

1 **New approach for the diagnosis of histamine intolerance based on the determination**
2 **of histamine and methylhistamine in urine**

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15 **Abstract**

16 Histamine intolerance is a disorder in the homeostasis of histamine due to a reduced
17 intestinal degradation of this amine, mainly caused by diamine oxidase (DAO) enzyme
18 deficiency, which provokes its accumulation in plasma and the appearance of adverse
19 health affects. A new approach for the diagnosis of this intolerance could be the
20 determination of histamine and its metabolites in urine. The aim of this work was to
21 develop and validate a rapid method to determine histamine and methylhistamine in
22 human urine by Ultra High Performance Liquid Chromatography and Fluorimetric
23 detection (UHPLC-FL). The proposed method is a consistent procedure to determine
24 histamine and methylhistamine in less than 11 minutes with adequate linearity and
25 sensitivity. Relative standard deviation was always lower than 5.5%, ensuring method
26 precision; and mean recovery was greater than 99% for both analytes. The structure of
27 histamine and methylhistamine conjugated with OPA were confirmed by UHPLC-ITD-
28 FTMS which enabled to unequivocally identify both analytes in standards and also in
29 urine samples. The analysis of histamine and methylhistamine in urine samples could be
30 a potential new approach for the routine diagnosis of histamine intolerance, more
31 patient-friendly and with clear advantages in terms of equipment and personnel
32 demand for sample collection in comparison with current plasmatic DAO activity
33 determination.

34

35 **Keywords**

36 Histamine; Methylhistamine; Histamine intolerance; Diamine oxidase; Solid phase
37 extraction; Ultra high pressure liquid chromatography.

38 **1. Introduction**

39 Histamine (2-[4-imidazolyl]ethylamine) is a bioactive amine discovered in 1910 by Dale
40 and Laidlaw [1] which is synthesized by decarboxylation of the amino acid histidine,
41 using pyridoxal phosphate (vitamin B6) as cofactor. It is mainly produced in mast cells,
42 basophils, platelets, histaminergic neurons and enterochromaffin cells; where it is
43 stored intracellularly in vesicles until its release upon stimulation. Histamine (HA) is
44 involved in the regulation of different physiological functions such as the secretion of
45 gastric juice, cell growth and cellular differentiation, the day-night rhythm,
46 neurotransmission and immunomodulation [2,3].

47 Two metabolic pathways for HA are known in humans. Histamine-*N*-methyltransferase
48 (HNMT) is the enzyme responsible for the ring methylation of HA and is mainly located
49 in the liver and kidney and it carries out the conversion of HA to 1-methylhistamine
50 (MHA), which will be finally converted to *N*-methylimidazoleacetic acid. As a cytosolic
51 protein, HNMT metabolizes HA only in the intracellular space of cells [4-6]. On the other
52 hand, diamine oxidase (DAO) is an enzyme of mainly intestinal location that performs
53 the oxidative deamination of HA producing imidazole acetaldehyde, which will later be
54 converted to imidazoleacetic acid and finally combined with ribose for its urinary
55 excretion. As a secretory protein, DAO is responsible for scavenging extracellular HA
56 after mediator release, being the main enzyme for the metabolism of intestinal HA [4-
57 6].

58 Histamine intolerance (HIT) is a disorder in the homeostasis of HA due to a reduced
59 intestinal degradation of this amine resulting in its accumulation in plasma and the
60 appearance of multi-faced clinical symptoms, mainly headaches, flatulence, diarrhea,

61 abdominal pain, sneezing, rhinorrhea, hypotonia, arrhythmias, idiopathic urticaria and
62 pruritus [2,3,7]. An enzymatic deficiency of DAO, key enzyme in the intestinal
63 degradation of histamine, can occur based on genetic predisposition, in inflammatory
64 and degenerative intestinal disorders or by pharmacological blockade [3,8,9]. The
65 incidence of HIT has been estimated to be 1% of the population although this percentage
66 may increase as a consequence of a better knowledge and diagnostic of this enzymatic
67 deficiency. Current therapy for HIT is the limitation of foods containing HA, which may
68 be complemented with encapsulated DAO enzyme to contribute to the degradation of
69 intestinal HA [3,10].

