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**Derivatization-targeted analysis of amino compounds in plant extracts in neutral loss acquisition mode by liquid chromatography-tandem mass spectrometry**

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(Article begins on next page)

1       **Derivatization-targeted analysis of amino compounds in plant**  
2               **extracts in neutral loss acquisition mode by liquid**  
3                       **chromatography-tandem mass spectrometry**

4  
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22       § Larissa Silva Maciel and Arianna Marengo gave an equivalent contribution to this study

23  
24  
25       **Abstract.** Amino compounds, such as amino acids and biogenic amines, are important  
26       metabolites that can be found in diverse natural matrices. The most common method for  
27       amino compound analysis nowadays is reversed-phase liquid chromatography tandem  
28       mass spectrometry (RPLC-MS/MS). However, due to the polar and the basic nature of  
29       amines, their RPLC retention is often insufficient or peaks are tailing. Derivatization is a  
30       way to overcome the issue and in the present work amino compounds are derivatized with  
31       diethyl ethoxymethylenemalonate (DEEMM) and analyzed by a RPLC triple quadrupole  
32       MS system in neutral loss scan (NLS) mode (loss of 46). This allows to target all  
33       compounds in the sample that undergo derivatization with DEEMM, so that the amino

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

44

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

47

## 48 **1. Introduction**

49

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

67 Methods for amino compound analysis include liquid chromatography (LC), gas  
68 chromatography (GC) and capillary electrophoresis (CE), coupled to different detectors  
69 (e.g. mass spectrometry, spectrophotometric or fluorescence detectors), where reverse-  
70 phase liquid chromatography tandem mass spectrometry (RPLC-MS/MS) is one of the  
71 leading analytical techniques nowadays. However, amino compounds' polar and basic  
72 nature do not favor the use of the commonly employed reverse-phase (RP) separation  
73 [4,9]. When electrospray ionization (ESI) is the ionization source of choice, ionization  
74 efficiency is also affected since the analyte should present a hydrophobic region and  
75 should be able to carry a charge in the gas-phase [10]. Therefore, if the analyte does not  
76 have these qualities, as in the case of amino acids and biogenic amines, it can be modified  
77 through derivatization.

78 Derivatization is a chemical reaction between an analyte and a derivatization reagent, to  
79 improve the chromatographic behavior (e.g. increased retention times, improved  
80 separation and peak shape) and other properties, such as stability [11]. Ideally, the  
81 derivatization reagent reacts only with a specific functional group (e.g. amino group) and is  
82 chosen to increase the sensitivity with: ultraviolet (UV), fluorescence or mass spectrometry  
83 (MS) detection. Examples of derivatization reagents specifically employed for amino  
84 compounds are: dansyl chloride (DASC), 2,5-dioxopyrrolidin-1-yl N-  
85 tri(pyrrolidino)phosphoranylideneamino carbamate (FOSF), 9-fluorenylmethyl  
86 chloroformate (FMOC-Cl), p-N,N,N-trimethylammonioanilyl N'-hydroxysuccinimidyl  
87 carbamate iodide (TAHS) and diethyl ethoxymethylenemalonate (DEEMM) [12,13].

88 Since for DEEMM-derivatives UV and MS detection can be used, it has been successfully  
89 employed for the detection and quantification of amino compounds in diverse matrices,  
90 such as tea extract [12], honey [13,14], milk [15], cheese [16], plant seeds [17], tobacco  
91 [6], saffron [18], strawberry purée [19] and biological samples [20,21]. The reaction  
92 between DEEMM and amino compounds is straightforward and robust, leading to the  
93 formation of an enamine and ethanol (Fig. 1), which is another advantage of such  
94 derivatization reagent.

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

101 mass spectrum from the precursor ion (Q1) that yielded the pre-selected neutral loss in Q2  
102 [23].

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

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

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

127

## 128 **2. Materials and Methods (Experimental)**

### 129 **2.1. Plant material**

130

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

134 voucher specimen for the species was deposited (CAG-802). In total, 13 specimens of *C.*  
135 *nutans* subsp. *macrocephalus* were collected randomly. All individuals were separated by  
136 1–50 m from one another. The fresh materials were dried at 40 °C until constant weight  
137 was reached.

