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Abstract. Amino compounds, such as amino acids and biogenic amines, are important metabolites that can be found in diverse natural matrices. The most common method for amino compound analysis nowadays is reversed-phase liquid chromatography tandem mass spectrometry (RPLC-MS/MS). However, due to the polar and the basic nature of amines, their RPLC retention is often insufficient or peaks are tailing. Derivatization is a way to overcome the issue and in the present work amino compounds are derivatized with diethyl ethoxymethylenemalonate (DEEMM) and analyzed by a RPLC triple quadrupole MS system in neutral loss scan (NLS) mode (loss of 46). This allows to target all compounds in the sample that undergo derivatization with DEEMM, so that the amino

compound profile of the sample is obtained. To the best of our knowledge, the NLS acquisition mode has never been employed to target amino compounds after DEEMM derivatization. In the first part of the study, eight amino acids (arginine, aspartic acid, threonine, proline, tyrosine, tryptophan, phenylalanine and isoleucine) were employed as model compounds for method optimization, with good results in terms of DEEMM derivatives detection and repeatability. The developed method was successfully applied to a complex extract from the plant species *Carduus nutans* subsp. *macrocephalus* (Desf.) Nyman, with 18 amino acids and 3 other amines being putatively identified. The proposed approach could be employed for straightforward identification of known and unknown amino compounds in different types of matrices.

Key words: amino compounds, pre-column derivatization, diethyl ethoxymethylenemalonate, neutral loss scan, *Carduus* spp.

1. Introduction

Amino compounds, such as amino acids and biogenic amines, are present in diverse natural matrices. Not only amino acids are the building blocks of proteins, but they can also exert important functions in the organisms' metabolism. On the other hand, biogenic amines, such as tyramine and histamine, are degradation products from the decarboxylation of amino acids and amination of aldehydes and ketones [1–3]. Therefore, many areas are interested in amino compounds analysis, such as clinical analysis, for the detection and treatment of diseases, dietary studies, food quality control and plants for different purposes [4,5]. Concerning plant metabolites, amino compounds are important for the vegetal organism itself and for humans as well. For instance, the analysis of these compounds can be useful in the evaluation of growth and development of plants and as discrimination markers for samples from different geographical sites [5,6]. Concerning edible vegetables, the analysis of both essential and non-essential amino acids is important to control proper protein levels in the diet, at the same time free amino acids can be exploited for their specific biological activities as therapeutic agents (*e.g*. L-theanine from tea) [4,5]. Free biogenic amines are endogenous compounds in plants (fruits and vegetables), being the precursor of some aroma compounds, while high amounts of these molecules can serve as markers for spoiled food [7,8].

Methods for amino compound analysis include liquid chromatography (LC), gas chromatography (GC) and capillary electrophoresis (CE), coupled to different detectors (*e.g*. mass spectrometry, spectrophotometric or fluorescence detectors), where reverse-phase liquid chromatography tandem mass spectrometry (RPLC-MS/MS) is one of the leading analytical techniques nowadays. However, amino compounds' polar and basic nature do not favor the use of the commonly employed reverse-phase (RP) separation [4,9]. When electrospray ionization (ESI) is the ionization source of choice, ionization efficiency is also affected since the analyte should present a hydrophobic region and should be able to carry a charge in the gas-phase [10]. Therefore, if the analyte does not have these qualities, as in the case of amino acids and biogenic amines, it can be modified through derivatization.

Derivatization is a chemical reaction between an analyte and a derivatization reagent, to improve the chromatographic behavior (*e.g*. increased retention times, improved separation and peak shape) and other properties, such as stability [11]. Ideally, the derivatization reagent reacts only with a specific functional group (*e.g.* amino group) and is chosen to increase the sensitivity with: ultraviolet (UV), fluorescence or mass spectrometry (MS) detection. Examples of derivatization reagents specifically employed for amino compounds are: dansyl chloride (DASC), 2,5-dioxopyrrolidin-1-yl N-tri(pyrrolidino)phosphoranylideneamino carbamate (FOSF), 9-fluorenylmethyl chloroformate (FMOC-Cl), p-N,N,N-trimethylammonioanilyl N'-hydroxysuccinimidyl carbamate iodide (TAHS) and diethyl ethoxymethylenemalonate (DEEMM) [12,13]. Since for DEEMM-derivatives UV and MS detection can be used, it has been successfully employed for the detection and quantification of amino compounds in diverse matrices, such as tea extract [12] , honey [13,14], milk [15], cheese [16], plant seeds [17], tobacco [6], saffron [18], strawberry purée [19] and biological samples [20,21]. The reaction between DEEMM and amino compounds is straightforward and robust, leading to the formation of an enamine and ethanol (Fig. 1), which is another advantage of such derivatization reagent.