70 Currently, the identification of individuals with HIT is based on plasmatic DAO activity
71 through a biochemical assay that measures the amount of HA that can be degraded by
72 this enzyme [11]. An alternative for the diagnosis of HIT by DAO deficiency could be the
73 determination of HA and its metabolites in urine, considering that individuals with
74 insufficient DAO activity would have a distribution profile of these compounds
75 significantly different from healthy individuals. In fact, individuals with symptoms
76 associated with HIT would show a higher urinary content of HA and its major metabolite
77 produced by the HNMT metabolic pathway (MHA) than healthy population.

78 The analytical approach for the simultaneous determination of HA and its metabolites
79 is complex. There are commercial kits that allow the determination of HA through ELISA
80 and of its metabolites through radioimmunoassay (RIA) techniques. However, these
81 immunological-based techniques do not allow simultaneous determination of HA and
82 its metabolites, while, in the case of RIA, they also involve complications related to the
83 use of radioactive material [12]. Alternatively, chromatographic methods, mainly based

84 on high performance liquid chromatography (HPLC) coupled with various detection
85 systems, appear to be the most appropriate for the simultaneous separation and
86 quantification of these compounds [13]. Few HPLC methods are available in the
87 literature for the simultaneous determination of HA and MHA, mainly focused on the
88 quantification of both compounds in laboratory animal plasma and other biological
89 specimens, such as brain or intestinal tissues [14-19]. Although UV detection has been
90 widely used, the high sensitivity and specificity necessary to detect these compounds in
91 samples such as blood makes fluorescence (FL) or mass spectrometry (MS) detection
92 systems more suitable for this purpose. More recently, ultra high performance liquid
93 chromatography (UHPLC) has been proposed for the simultaneous determination of HA
94 and MHA in mice hair and cerebrospinal fluid [20-22]. A fast chromatographic procedure
95 (UHPLC) coupled with FL detection could be an advantageous approach for the routine
96 determination of these analytes in human urine.

97 In order to have a new approach for the diagnosis of histamine intolerance, the aim of
98 this work was to develop and validate a rapid and reliable method to quantify HA and
99 MHA in urine. An UHPLC procedure coupled with an on-line *o*-phthaldehyde (OPA) post-
100 column derivatization and FL detection has been validated in terms of linearity,
101 sensitivity, precision and recovery. Structural analysis of HA and MHA OPA derivatives
102 using UHPLC-ITD-FTMS was carried out to unequivocally identify these compounds in
103 urine samples.

104

105 **2. Material and methods**

106 *2.1. Reagents and chemicals*

107 Histamine dihydrochloride and 1-methylhistamine dihydrochloride were purchased
108 from Sigma (St. Louis, MO, USA). Ultra pure water (18.2 MΩcm) was produced using a
109 LaboStar System from Evoqua Water Technologies (Warrendale, PA, USA). Methanol
110 and acetonitrile (both HPLC grade) were obtained from Fisher Scientific (Loughborough,
111 UK). The other reagent-grade chemicals used were: acetic acid, formic acid, sodium
112 acetate anhydrous, OPA, 2-mercaptoethanol and Brij 35 from Merck (Darmstadt,
113 Germany); hydrochloric acid 0.1M (HCl), potassium hydroxide, boric acid and
114 ammonium 30% from Panreac (Barcelona, Spain); and sodium octanesulphonate from
115 Romil Chemicals (Cambridge, UK).

116

117 *2.2. Standard solutions*

118 Stock solutions of HA and MHA were prepared in 0.1 M HCl for a given concentration of
119 20 mg/L. Pooled working standard solutions (ranging from 0.05 to 10 mg/L) were
120 prepared diluting and mixing aliquot of each compound stock solution with 0.1 M HCl.
121 Standard solutions were protected from light and stored at 4°C until use and filtered
122 through a 0.22 µm membrane filter (GHP, Waters Corp., Milford, MA, USA).