138

## 139 **2.2. Chemicals**

140

141 HPLC-grade acetonitrile, methanol and ethanol were purchased from Merck. Amino acid  
142 standards (L-alanine, L-arginine hydrochloride, L-asparagine, L-aspartic acid, L-glutamine,  
143 L-glutamic acid, L-glycine, L-histidine, L-isoleucine, L-leucine, L-lysine, L-methionine, L-  
144 phenylalanine, L-proline, L-serine, L-threonine, L-tryptophan, L-tyrosine and L-valine,  
145  $\geq 99.5\%$ ),  $\gamma$ -aminobutyric acid ( $\geq 99\%$ ), formic acid ( $\geq 95\%$ ), diethylamine ( $\geq 99.5\%$ ),  
146 butylamine (99.5%), putrescine ( $\geq 99.5\%$ ), phenylethylamine ( $\geq 99.5\%$ ), tyramine  
147 hydrochloride ( $\geq 98\%$ ), ( $\pm$ )-octopamine hydrochloride ( $\geq 95\%$ ) purchased from Sigma.  
148 Hydrochloric acid (HCl) was purchased from Reakhim. DEEMM ( $\geq 99\%$ ) and L-ornithine  
149 monohydrochloride ( $\geq 99\%$ ) purchased from Fluka. Boric acid ( $\geq 99\%$ ) was purchased from  
150 Hopkin & Williams. 2-amino-2-(hydroxymethyl)propane-1,3-diol was purchased from  
151 Schuchardt. Hydroxylamine ( $> 97.5\%$ ) was purchased from Reakhim.

152 0.75 M borate buffer was prepared in deionized water and pH was adjusted to 9.00 with a  
153 saturated sodium hydroxide solution.

154 All aqueous solutions were prepared with ultrapure water by Millipore Milli-Q Advantage  
155 A10 (Millipore).

156

## 157 **2.3. Preparation of standard solution**

158

159 Stock solutions of each of the eight amino acids (c.a. 300 mg/L) were individually prepared  
160 in 0.3 M HCl and stored at  $-20^{\circ}\text{C}$ . The final concentration of each amino acid in the  
161 standard solution was ca 10 mg/L either in 0.1 M HCl in 30% methanol, ethanol or  
162 acetonitrile.

163 Hydroxylamine, diethylamine, butylamine, 2-amino-2-(hydroxymethyl)propane-1,3-diol and  
164 glycine were diluted, according to their solubility, in 0.1 M HCl in 30% methanol to a final  
165 concentration of 1.5 M for hydroxylamine and 2-amino-2-(hydroxymethyl)propane-1,3-diol,

166 0.5 M for glycine and 10 M for diethylamine and butylamine. Hydroxylamine was prepared  
167 in 0.1 M HCl in 30% ethanol and acetonitrile as well.

168

#### 169 **2.4. Pre-column derivatization**

170

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

174 Derivatization was carried out in a 1.5 ml glass vial by addition of solutions in the following  
175 order and mixing after each addition: 588 µl of the sample in 0.1 M HCl 30% methanol,  
176 875 µl of borate buffer (0.75 M, pH 9), 7 µl of DEEMM and 30 µl of the quenching reagent  
177 (hydroxylamine, 1.5M) added after 2 h.

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

180

181

#### 182 **2.5. Plant extraction**

183

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

194

#### 195 **2.6. LC-MS/MS analysis**

196

197 LC-MS system equipped with Agilent 1290 Infinity II quaternary pump, column thermostat,  
198 an autosampler and an Agilent 6460 Triple Quadrupole (QqQ) mass spectrometer (MS)  
199 with Agilent Jet Stream Technology electrospray ionization source (ESI) was used.

200 Chromatographic analysis was performed in a Zorbax Eclipse Plus C18 (3.0 x 100 mm,  
201 1.8 µm) column, which was maintained at 40 °C and 5 µL of the sample was injected. The  
202 mobile phase was composed of 0.1% aqueous formic acid (A) and acetonitrile (B). For  
203 derivatization method development, the following gradient was used (gradient 1): 0 – 2  
204 min, 10% B; 2 – 17 min, 10-100% B; 17 – 19 min, 100% B; 19 – 21 min, 100-10% B; the  
205 total pre-running and post-running time was 25 min. The plant extract was analyzed with  
206 the following gradient (gradient 2): 0 – 2 min, 10% B; 2 – 27 min, 10-100% B; 27 – 29 min,  
207 100% B; 29 – 31 min, 100-10% B; total pre-running and post-running time was 35 min.  
208 Eluent flow rate was 0.5 mL/min in both cases.

209 The following ESI and MS parameters were used: drying gas temperature 320°C, drying  
210 gas flow 9 L/min, nebulizer gas pressure 45 psi, sheath gas temperature 400°C, sheath  
211 gas flow 12 L/min, capillary voltage 3000 V and nozzle voltage 0 V. Neutral loss scan  
212 mode was performed with the neutral loss of 46 in the *m/z* ranges from 50 to 500, 500 to  
213 1000, 1000 to 1500 and 1500 to 2000, fragmentor 90 V and collision energy 8 V.  
214 Data was processed using the Agilent MassHunter Qualitative Analysis Navigator B.08.00  
215 software.