The ionization and fragmentation pattern of DEEMM-derivatives in ESI MS detection is important. DEEMM-derivatives ionize via protonation $[M+H]^+$ or sodium adduct $[M+Na]^+$ formation. Collision induced dissociation (CID) of the precursor ions leads to the product ion corresponding to the loss of a neutral ethanol molecule $[M+H-46]^+$ [6,22]. This enables the use of neutral loss scan (NLS) mode of the triple quadrupole mass analyzer. In this mode, quadrupoles Q1 and Q3 scan the ions in a synchronized manner, and the result is a

mass spectrum from the precursor ion (Q1) that yielded the pre-selected neutral loss in Q2 [23].

NLS mode has been employed in the screening of different metabolites, such as, identification of prenylated dihydrostilbenes in *Glycyrrhiza uralensis* Fisch. leaves [24], detection and quantification of sulfated flavonoids in plants [25], detection of diacylglycerols by 2,4-difluorophenyl isocyanate derivatization in cells [26] and of metabolites with carboxyl group by N,N-dimethylethylenediamine derivatization in plasma of smokers and non-smokers [27]. To the best of our knowledge, there are no studies about DEEMM derivatives detected with NLS mode. Therefore, the aim of this work is the analysis of DEEMM-derivatized amino compounds in NLS mode that would enable the profiling of amino compounds in a diversity of samples.

LC-MS analyses of known amino compounds are usually performed in single reaction monitoring acquisition mode (SRM) with the monitoring of pre-defined ion transitions, while high resolution mass spectrometers are employed in untargeted analysis, to obtain an exhaustive profile including unknown compounds [4,6,28]. The NLS approach proposed in this study could be useful in the detection of both known and unknown amino compounds derivatives leading to amino compound profiling, while at the same time employing a conventional and widespread triple quadrupole mass spectrometer and having an easy and straightforward procedure.

A plant extract was selected as a real-world sample to test the feasibility of the method. *Carduus nutans* subsp. *macrocephalus* (Desf.) Nyman (Compositae) was employed as a plant model. This is a wild edible species, widely distributed in the Mediterranean countries, traditionally used for its healthy and nutritional properties. *C. nutans* subsp. *macrocephalus* phytochemical information is limited to the polyphenolic profile of its hydroalcoholic extract [29], which makes this species a good candidate for derivatization-targeted analysis of amino compounds.

2. Materials and Methods (Experimental)

2.1. Plant material

Aerial parts of the wild species *Carduus nutans* subsp*. macrocephalus* were collected from Gennargentu, Sardinia, Italy (39°57'35.77"N - 9°19'12.46"E). They were identified at the Department of Life and Environmental Sciences, University of Cagliari, Italy, where a

voucher specimen for the species was deposited (CAG-802). In total, 13 specimens of *C. nutans* subsp. *macrocephalus* were collected randomly. All individuals were separated by 1–50 m from one another. The fresh materials were dried at 40 °C until constant weight was reached.

2.2. Chemicals

HPLC-grade acetonitrile, methanol and ethanol were purchased from Merck. Amino acid standards (L-alanine, L-arginine hydrochloride, L-asparagine, L-aspartic acid, L-glutamine, L-glutamic acid, L-glycine, L-histidine, L-isoleucine, L-leucine, L-lysine, L-methionine, L-phenylalanine, L-proline, L-serine, L-threonine, L-tryptophan, L-tyrosine and L-valine, ≥99.5%), γ-aminobutyric acid (≥99%), formic acid (≥95%), diethylamine (≥99.5%), butylamine (99.5%), putrescine (≥99.5%), phenyethylamine (≥99.5%), tyramine hydrochloride (≥98%), (±)-octopamine hydrochloride (≥95%) purchased from Sigma. Hydrochloric acid (HCl) was purchased from Reakhim. DEEMM (≥99%) and L-ornithine monohydrochloride (≥99%) purchased from Fluka. Boric acid (≥99%) was purchased from Hopkin & Williams. 2-amino-2-(hydroxymethyl)propane-1,3-diol was purchased from Schuchardt. Hydroxylamine (>97.5%) was purchased from Reakhim.
0.75 M borate buffer was prepared in deionized water and pH was adjusted to 9.00 with a saturated sodium hydroxide solution.
All aqueous solutions were prepared with ultrapure water by Millipore Milli-Q Advantage A10 (Millipore).