123

124 *2.3. Samples preparation and MCX SPE procedure*

125 Urine samples from twelve subjects aged 22-40 years were collected for 24 hours
126 without the addition of preservatives. All volunteers received an information kit,
127 including an informed consent form and a questionnaire to record any symptomatology
128 associated with HIT. Samples were stored in a freezer (-20 °C) until analysis. Prior to
129 experiments, samples were thawed at room temperature and completely homogenized.

130 Sample preparation consisted of an acidic hydrolysis by adding 750 μL of 0.1 M HCl to
131 10 mL of urine sample. The solution was placed in a heater at 90 $^{\circ}\text{C}$ for 30 minutes and
132 subsequently cooled at room temperature. Later, a purification and concentration
133 procedure was performed by solid phase extraction (SPE) using mixed cation exchange
134 (MCX, 3 mL, 60 mg) cartridges acquired from Waters Corporation (Milford, MA, USA).
135 The cartridges were conditioned with 2 mL of methanol, followed by 2 mL of distilled
136 water. After adsorption of the sample, the cartridge was washed with 10 mL of 0.1 M
137 HCl and dried for 1 minute under vacuum to remove the excess of water. The elution of
138 HA and MHA was performed with 2 mL of 5% NH_4OH in methanol (v/v). The eluate was
139 evaporated to dryness with a centrifugal vacuum concentrator (30 $^{\circ}\text{C}$, 1465 rpm) and
140 later redissolved in 200 μL of 0.1 M HCl and filtered through a 0.22 μm filter (GHP,
141 Waters Corp., Milford, MA, USA) before UHPLC injection.

142

143 *2.4. UHPLC-FL determination of HA and MHA*

144 *2.4.1. Equipment*

145 Chromatographic separation was performed using an UHPLC-FL system consisting of a
146 Waters Acquity™ Ultra Performance Liquid Chromatography equipment, which involved
147 a quaternary pump, an auto-sampler and a FL detector; accomplished with a post-
148 column reagent manager (Waters 510). The post-column pump was connected to a zero
149 dead volume installed between the column outlet and the detector. An Acquity UPLC™
150 BEH C_{18} column (1.7 μm , 2.1 mm x 50 mm) (Waters Corp., Milford, MA, USA) was used
151 as the analytic column. Data acquisition was managed with the Empower version 3
152 system.

153

154 *2.4.2. Chromatographic conditions*

155 Mobile phase consisted of the eluent A as a solution of 0.1 M sodium acetate and 10
156 mM sodium octanesulphonate adjusted to pH 4.8 with acetic acid, and the eluent B as
157 a mixture of solvent B-acetonitrile (6.6:3.4), where solvent B was a solution of 0.2 M
158 sodium acetate and 10 mM sodium octanesulphonate adjusted to pH 4.5 with acetic
159 acid. The mobile phase was filtered through a 0.22 μm filter. The flow rate of the mobile
160 phase was 0.8 mL/min. A linear gradient was applied: 0 min, 80% A; 6 min, 70% A; 6.5
161 min, 0% A; 8.5 min 0% A; 9 min, 80% A. Finally, the system was re-equilibrated for 2 min
162 at the initial conditions before the next injection. Derivatization reagent was prepared
163 by mixing an aqueous solution of 31 g of boric acid and 26.2 g of potassium hydroxide
164 with 0.2 g of OPA dissolved in 5 mL of methanol. To the above solution, 3 mL of 30% Brij
165 and 3 mL of 2-mercaptoethanol as a reducing agent were added and final volume was
166 brought to 1 L with water. The daily prepared post-column derivatization reagent was
167 filtered through a 0.22 μm membrane filter and protected from light. The flow rate of
168 the derivatization reagent was 0.4 mL/min. Automatic injection of 1 μL of the standard
169 solution and samples was carried out. Vials filled with standard solutions or samples
170 were cooled to 4 $^{\circ}\text{C}$ in the auto sampler, the column was kept at 42 $^{\circ}\text{C}$ and post-column
171 reaction equipment was maintained at room temperature. Fluorescence detection at
172 340 nm for excitation and 445 nm for emission was applied.