216

## 217 **2.7. Statistical analysis**

218

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

221

## 222 **3. Results and discussion**

223

### 224 **3.1. Optimization of the derivatization procedure**

225

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



234 as a neutral fragment. DEEMM causes one of the three main interfering peaks (RT 9.460  
235 minutes) characterizing the profile, together with other byproducts of the reaction (e.g.  $m/z$   
236 188, 161, 203, 189, 285 and 217 at RT 6.7, 7.9, 8.3, 9.3, 10.72, 12.12 minutes,  
237 respectively), for example, transesterification product or enol ether product between  
238 DEEMM and methanol ( $m/z$  203) and DEEMM hydrolysis product ( $m/z$  161).

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

247 As a first step, the NLS profile of the hydroxylamine derivative alone was monitored at  
248 different conditions: methanol, ethanol and acetonitrile were tested as diluting solvents (*i.e.*  
249 0.1 M HCl in 30% of organic solvent as reported by Oldekop et al., 2017) to detect  
250 potential differences. At the same time, both pre-diluted DEEMM in solvent [33] and  
251 undiluted DEEMM (referred to as “pure DEEMM”) [14] were employed, and analysis was  
252 carried out right away or after 24 h as suggested by Rebane et al., 2010 [6,14,18,31,33].  
253 When DEEMM reacts with hydroxylamine the chromatographic profile becomes cleaner, in  
254 particular when pure DEEMM is added, regardless of the organic solvent employed (an  
255 example with methanol is shown in Fig. S1). Regular analysis of the samples revealed a  
256 considerable interference, between 6-8 min in the chromatogram, that is less noticeable  
257 over time *i.e.* after 160, 80, 440 min for methanol, ethanol and acetonitrile, respectively (an  
258 example is shown in Fig. S2 for methanol). Based on the obtained results, the  
259 derivatization of the 8 selected amino acids was performed in the three selected organic  
260 solvents, using pure DEEMM with and without the addition of hydroxylamine as a  
261 quenching reagent. The analysis was performed both right away and after 24 hours to  
262 detect potential differences. As mentioned before, all amino acid peaks are detectable in  
263 NLS mode, however, since sensitivity towards proline was lower, its derivatization  
264 behavior will be discussed in a dedicated section.

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

268 no longer present (Fig. S3 A and E). Different quenching reagent addition times - right-  
269 away, 2 h and 24 h - were investigated as well. The right-away quenching reagent  
270 addition, which prevents the complete derivatization of the selected amino acids, as  
271 evidenced by lower peak areas compared to samples without hydroxylamine addition or  
272 with delayed (2 h, 24 h) quenching reagent addition, was not taken into consideration (Fig.  
273 S3 B, C and D). Since the 2 h and 24 h hydroxylamine addition gave consistent results,  
274 the 2 h procedure was selected to reduce the sample preparation time.

275 The LC-MS analysis was performed both right away and after 24 h from the sample  
276 preparation and, as before, 24 h analysis gave a cleaner NLS profile in comparison to the  
277 right-away analysis time (Fig.S3 C and E).

278

279 The influence of the quenching reagent to the amino acid derivatives signal intensity was  
280 also evaluated comparing the samples without or with the 2 h quenching reagent addition  
281 (Table S1). Overall, there are significant statistical differences between the two treatments,  
282 with peak areas being higher when the quenching reagent is employed. A possible reason  
283 is that the chromatographic profile is cleaner and, therefore, leads to less ionization-  
284 suppressing interference from co-eluting compounds.

285 These results confirm the advantages of adding a quenching reagent to the DEEMM  
286 derivatives solution after 2 h. With regards to the analysis time and its effect on peak area,  
287 there is a small decrease in sensitivity after 24 h overall (Table S2). One-way ANOVA ( $p <$   
288  $0.05$ ) showed there are statistically significant differences for aspartic acid and tyrosine  
289 (ethanol), arginine (methanol) and aspartic acid, tyrosine and isoleucine (acetonitrile).

290 Although the results obtained with both analysis times are consistent (Table S3), analysis  
291 after 24 h was chosen for further experiments, due to the cleanliness of the chromatogram.  
292 Apart from proline that will be discussed in section 3.2, the peak areas are similar  
293 regardless of the solvent employed, with RSD% below 5%. When one-way ANOVA is  
294 employed ( $p < 0.05$ ) for the three different solvents and pure DEEMM, there are  
295 statistically significant differences only for aspartic acid and tryptophan. The only amino  
296 acid that has a statistically highly significant difference ( $p < 0.001$ ) in sensitivity among the  
297 solvents is aspartic acid, with a decrease in peak area in the case of acetonitrile. Since  
298 there are no large differences in the sensitivity among the solvents and methanol has been  
299 employed as a solvent for DEEMM derivatization in the literature, it was chosen for further  
300 experiments.