2.3. Preparation of standard solution

Stock solutions of each of the eight amino acids (c.a. 300 mg/L) were individually prepared in 0.3 M HCl and stored at -20ºC. The final concentration of each amino acid in the standard solution was ca 10 mg/L either in 0.1 M HCl in 30% methanol, ethanol or acetonitrile.
Hydroxylamine, diethylamine, butylamine, 2-amino-2-(hydroxymethyl)propane-1,3-diol and glycine were diluted, according to their solubility, in 0.1 M HCl in 30% methanol to a final concentration of 1.5 M for hydroxylamine and 2-amino-2-(hydroxymethyl)propane-1,3-diol,

0.5 M for glycine and 10 M for diethylamine and butylamine. Hydroxylamine was prepared in 0.1 M HCl in 30% ethanol and acetonitrile as well.

2.4. Pre-column derivatization

The optimized derivatization procedure follows the indications of Rebane et al., 2010 [14] with some modifications. The optimized procedure is presented below, other tested conditions are discussed in Results and discussion section.

Derivatization was carried out 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 in 0.1 M HCl 30% methanol, 875 µl of borate buffer (0.75 M, pH 9), 7 µl of DEEMM and 30 µl of the quenching reagent (hydroxylamine, 1.5M) added after 2 h.

Reaction solution was filtered with a 4 mm diameter, 0.20 µm pore diameter hydrophilic regenerated cellulose syringe filter (Sartorius).

2.5. Plant extraction

Plant extract was prepared through a conventional extraction method for primary and specialized metabolites, with the same solvents employed for the standard solutions preparation [6,29,30]. Five mL of 0.1 M HCl in 30% methanol, ethanol or acetonitrile solution were added to 100 mg of plant material and the extraction was carried out for 20 minutes in an ultrasonic bath (Bandelin Sonorex) at room temperature. The sample was centrifuged at maximum speed (MTS MPW 340 centrifuge) for 10 minutes and the supernatant was brought to a volume of 5 mL and filtered with a 25 mm diameter, 0.20 µm pore diameter hydrophilic regenerated cellulose syringe filter Chromafil®Xtra. Thereafter, the plant extract was submitted to derivatization according to the procedure reported in paragraph 2.4. Analyses by LC-MS/MS were carried out right away, after 24 and 48 h.

2.6. LC-MS/MS analysis

LC-MS system equipped with 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 electrospray ionization source (ESI) was used.

Chromatographic analysis was performed in a Zorbax Eclipse Plus C18 (3.0 x 100 mm, 1.8 µm) column, which was maintained at 40 °C and 5 µL of the sample was injected. The mobile phase was composed of 0.1% aqueous formic acid (A) and acetonitrile (B). For derivatization method development, the following gradient was used (gradient 1): 0 – 2 min, 10% B; 2 – 17 min, 10-100% B; 17 – 19 min,100% B; 19 – 21 min, 100-10% B; the total pre-running and post-running time was 25 min. The plant extract was analyzed with the following gradient (gradient 2): 0 – 2 min, 10% B; 2 – 27 min, 10-100% B; 27 – 29 min, 100% B; 29 – 31 min, 100-10% B; total pre-running and post-running time was 35 min. Eluent flow rate was 0.5 mL/min in both cases.

The following ESI and MS parameters were used: drying gas temperature 320ºC, drying gas flow 9 L/min, nebulizer gas pressure 45 psi, sheath gas temperature 400ºC, sheath gas flow 12 L/min, capillary voltage 3000 V and nozzle voltage 0 V. Neutral loss scan mode was performed with the neutral loss of 46 in the *m/z* ranges from 50 to 500, 500 to 1000, 1000 to 1500 and 1500 to 2000, fragmentor 90 V and collision energy 8 V.

Data was processed using the Agilent MassHunter Qualitative Analysis Navigator B.08.00 software.

2.7. Statistical analysis

One-way ANOVA t-test (p-value < 0.05) was performed in Microsoft Excel 2017 Data Analysis Add-in.

