://doi.org/10.3390/plants12020319
5. **Maciel, L. S.**, Marengo, A., Rubiolo, P., Leito, I., & Herodes, K. (2021). Derivatization-targeted analysis of amino compounds in plant extracts in neutral loss acquisition mode by liquid chromatography-tandem mass spectrometry. *Journal of Chromatography A*, *1656*, 462555. https://doi.org/10.1016/j.chroma.2021.462555
6. Goulart, B. V., Nascimento, W. N. dos S., Pinto, A. C., Fagundes, P. M. L. L., **Maciel, L. S.**, Alvarenga, M. I. N., & Kondo, M. M. (2020). Sorption and mobility of thiabendazole in a Brazilian Oxisol. *Ciência e Natura*, *42*, e44. https://doi.org/10.5902/2179460X42330

Attended conferences

17–22.09.23	6th International Mass Spectrometry School. Cagliari, Italy. Poster presentation.
10–15.12.22	3rd Iberoamerican Conference on Mass Spectrometry. Rio de Janeiro, Brazil. Poster presentation.
13–15.09.22	1st summer School on automation and miniaturization in sample preparation provided by Charles University. Hradec Králové, Czechia.
27.08–02.09.22	24th International Mass Spectrometry Conference. Maastricht, the Netherlands. Poster presentation.

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2012–2017	Keemia bakalaureuseõppekava, keemia bakalaureusekraad Itajubá föderaalne ülikool, Itajubá, Brasiilia

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Jan/16 – Jul/16	Struktuuri iseloomustamise laboris praktikant Solvay Group, Paulinia, Brasiilia

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