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

305

### 306 **3.2. Proline case**

307

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

325

### 326 **3.3. Case study**

327

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

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

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

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

354 The repeatability of the extraction procedure and derivatization method applied to this  
355 complex plant sample was also evaluated, with RSD% of the peak areas not exceeding  
356 15%.

357 The derivatized extract was analyzed in NLS mode in different  $m/z$  ranges, from  $m/z$  50 to  
358 2000, with no signal registered from  $m/z$  1000 to 2000 and all the detected compounds  
359 below  $m/z$  500. The non-derivatized extract NLS analysis, performed in the same  $m/z$   
360 ranges, showed no significant peaks, confirming the selectivity of the NLS mode in the  
361 detection of DEEMM derivatives (Fig. 3).

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

367

#### 368 **4. Conclusions**

369

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

384

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386

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393

### 394 **Authors contributions**

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

400

### 401 **References**

402

- 403 [1] R.E. Grier, W.A. Gahl, T. Cowan, I. Bernardini, G.A. McDowell, P. Rinaldo, Revised  
404 Sections F7.5 (Quantitative Amino Acid Analysis) and F7.6 (Qualitative Amino Acid  
405 Analysis): American College of Medical Genetics Standards and Guidelines for  
406 Clinical Genetics Laboratories, 2003, *Genet. Med.* 6 (2004) 66–68.  
407 <https://doi.org/10.1097/01.GIM.0000106163.35058.7D>.
- 408 [2] A. Jain, K.K. Verma, Strategies in liquid chromatographic methods for the analysis  
409 of biogenic amines without and with derivatization, *TrAC - Trends Anal. Chem.* 109  
410 (2018) 62–82. <https://doi.org/10.1016/j.trac.2018.10.001>.
- 411 [3] A. Önal, A review: Current analytical methods for the determination of biogenic  
412 amines in foods, *Food Chem.* 103 (2007) 1475–1486.  
413 <https://doi.org/10.1016/j.foodchem.2006.08.028>.
- 414 [4] J.P. Violi, D.P. Bishop, M.P. Padula, J.R. Steele, K.J. Rodgers, Considerations for  
415 amino acid analysis by liquid chromatography-tandem mass spectrometry: A tutorial  
416 review, *TrAC - Trends Anal. Chem.* 131 (2020) 116018.  
417 <https://doi.org/10.1016/j.trac.2020.116018>.
- 418 [5] R. Dahl-Lassen, J. van Hecke, H. Jørgensen, C. Bukh, B. Andersen, J.K.  
419 Schjoerring, High-throughput analysis of amino acids in plant materials by single  
420 quadrupole mass spectrometry, *Plant Methods.* 14 (2018) 1–9.  
421 <https://doi.org/10.1186/s13007-018-0277-8>.
- 422 [6] K. Cai, Z. Xiang, H. Li, H. Zhao, Y. Lin, W. Pan, B. Lei, Free amino acids, biogenic  
423 amines, and ammonium profiling in tobacco from different geographical origins  
424 using microwave-assisted extraction followed by ultra high performance liquid  
425 chromatography, *J. Sep. Sci.* 40 (2017) 4571–4582.  
426 <https://doi.org/10.1002/jssc.201700608>.
- 427 [7] S. Moret, D. Smela, T. Populin, L.S. Conte, A survey on free biogenic amine content  
428 of fresh and preserved vegetables, *Food Chem.* 89 (2005) 355–361.  
429 <https://doi.org/10.1016/j.foodchem.2004.02.050>.
- 430 [8] M. Papageorgiou, D. Lambropoulou, C. Morrison, E. Kłodzińska, J. Namieśnik, J.  
431 Płotka-Wasyłka, Literature update of analytical methods for biogenic amines  
432 determination in food and beverages, *TrAC - Trends Anal. Chem.* 98 (2018) 128–  
433 142. <https://doi.org/10.1016/j.trac.2017.11.001>.
- 434 [9] T. Santa, Derivatization reagents in liquid chromatography/electrospray ionization  
435 tandem mass spectrometry, *Biomed. Chromatogr.* 25 (2011) 1–10.  
436 <https://doi.org/10.1002/bmc.1548>.