3. Results and discussion

3.1. Optimization of the derivatization procedure

A UHPLC-MS method was developed for the detection of derivatized amino compounds in neutral loss scan mode, by exploiting the characteristic DEEMM fragmentation pattern. All analyses were performed in positive ionization mode, due to the lower MS intensity of DEEMM derivatives (especially biogenic amines) in negative ESI mode, and because the investigated neutral fragment originates from the positively charged compounds [6,12,31]. The NLS profile of a standard solution containing 8 amino acids, obtained following the same procedure adopted by Oldekop et al., 2014, is reported in Fig. 2, revealing the presence of enamines (Table 1) and other interfering peaks characterized by the loss of 46

as a neutral fragment. DEEMM causes one of the three main interfering peaks (RT 9.460 minutes) characterizing the profile, together with other byproducts of the reaction (*e.g*. *m/z* 188, 161, 203, 189, 285 and 217 at RT 6.7, 7.9, 8.3, 9.3, 10.72, 12.12 minutes, respectively), for example, transesterification product or enol ether product between DEEMM and methanol (*m/z* 203) and DEEMM hydrolysis product (*m/z* 161).

A method to eliminate the excess of DEEMM and its by-products was attempted. Several authors have heated the derivatized sample up to 80 °C to overcome this problem [6,32], however, since submitting the sample to high temperatures may cause faster degradation of compounds, a different approach, based on the addition of a quenching reagent, was tested in this work [14]. Hydroxylamine was selected as quenching reagent among several other amino compounds (*i.e*. diethylamine, butylamine, 2-amino-2-(hydroxymethyl)propane-1,3-diol and glycine) because of the short retention time (1.5 minutes) of its DEEMM-derivative and its high solubility in the employed solvent.

As a first step, the NLS profile of the hydroxylamine derivative alone was monitored at different conditions: methanol, ethanol and acetonitrile were tested as diluting solvents (*i.e.* 0.1 M HCl in 30% of organic solvent as reported by Oldekop et al., 2017) to detect potential differences. At the same time, both pre-diluted DEEMM in solvent [33] and undiluted DEEMM (referred to as “pure DEEMM”) [14] were employed, and analysis was carried out right away or after 24 h as suggested by Rebane et al., 2010 [6,14,18,31,33]. When DEEMM reacts with hydroxylamine the chromatographic profile becomes cleaner, in particular when pure DEEMM is added, regardless of the organic solvent employed (an example with methanol is shown in Fig. S1). Regular analysis of the samples revealed a considerable interference, between 6-8 min in the chromatogram, that is less noticeable over time *i.e.* after 160, 80, 440 min for methanol, ethanol and acetonitrile, respectively (an example is shown in Fig. S2 for methanol). Based on the obtained results, the derivatization of the 8 selected amino acids was performed in the three selected organic solvents, using pure DEEMM with and without the addition of hydroxylamine as a quenching reagent. The analysis was performed both right away and after 24 hours to detect potential differences. As mentioned before, all amino acid peaks are detectable in NLS mode, however, since sensitivity towards proline was lower, its derivatization behavior will be discussed in a dedicated section.

As expected, for all the tested solvents (an example is shown in Fig. S3 for methanol), the NLS chromatographic profile of the standard solution in which the hydroxylamine was added as a quenching reagent appears cleaner, DEEMM peak and other by-products are

no longer present (Fig. S3 A and E). Different quenching reagent addition times - right-away, 2 h and 24 h - were investigated as well. The right-away quenching reagent addition, which prevents the complete derivatization of the selected amino acids, as evidenced by lower peak areas compared to samples without hydroxylamine addition or with delayed (2 h, 24 h) quenching reagent addition, was not taken into consideration (Fig. S3 B, C and D). Since the 2 h and 24 h hydroxylamine addition gave consistent results, the 2 h procedure was selected to reduce the sample preparation time.
The LC-MS analysis was performed both right away and after 24 h from the sample preparation and, as before, 24 h analysis gave a cleaner NLS profile in comparison to the right-away analysis time (Fig.S3 C and E).