173

174 *2.5. LC-MS system*

175 An LTQ Orbitrap Velos mass spectrometer (Thermo Scientific, Hemel Hempstead, UK)
176 equipped with an ESI source working in positive mode was used for accurate mass
177 measurements. Mass spectra were acquired in profile mode with a setting of 30,000
178 resolution at m/z 400. Operation parameters were as follows: source voltage, 3.5kV;
179 sheath gas, 50 (arbitrary units); auxiliary gas, 20 (arbitrary units); sweep gas, 2 (arbitrary
180 units); and capillary temperature, 375 °C. Default values were used for most other
181 acquisition parameters (FT Automatic gain control (AGC) target $5 \cdot 10^5$ for MS mode and
182 $5 \cdot 10^4$ for MSⁿ mode). Samples were analysed in full scan mode at a resolving power of
183 30,000 at m/z 400 and MS² events acquired at a resolving power of 15,000. An isolation
184 width of 1 amu was used and precursors were fragmented by collision-induced
185 dissociation C-trap (CID) with normalised collision energy of 35 V and an activation time
186 of 10 ms. The mass range in FTMS mode was from m/z 100 to 1000. Data analysis was
187 achieved using XCalibur software v2.0.7 (Thermo Fisher Scientific). An external
188 calibration for mass was carried out before the analysis.

189 Liquid chromatography analysis was performed using an Accela chromatograph
190 (Thermo Scientific, Hemel Hempstead, UK) equipped with a quaternary pump and a
191 thermostated autosampler. Chromatographic separation was accomplished with an
192 Acquity UPLC™ BEH C₁₈ column (1.7 μm, 2.1 mm x 50 mm) (Waters Corp., Milford, MA,
193 USA) kept at 40 °C. The mobile phase consisted of 0.1% formic acid in water (solvent A)
194 and 0.1% formic acid in acetonitrile (solvent B), and was delivered at a constant flow
195 rate of 0.8 ml/min following a gradient elution: 0 min, 100% A; 3 min, 80% A and 20% B;
196 3.5 min, 80% A and 20% B; 3.6 min 40% A and 60% B; 4.6 min, 40% A and 60% B; 5 min,
197 100% A. Finally, the system was re-equilibrated for 2 min at the initial conditions before

198 the next injection. The autosampler plate was held at 10 °C and the injection volume
199 was 1 µL.

200 For the pre-column derivatization, 2% acid formic in water was used for the preparation
201 of standard solutions and to resuspended urine samples. To 100 µL of standard solution,
202 or redissolved urine sample, 400 µL of the derivatization reagent were added and
203 thoroughly mixed using a vortex. UHPLC-MS analysis was performed immediately after
204 the derivatization reaction due to the low stability of OPA derivatives.

205

206 *2.6. Statistical analysis*

207 The statistical analysis of data was performed with Statistical Software Package for
208 Windows SPSS, version 22 (SPSS, Chicago, IL, USA). Analysis of the variance for linear
209 regression was used to test the reliability of the method. The Student's t-test was used
210 to compare between data sets and the homogeneity of variances was assessed through
211 Cochran's C test.

212

213 **3. Results and discussion**

214 To properly determine total content of HA and MHA in urine, sample preparation
215 consisted in the acid hydrolysis (0.1M HCl) combined with heat treatment. As reported
216 in previous works, higher levels of these compounds were obtained in hydrolysed urine
217 than in non-hydrolysed samples [17]. In order to set final sample hydrolysis parameters,
218 several time and temperature conditions were assayed. Total conversion of conjugated

219 analytes to free HA and MHA was achieved after submitting the sample at 90 °C for 30
220 minutes.

221 Purification and concentration procedure through MCX SPE cartridges was implemented
222 attending the low concentration of HA and MHA in urine and the need to minimize
223 potential interferences of this matrix. When loading urine sample onto the SPE
224 cartridge, acid pH values achieved by previous acidic hydrolysis facilitated the strong
225 interaction of positively charged amine groups of HA and MHA with the sulfonic anion
226 of MCX sorbent. A proper cleanup step washing with 0.1M HCl allowed the reduction of
227 matrix interferences. Later elution with a basic 5% NH₄OH in methanol (v/v) solution
228 ensured cleavage of the electrostatic interactions between ammonium ion of HA and
229 MHA and sulfonic anion of the sorbent. The use of MCX SPE cartridges allowed five-fold
230 pre-concentration of analytes and greater sample concentration was achieved by eluate
231 evaporation to dryness with a centrifugal vacuum concentrator. Overall analytical
232 procedure achieved a final concentration of the analytes of fifty-fold in relation to initial
233 urine content.