- 437 [10] M. Wilm, Principles of electrospray ionization, *Mol. Cell. Proteomics*. 10 (2011) 1–8.  
438 <https://doi.org/10.1074/mcp.M111.009407>.
- 439 [11] F. Xu, L. Zou, Y. Liu, Z. Zhang, C.N. Ong, Enhancement of the capabilities of liquid  
440 chromatography–mass spectrometry with derivatization: General principles and  
441 applications, *Mass Spectrom. Rev.* 30 (2011) 1143–1172.  
442 <https://doi.org/10.1002/mas.20316>.
- 443 [12] M.L. Oldekop, K. Herodes, R. Rebane, Comparison of amino acid derivatization  
444 reagents for liquid chromatography atmospheric pressure chemical ionization mass  
445 spectrometric analysis of seven amino acids in tea extract, *Int. J. Mass Spectrom.*  
446 421 (2017) 189–195. <https://doi.org/10.1016/j.ijms.2017.07.004>.
- 447 [13] J.L. Bernal, M.J. Nozal, L. Toribio, J.C. Diego, A. Ruiz, A comparative study of  
448 several HPLC methods for determining free amino acid profiles in honey, *J. Sep.*  
449 *Sci.* 28 (2005) 1039–1047. <https://doi.org/10.1002/jssc.200500008>.
- 450 [14] R. Rebane, K. Herodes, A sensitive method for free amino acids analysis by liquid  
451 chromatography with ultraviolet and mass spectrometric detection using precolumn  
452 derivatization with diethyl ethoxymethylenemalonate: Application to the honey  
453 analysis, *Anal. Chim. Acta.* 672 (2010) 79–84.  
454 <https://doi.org/10.1016/j.aca.2010.04.014>.
- 455 [15] A.T. Mazhitova, A.A. Kulmyrzaev, Determination of amino acid profile of mare milk  
456 produced in the highlands of the Kyrgyz Republic during the milking season, *J.*  
457 *Dairy Sci.* 99 (2016) 2480–2487. <https://doi.org/10.3168/jds.2015-9717>.
- 458 [16] B. Redruello, V. Ladero, I. Cuesta, J.R. Álvarez-Buylla, M.C. Martín, M. Fernández,  
459 M.A. Alvarez, A fast, reliable, ultra high performance liquid chromatography method  
460 for the simultaneous determination of amino acids, biogenic amines and ammonium  
461 ions in cheese, using diethyl ethoxymethylenemalonate as a derivatising agent,  
462 *Food Chem.* 139 (2013) 1029–1035.  
463 <https://doi.org/10.1016/j.foodchem.2013.01.071>.
- 464 [17] C. Megías, I. Cortés-Giraldo, M. Alaiz, J. Girón-Calle, J. Vioque, O. Santana-  
465 Méridas, D. Herraiz-Peñalver, R. Sánchez-Vioque, Determination of the Neurotoxin  
466 3-N-Oxalyl-2,3-Diaminopropionic Acid and Other Free Amino Acids in *Lathyrus*  
467 *cicera* and *L. sativus* Seeds by Reversed-Phase High-Performance Liquid  
468 Chromatography, *Food Anal. Methods.* 8 (2015) 1953–1961.  
469 <https://doi.org/10.1007/s12161-014-0084-4>.
- 470 [18] C. Priscila del Campo, T. Garde-Cerdán, A.M. Sánchez, L. Maggi, M. Carmona,