The influence of the quenching reagent to the amino acid derivatives signal intensity was also evaluated comparing the samples without or with the 2 h quenching reagent addition (Table S1). Overall, there are significant statistical differences between the two treatments, with peak areas being higher when the quenching reagent is employed. A possible reason is that the chromatographic profile is cleaner and, therefore, leads to less ionization-suppressing interference from co-eluting compounds.
These results confirm the advantages of adding a quenching reagent to the DEEMM derivatives solution after 2 h. With regards to the analysis time and its effect on peak area, there is a small decrease in sensitivity after 24 h overall (Table S2). One-way ANOVA ($p < 0.05$) showed there are statistically significant differences for aspartic acid and tyrosine (ethanol), arginine (methanol) and aspartic acid, tyrosine and isoleucine (acetonitrile). Although the results obtained with both analysis times are consistent (Table S3), analysis after 24 h was chosen for further experiments, due to the cleanliness of the chromatogram. Apart from proline that will be discussed in section 3.2, the peak areas are similar regardless of the solvent employed, with RSD% below 5%. When one-way ANOVA is employed ($p < 0.05$) for the three different solvents and pure DEEMM, there are statistically significant differences only for aspartic acid and tryptophan. The only amino acid that has a statistically highly significant difference ($p < 0.001$) in sensitivity among the solvents is aspartic acid, with a decrease in peak area in the case of acetonitrile. Since there are no large differences in the sensitivity among the solvents and methanol has been employed as a solvent for DEEMM derivatization in the literature, it was chosen for further experiments.

These findings confirm that pure DEEMM in a methanol containing solution with the addition of a quenching reagent after 2 h is the best option for NLS mode analysis within the scope of this work. The analysis time is not a crucial variable to be considered based on the obtained results.

3.2. Proline case

As reported by Rebane et al. [14], proline has a slow reaction rate, since it reaches its maximum intensity by 24 h from DEEMM addition. Since proline was one of the selected amino acids for the test solution, its derivatization product was monitored in the different sample preparations mentioned before. The 24 h analysis of the sample without quenching reagent addition resulted in a peak area of 76243, 44587 and 104728 for ethanol, methanol and acetonitrile, respectively, which are much higher than when quenching reagent is employed (Table S1). As expected, the hydroxylamine addition right away or after 2 h from the sample preparation interferes with the proline derivatization process. On the other hand, a 24 h quenching reagent addition followed by right away UHPLC-MS analysis gives results similar to the sample without the hydroxylamine addition (data now shown), nevertheless proline derivative probably undergoes degradation when the same sample is analyzed after 24 h. For this reason, when proline is the object of the study, it is suggested to carefully evaluate the method to be employed, *i.e*. no quenching reagent addition or its addition after a period longer than 2 h can be good options. Moreover, alternative mass spectrometers' acquisition methods can be employed as confirmation, *i.e.* SRM. This indicates the method should be further optimized when it comes to secondary amines.

3.3. Case study

Plant extracts are complex matrices with several compounds belonging to different chemical classes. The proposed DEEMM derivatization-targeted analysis using UHPLC with MS detection in NLS mode was therefore applied to a plant species, namely *Carduus nutans* subsp. *macrocephalus*, to determine its amino compounds profile. Few works, mostly on the polyphenols profile, have been published on the chemical composition of the selected species and its amino compounds content has never been investigated [29]. For this reason, it was interesting to exploit the NLS mode to separate and identify unknown

amines by monitoring the loss of the characteristic neutral ethanol fragment from DEEMM derivatized compounds.

The derivatization was carried out considering the optimized derivatization procedure and the UHPLC separation was performed under the same conditions as previously reported with a slight modification of the linear gradient due to the complexity of the sample and in order to obtain better separation of the peaks. The absence of evident differences in the profile of the extracts obtained with 0.1 M HCl in methanol, ethanol or acetonitrile, as solvents, determined the use of 0.1 M HCl in 30% methanol for all the experiments with *C. nutans* subsp. *macrocephalus* (data not shown). As previously observed, DEEMM and by-products interferences were detectable in the derivatized extract without the quenching reagent addition, together with two more peaks (RT 4.6 and 5.3 min; *m/z* 350) not present in the samples to which the hydroxylamine was added after 2 h (Fig. S4).

In order to avoid interfering peaks in the middle of the chromatogram, the quenching reagent addition approach was employed. The UHPLC-MS analysis of the derivatized extracts in NLS mode was performed right away, after 24 and 48 h with 24 hours still being optimal considering the overall profile (Fig. S5). However, in a routine study of several samples, reducing the 24-hour analysis time could be a good compromise to shorten the overall procedure time that involves extraction, derivatization and LC-MS analysis, since the NLS profiles analyzed at the three different times are consistent.