234 Due to the low natural fluorescence of the analytes, the use of a derivatizing reagent in
235 order to detect HA and MHA and increase the sensitivity of the method was required.
236 The presence of amino groups in the structures of HA and MHA makes both compounds
237 suitable for the derivatization with a large number of fluorogenic reagents, being OPA,
238 fluorescamina and DBD-F (4-(*N,N*-dimethylaminosulfonyl)-7-fluoro-2,1,3-
239 *benzoxadiazole*) the most commonly used [12,13,20]. In this method, an online post-
240 column OPA derivatization procedure was used, which ensured a high reproducibility by
241 minimizing sample manipulation prior to the injection. Moreover, OPA reacts rapidly

242 with amines in the presence of a reducing agent, improving detection sensitivity,
243 reducing the polarity of original amino compounds and increasing method selectivity
244 [23]. The current method provides a significant improvement in comparison with some
245 previous methods that mostly used pre-column derivatization techniques, which could
246 face problems related to the low stability of OPA-amine derivatives [12,13].

247 According to previous methods, several mobile phase compositions and gradient
248 programmes were assayed to get the best resolved peaks for HA and MHA [12,13,24].
249 Considering the slightly structural differences of the analytes, a mobile phase consisting
250 in two eluents of different polarity and pH permitted to establish the necessary gradient
251 in order to properly separate HA and MHA. This was achieved by a gradual and linear
252 slight increase of eluent B, the less polar eluent of the mobile phase, during the first
253 minutes of the chromatographic run. Just after the separation of both analytes, the
254 proportion of acetonitrile was markedly incremented aiming to completely elute less
255 polar urinary compounds, which consequently incremented the chromatographic run
256 up to the final 11 minutes. Moreover, sodium octanesulphonate as ion-pairing reagent
257 was added to the mobile phase in order to improve chromatographic separation of
258 these hydrophilic and polar compounds. Fig. 1 shows the chromatograms of the
259 standard solutions and of urine samples. The proposed method accomplished an
260 acceptable separation between HA and MHA with a chromatographic resolution (R) of
261 1.5. HA and MHA were identified on the basis of the retention time by comparison with
262 the standard.

263 The present UHPLC method reduces considerably the time required for urinary
264 determination of HA and MHA in comparison with previously published HPLC-FL

265 methods, resulting in turn in decreased reagent costs and reduced environmental
266 impact [14,15,17]. The reliability of this UHPLC method for routine analysis of urine
267 samples was assessed in terms of linearity, sensitivity, precision and recovery.

268

269 *3.1. Linearity*

270 Linearity was tested at twelve different concentrations between 0.05 and 10 mg/L,
271 performing seven measurements at each level. Analysis of the variance of the regression
272 allowed assessing the linearity of the UHPLC method. Least-squares analysis resulted in
273 a correlation coefficient of $r \geq 0.9999$ for both analytes ($p < 0.001$). Calibrations data fit a
274 linear regression model with determination coefficients (r^2) higher than 99.99% for all
275 standard curves.

276

277 *3.2. Sensitivity*

278 The chromatographic limit of detection (LOD) and the limit of quantification (LOQ) were
279 obtained following the IUPAC guidelines and using low-concentration HA and MHA
280 standard regression curves ranging from 0.05 to 0.8 mg/L [25]. A blank consisting in 0.1
281 M HCl was used to determine baseline noise. LODs were 0.025 mg/L for HA and 0.028
282 mg/L for MHA. LOQs were 0.035 mg/L and 0.045 mg/L for HA and MHA, respectively. To
283 confirm the established LODs and LOQs a standard solution at those level
284 concentrations was analysed (Table 1).