- 471 G.L. Alonso, Determination of free amino acids and ammonium ion in saffron  
472 (*Crocus sativus* L.) from different geographical origins, *Food Chem.* 114 (2009)  
473 1542–1548. <https://doi.org/10.1016/j.foodchem.2008.11.034>.
- 474 [19] J.L. Ordóñez, F. Sainz, R.M. Callejón, A.M. Troncoso, M.J. Torija, M.C. García-  
475 parrilla, Impact of gluconic fermentation of strawberry using acetic acid bacteria on  
476 amino acids and biogenic amines profile, 178 (2015) 221–228.  
477 <https://doi.org/10.1016/j.foodchem.2015.01.085>.
- 478 [20] Y.H. Kim, H.J. Kim, J.H. Shin, S.K. Bhatia, H.M. Seo, Y.G. Kim, Y.K. Lee, Y.H.  
479 Yang, K. Park, Application of diethyl ethoxymethylenemalonate (DEEMM)  
480 derivatization for monitoring of lysine decarboxylase activity, *J. Mol. Catal. B Enzym.*  
481 115 (2015) 151–154. <https://doi.org/10.1016/j.molcatb.2015.01.018>.
- 482 [21] D.L. Vu, K. Ranglová, J. Hájek, P. Hrouzek, Quantification of methionine and  
483 selenomethionine in biological samples using multiple reaction monitoring high  
484 performance liquid chromatography tandem mass spectrometry (MRM-HPLC-  
485 MS/MS), *J. Chromatogr. B Anal. Technol. Biomed. Life Sci.* 1084 (2018) 36–44.  
486 <https://doi.org/10.1016/j.jchromb.2018.03.012>.
- 487 [22] R. Rebane, M.L. Oldekop, K. Herodes, Comparison of amino acid derivatization  
488 reagents for LC-ESI-MS analysis. Introducing a novel phosphazene-based  
489 derivatization reagent, *J. Chromatogr. B Anal. Technol. Biomed. Life Sci.* 904 (2012)  
490 99–106. <https://doi.org/10.1016/j.jchromb.2012.07.029>.
- 491 [23] W.M.A. Niessen, D. Fakck, Introduction to Mass Spectrometry, a Tutorial, in: *Anal.*  
492 *Biomol. Interact. by Mass Spectrom.*, 2015: p. Chapter 1.
- 493 [24] H. Meng, S. Zhu, Y. Fan, R. Ye, M. Hattori, K. Komatsu, C. Ma, Discovery of  
494 prenylated dihydrostilbenes in *Glycyrrhiza uralensis* leaves by UHPLC-MS using  
495 neutral loss scan, *Ind. Crops Prod.* 152 (2020) 112557.  
496 <https://doi.org/10.1016/j.indcrop.2020.112557>.
- 497 [25] N. Kleinenkuhnen, F. Büchel, S.C. Gerlich, S. Kopriva, S. Metzger, A Novel Method  
498 for Identification and Quantification of Sulfated Flavonoids in Plants by Neutral Loss  
499 Scan Mass Spectrometry, *Front. Plant Sci.* 10 (2019) 885.  
500 <https://doi.org/10.3389/fpls.2019.00885>.
- 501 [26] T.J. Leiker, R.M. Barkley, R.C. Murphy, Analysis of diacylglycerol molecular species  
502 in cellular lipid extracts by normal-phase LC-electrospray mass spectrometry, *Int. J.*  
503 *Mass Spectrom.* 305 (2011) 103–108. <https://doi.org/10.1016/j.ijms.2010.09.008>.
- 504 [27] Y. He, Y. Luo, H. Chen, J. Chen, Y. Fu, H. Hou, Q. Hu, Profiling of carboxyl-



505 containing metabolites in smokers and non-smokers by stable isotope labeling  
506 combined with LC-MS/MS, *Anal. Biochem.* 569 (2019) 1–9.  
507 <https://doi.org/10.1016/j.ab.2018.12.006>.

508 [28] B.A. Boughton, D.L. Callahan, C. Silva, J. Bowne, A. Nahid, T. Rupasinghe, D.L.  
509 Tull, M.J. McConville, A. Bacic, U. Roessner, Comprehensive profiling and  
510 quantitation of amine group containing metabolites., *Anal. Chem.* 83 (2011) 7523–  
511 7530. <https://doi.org/10.1021/ac201610x>.

512 [29] A. Marengo, A. Maxia, C. Sanna, C.M. Bertea, C. Bicchi, M. Ballero, C. Cagliari, P.  
513 Rubiolo, Characterization of four wild edible *Carduus* species from the  
514 Mediterranean region via phytochemical and biomolecular analyses, *Food Res. Int.*  
515 100 (2017) 822–831. <https://doi.org/10.1016/j.foodres.2017.07.071>.

516 [30] A.A.G. Ibarra, K. Wrobel, A.R.C. Escobosa, J.C.T. Elguera, M.E. Garay-Sevilla, K.  
517 Wrobel, Determination of putrescine, cadaverine, spermidine and spermine in  
518 different chemical matrices by high performance liquid chromatography-electrospray  
519 ionization-ion trap tandem mass spectrometry (HPLC-ESI-ITMS/MS), *J.*  
520 *Chromatogr. B Anal. Technol. Biomed. Life Sci.* 1002 (2015) 176–184.  
521 <https://doi.org/10.1016/j.jchromb.2015.08.036>.

522 [31] M.L. Oldekop, R. Rebane, K. Herodes, Dependence of matrix effect on ionization  
523 polarity during LC–ESI–MS analysis of derivatized amino acids in some natural  
524 samples, *Eur. J. Mass Spectrom.* 23 (2017) 245–253.  
525 <https://doi.org/10.1177/1469066717711026>.

526 [32] S. Gómez-Alonso, I. Hermosín-Gutiérrez, E. García-Romero, Simultaneous HPLC  
527 analysis of biogenic amines, amino acids, and ammonium ion as aminoenone  
528 derivatives in wine and beer samples, *J. Agric. Food Chem.* 55 (2007) 608–613.  
529 <https://doi.org/10.1021/jf062820m>.