The repeatability of the extraction procedure and derivatization method applied to this complex plant sample was also evaluated, with RSD% of the peak areas not exceeding 15%.

The derivatized extract was analyzed in NLS mode in different *m/z* ranges, from *m/z* 50 to 2000, with no signal registered from *m/z* 1000 to 2000 and all the detected compounds below m/z 500. The non-derivatized extract NLS analysis, performed in the same m/z ranges, showed no significant peaks, confirming the selectivity of the NLS mode in the detection of DEEMM derivatives (Fig. 3).

Finally, the main peaks in the NLS profile of the derivatized extract were putatively identified based on the literature data and confirmed with the injection of the pure commercial standards, when available (Table 2). Eighteen amino acids were identified in *C. nutans* subsp. *macrocephalus* extract and few other amino compounds were also detected (*e.g.* tyramine, putrescine and phenylethylamine).

4. Conclusions

UHPLC-MS in neutral loss scan was successfully employed in the analysis of DEEMM-derivatized amino compounds. The derivatization procedure was optimized considering the solvent employed, the analysis time and the removal of DEEMM excess by the addition of a quenching reagent, namely hydroxylamine. The optimized method, which involves the employment of a 0.1 M HCl in 30% methanol solution, addition of pure DEEMM reagent and quenching the reaction with hydroxylamine after 2 h, is reproducible for primary amines (RSD between 1.6 – 4.1%) and can be applied to very complex matrices, such as plant extract, to detect known and unknown amine derivatives by monitoring the presence of a specific neutral fragment loss in the LC-MS analysis. Future perspectives include the quantitation of DEEMM derivatized compounds, with a comparison with the more popular multiple reaction monitoring acquisition mode, and the application to further plant species and different types of samples. Moreover, it would be interesting to test this acquisition mode with other derivatization reagents that exhibit a similar fragmentation pattern, to compare their selectivity, sensitivity and matrix effect.

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Authors contributions

Larissa Silva Maciel: investigation, data curation, writing-original draft, methodology, visualization; Arianna Marengo: investigation, data curation, writing-original draft, methodology, visualization; Koit Herodes: conceptualization, methodology, writing-review and editing, supervision, funding acquisition; Patrizia Rubiolo: writing-review and editing; Ivo Leito: writing-review and editing

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Figure captions:

Figure 1. Derivatization reaction of amino compounds with DEEMM.

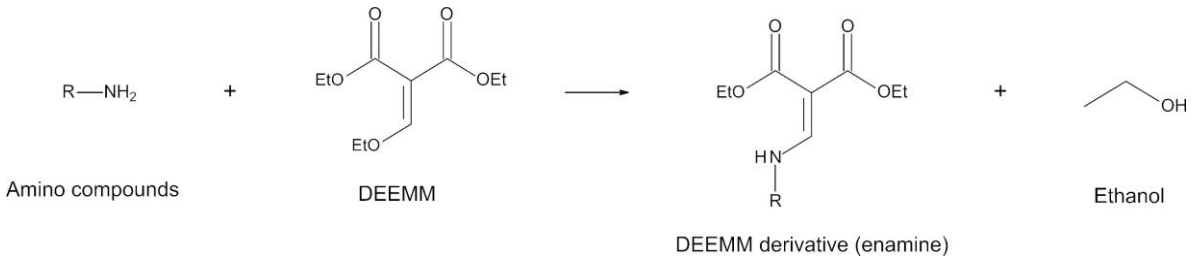


Figure 2. NLS profile of amino acid derivatives and other compounds: 1 – arginine, 2 – aspartic acid, 3 – m/z 147, 4 – threonine, 5 – proline, 6 – m/z 161 (DEEMM hydrolysis product), 7 – tyrosine, 8 – m/z 203 (product between DEEMM and methanol), 9 – m/z 189 (transesterification product), 10 – DEEMM, 11 – tryptophan, 12 – phenylalanine, 13 – isoleucine, 14 – m/z 285 and 15 – m/z 217.