530 [33] M.L. Oldekop, K. Herodes, R. Rebane, Study of the matrix effects and sample  
531 dilution influence on the LC-ESI-MS/MS analysis using four derivatization reagents,  
532 *J. Chromatogr. B Anal. Technol. Biomed. Life Sci.* 967 (2014) 147–155.  
533 <https://doi.org/10.1016/j.jchromb.2014.07.027>.

534 [34] R. Dosi, A. Daniele, V. Guida, L. Ferrara, V. Severino, A. Di Maro, Nutritional and  
535 metabolic profiling of the globe artichoke (*cynara scolymus* L. cv. capuanella heads)  
536 in province of caserta, Italy, *Aust. J. Crop Sci.* 7 (2013) 1927–1934.

537 [35] D. Thevenet, V. Pastor, I. Baccelli, A. Balmer, A. Vallat, R. Neier, G. Glauser, B.  
538 Mauch-Mani, The priming molecule  $\beta$  -aminobutyric acid is naturally present in

539 plants and is induced by stress, *New Phytol.* 213 (2017) 552–559.

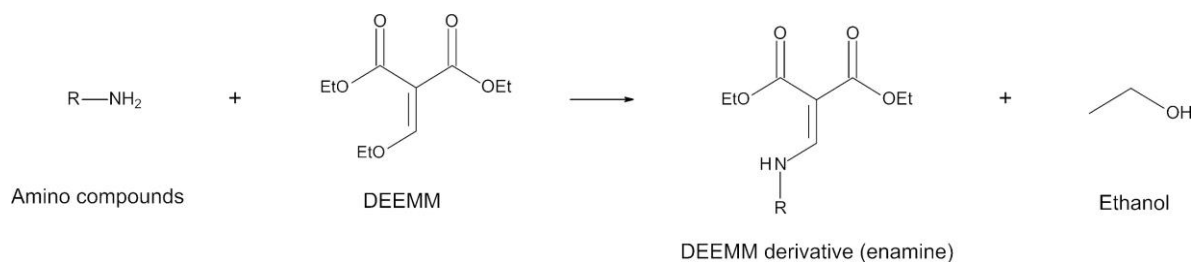
540 <https://doi.org/10.1111/nph.14298>.

541

542 **Figure captions:**

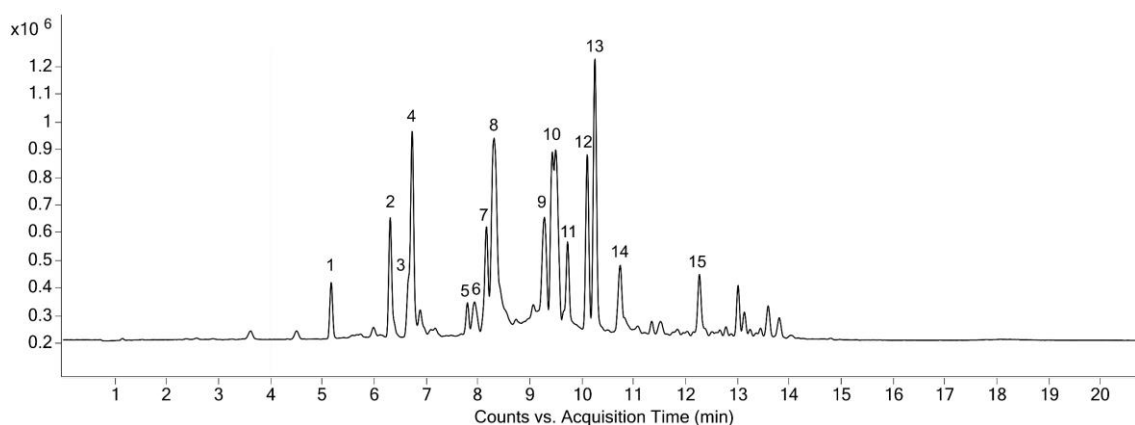
543

544 **Figure 1.** Derivatization reaction of amino compounds with DEEMM.