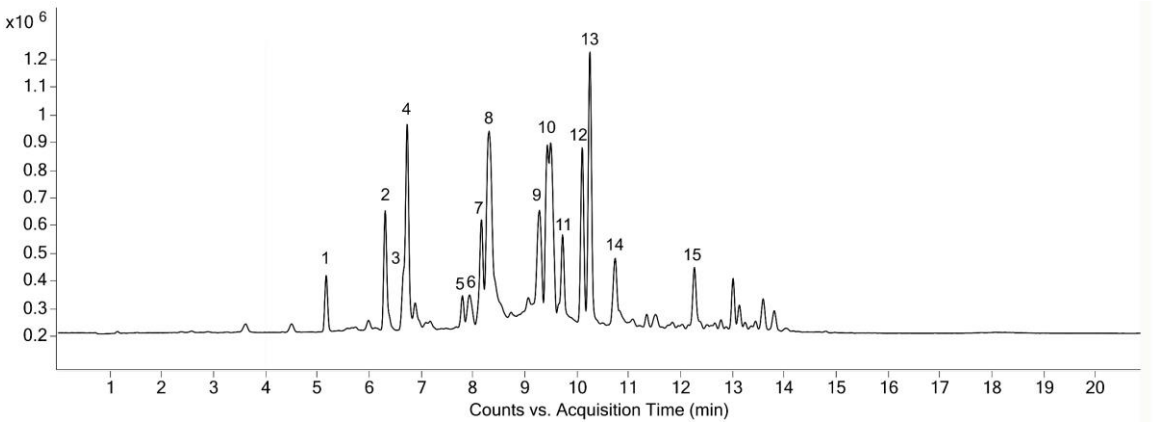
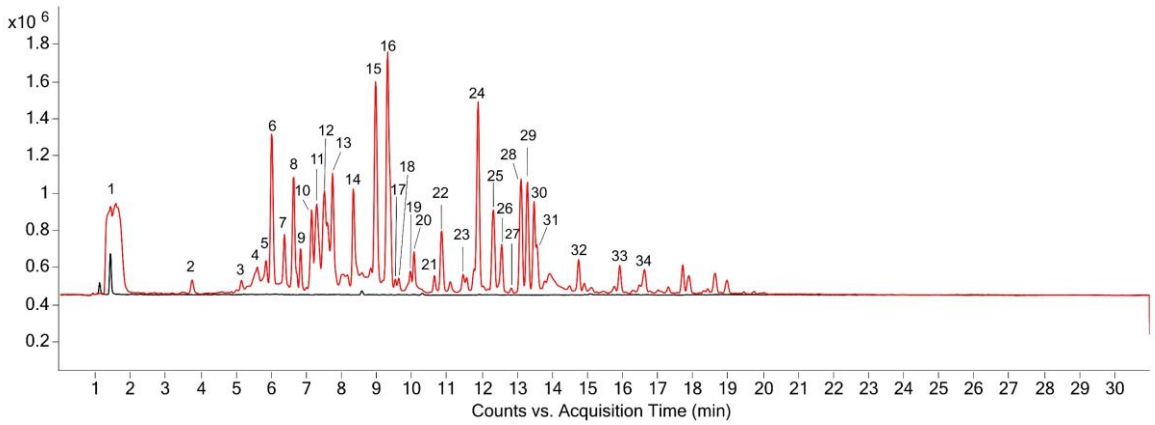


Figure 3. Comparison of NLS profile of *C. nutans* native (black line) and derivatized (red line) extracts: 1 – hydroxylamine derivative, 2 – histidine, 3 – m/z 282, 4 – arginine, 5 – m/z 365, 6 – asparagine, 7 – glutamine, 8 – serine, 9 – m/z 258.9, 10 – aspartic acid, 11 – m/z 232, 12 – m/z 188 (from blank), 13 – threonine, 14 – m/z 259.9, 15 – γ-aminobutyric acid, 16 – alanine, 17 – proline, 18 – m/z 274, 19 – m/z 288, 20 – tyrosine, 21 – m/z 274, 22 – m/z 324, 23 – m/z 242, 24 – valine, 25 – tyramine, 26 – tryptophan, 27 – ornithine, 28 – phenylalanine, 29 – isoleucine, 30 – leucine, 31 – lysine, 32 – putrescine, 33 – phenylethylamine, 34 – m/z 353. Only compounds that have been identified with a standard substance are written. Absence of any significant peaks in the chromatogram of underivatized sample extract demonstrates the selectivity of the combination of DEEMM derivatization and NLS mode detection.