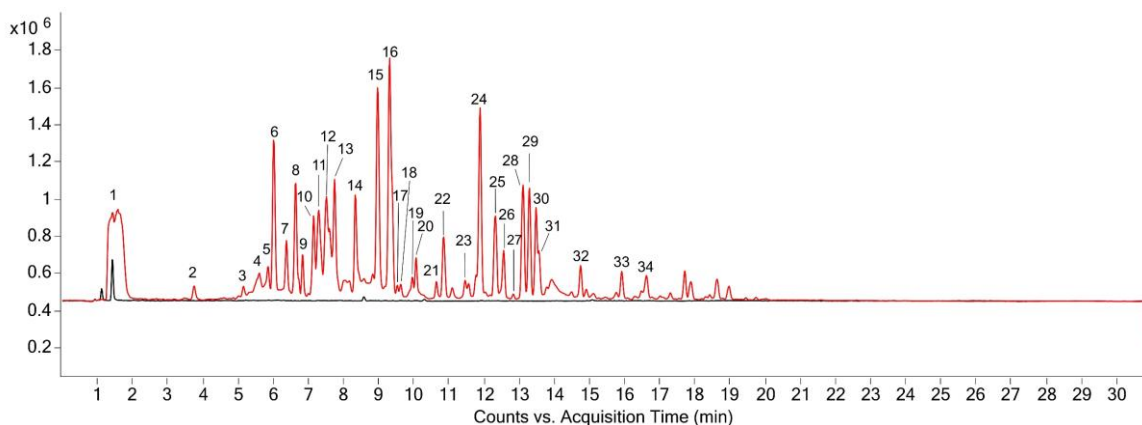
545

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



551

552 **Figure 3.** Comparison of NLS profile of *C. nutans* native (black line) and derivatized (red  
553 line) extracts: 1 – hydroxylamine derivative, 2 – histidine, 3 – m/z 282, 4 – arginine, 5 –  
554 m/z 365, 6 – asparagine, 7 – glutamine, 8 – serine, 9 – m/z 258.9, 10 – aspartic acid, 11  
555 – m/z 232, 12 – m/z 188 (from blank), 13 – threonine, 14 – m/z 259.9, 15 –  $\gamma$ -  
556 aminobutyric acid, 16 – alanine, 17 – proline, 18 – m/z 274, 19 – m/z 288, 20 – tyrosine,  
557 21 – m/z 274, 22 – m/z 324, 23 – m/z 242, 24 – valine, 25 – tyramine, 26 – tryptophan,  
558 27 – ornithine, 28 – phenylalanine, 29 – isoleucine, 30 – leucine, 31 – lysine, 32 –  
559 putrescine, 33 – phenylethylamine, 34 – m/z 353. Only compounds that have been  
560 identified with a standard substance are written. Absence of any significant peaks in the  
561 chromatogram of underivatized sample extract demonstrates the selectivity of the  
562 combination of DEEMM derivatization and NLS mode detection.



563

564 **Tables captions**

565

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

Amino acid derivative	Molecular formula of DEEMM derivative	Monoisotopic mass (g/mol)	RT (min)	Protonated molecule <i>m/z</i> in ESI- (detected fragment in the second mass analyzer)
Arginine	C <sub>14</sub> H <sub>24</sub> N <sub>4</sub> O <sub>6</sub>	344	5.20	345 (299)
Aspartic acid	C <sub>12</sub> H <sub>17</sub> NO <sub>8</sub>	303	6.35	304 (258)
Threonine	C <sub>12</sub> H <sub>19</sub> NO <sub>7</sub>	289	6.74	290 (244)
Proline	C <sub>13</sub> H <sub>19</sub> NO <sub>6</sub>	285	7.81	286 (240)
Tyrosine	C <sub>17</sub> H <sub>21</sub> NO <sub>7</sub>	351	8.19	352 (306)
Tryptophan	C <sub>19</sub> H <sub>22</sub> N <sub>2</sub> O <sub>6</sub>	374	9.75	375 (329)
Phenylalanine	C <sub>17</sub> H <sub>21</sub> NO <sub>6</sub>	335	10.13	336 (290)
Isoleucine	C <sub>14</sub> H <sub>23</sub> NO <sub>6</sub>	301	10.27	302 (256)

570

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

N°	RT (min)	<i>m/z</i>	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 <sup>3</sup>	111.0/89 <sup>3</sup>	Unknown		
4	5.546	345.0	344.0	174.0	Arginine	1	[6]
5	5.846	365.0	364.0/342.0 <sup>3</sup>	194.0/172.0 <sup>3</sup>	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 <sup>3</sup>	87.9/65.9 <sup>3</sup>	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 <sup>3</sup>	88.9/66.9 <sup>3</sup>	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 <sup>3</sup>	117.0/95.0 <sup>3</sup>	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 <sup>3</sup>	153.0/131.0 <sup>3</sup>	Unknown		
23	11.459	242.0	241.0/219.0 <sup>3</sup>	71.0/49.0 <sup>3</sup>	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 <sup>1</sup>	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 <sup>1</sup> / 509.0 <sup>2</sup>	486.0	146.0	Lysine	1	[6]
32	14.733	383.0 <sup>1</sup>	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 <sup>3</sup>	182.0/160.0 <sup>3</sup>	Unknown		

575