Tables captions

Table 1. LC-MS information on the amino acid DEEMM derivatives concerning the molecular formula, the monoisotopic mass, the retention time and the protonated molecule found in the NL mass spectrum. Retention time (RT) corresponds to the method used for method development (gradient 1).

Amino acid derivative	Molecular formula of DEEMM derivative	Monoisotopic mass (g/mol)	RT (min)	Protonated molecule *m/z* in ESI^+ (detected fragment in the second mass analyzer)
Arginine	$C_{14}H_{24}N_4O_6$	344	5.20	345 (299)
Aspartic acid	$C_{12}H_{17}NO_8$	303	6.35	304 (258)
Threonine	$C_{12}H_{19}NO_7$	289	6.74	290 (244)
Proline	$C_{13}H_{19}NO_6$	285	7.81	286 (240)
Tyrosine	$C_{17}H_{21}NO_7$	351	8.19	352 (306)
Tryptophan	$C_{19}H_{22}N_2O_6$	374	9.75	375 (329)
Phenylalanine	$C_{17}H_{21}NO_6$	335	10.13	336 (290)
Isoleucine	$C_{14}H_{23}NO_6$	301	10.27	302 (256)

Table 2. List of identified and putatively identified derivatives in the Carduus nutans. Each compound is described by retention time (gradient 2), ESI^+ protonated molecule (*m/z*), molecular weight of the derivative and amino compound (g/mol) and identified or tentatively identified compound names. The Identification Confidence (IC) value and the references are also indicated.

N°	RT (min)	*m/z*	Derivative molecular weight (g/mol)	Molecular weight (g/mol)	Compound name	IC	Reference
1	1.540	159.0	158.0	33.0	Hydroxylamine derivative		
2	3.742	325.9	324.9	154.9	Histidine	1	[6]
3	5.145	282.0	281.0/259[3]	111.0/89[3]	Unknown		
4	5.546	345.0	344.0	174.0	Arginine	1	[6]
5	5.846	365.0	364.0/342.0[3]	194.0/172.0[3]	Unknown		
6	6.014	302.9	301.9	131.9	Asparagine	1	[6]

7	6.364	317.0	316.0	146.0	Glutamine	1	[6]
8	6.632	275.9	274.9	104.9	Serine	1	[6]
9	6.832	258.9	257.9/235.9[3]	87.9/65.9[3]	Unknown		
10	7.116	303.9	302.9	132.9	Aspartic acid	1	[6]
11	7.283	232.0	231.0	61.0	Ethanolamine	2	[34]
12	7.500	188.0	-	-	Unknown (from blank)		
13	7.734	290.0	289.0	119.0	Threonine	1	[6]
14	8.319	259.9	258.9/236.9[3]	88.9/66.9[3]	Unknown		
15	8.970	274.0	273.0	103.0	γ-aminobutyric acid	1	[6]
16	9.304	260.0	259.0	89.0	Alanine	1	[6]
17	9.388	286.0	285.0	115.0	Proline	1	[6]
18	9.668	274.0	273.0	103.0	α-aminobutyric acid/β-aminobutyric acid	2	[35]
19	9.956	288.0	287.0/265.0[3]	117.0/95.0[3]	Unknown		
20	10.056	352.0	351.0	181.0	Tyrosine	1	[6]
21	10.641	274.0	273.0	103.0	α-aminobutyric acid/β-aminobutyric acid	2	[35]
22	10.858	324.0	323.0/301.0[3]	153.0/131.0[3]	Unknown		
23	11.459	242.0	241.0/219.0[3]	71.0/49.0[3]	Unknown		
24	11.894	288.0	287.0	117.0	Valine	1	[6]
25	12.311	308.0	307.0	137.0	Tyramine	1	[6]
26	12.545	375.0	374.0	204.0	Tryptophan	1	[6]
27	12.829	427.0[1]	472.0	132.0	Ornithine	1	[34]
28	13.096	336.0	335.0	165.0	Phenylalanine	1	[6]
29	13.280	302.0	301.0	131.0	Isoleucine	1	[6]
30	13.481	302.0	301.0	131.0	Leucine	1	[6]
31	13.547	441.0[1]/ 509.0[2]	486.0	146.0	Lysine	1	[6]
32	14.733	383.0[1]	428.0	88.0	Putrescine	1	[6]
33	15.903	292.0	291.0	121.0	Phenylethylamine	1	[6]
34	16.454	353.0	352.0/330.0[3]	182.0/160.0[3]	Unknown		

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