285

286 *3.3. Precision*

287 Method precision was evaluated through repeatability by carrying out seven
288 independent determinations of a urine sample from a volunteer using the same
289 analytical conditions (Table 1). Urine and spiked urine with known amounts of HA and
290 MHA (0.01, 0.04 and 0.15 mg/L) were studied in septuplicate in order to test the
291 precision at different levels. The relative standard deviation (RSD) for HA and MHA at all
292 concentration levels was lower than 5.5%, representing a satisfactory level of precision.
293 The Horwitz equation for intra-laboratory studies confirmed the acceptability of these
294 precision results.

295

296 *3.4. Recovery*

297 Method recovery was determined via accuracy evaluation by the standard addition
298 procedure using urine samples spiked with three addition levels (0.01, 0.04 and 0.15
299 mg/L of HA and MHA). Seven determinations were performed for each addition level
300 (Table 1). The mean recovery of HA was 99.25% (SD=1.86), which was not statistically
301 different from the theoretical value of 100% ($p>0.005$ according to the Student's t-test).
302 For MHA, mean recovery was 99.8% (SD=0.21), which neither was statistically different
303 from the theoretical value of 100% ($p>0.005$). The assumption of homogeneity of
304 variances among the three spiking levels was tested using Cochran's C test. Experimental
305 values for both analytes remained under the Cochran's test tabled value, confirming
306 that the variance of recovery values was not dependent on the analyte content
307 ($p>0.005$).

308

309 *3.5. Structural analysis of HA and MHA OPA derivatives using UHPLC-HRMS*

310 The structure of HA and MHA conjugated with OPA were confirmed by UHPLC-ITD-FTMS
311 which enabled specific detection of both compounds in a complex matrix such as urine.
312 The spectra showed the protonated molecule with a mass error of less than 2 ppm.
313 The mass spectra shown in Fig. 3 and Fig. 4 confirmed that the OPA complex for HA was
314 formed with a molecular ion $[M+H]^+$ at $m/z = 288.1162$ (-1.2 ppm error) in the standard
315 and a molecular ion $[M+H]^+$ at $m/z = 288.1165$ (0.0 ppm error) in the urine sample. For
316 MHA, OPA conjugated complex was observed with a molecular ion $[M+H]$ at $m/z =$
317 302.1319 (-1.1 ppm error) and at $m/z = 302.1318$ (-0.8 ppm error) in the standard and
318 urine sample, respectively.
319 Moreover, injection in MS² mode was performed. The MS² spectra gave as result an ion
320 with m/z 228.1132 for HA and an ion with m/z 242.1290 for MHA. Confirmation in
321 samples was accomplished by injection of urine samples in the same conditions. Results
322 are shown in Fig. 3 and Fig. 4.

323

324 3.6. UHPLC-FL sample analysis

325 Urine samples from twelve volunteers were analysed using the proposed method (**Fig.**
326 **2**). No significant differences were found in HA urinary content from different volunteers
327 with a mean value of 0.143 ± 0.08 $\mu\text{mol/day}$, which is in good agreement with the values
328 obtained by other works [17,26]. On the contrary, greater differences in MHA content
329 were found in analysed urine samples. Concretely, two individuals who reported
330 symptomatology related to HIT showed a significantly higher urinary content of MHA.
331 Moreover, the increased MHA contents exceeded the threshold established as normal
332 level of this analyte in urine [27]. These results support the initial hypothesis according

333 to which the decrease in DAO activity would cause an accumulation of MHA, the
334 metabolite produced by HNMT. Therefore, this method could be an advantageous and
335 more patient-friendly alternative to current plasmatic DAO activity determination used
336 to diagnose histamine intolerance, being less invasive and avoiding the need for specific
337 equipment and qualified personnel required for plasma sample extraction. Additionally,
338 the distribution profile of histamine and its main metabolite in urine could provide a
339 complementary evaluation of DAO activity, specifically considering that some authors
340 have reported a wide variability in DAO activity both in healthy volunteers and patients
341 diagnosed with HIT according to their symptomatology [28,29].

342

343 **4. Conclusion**

344 The proposed UHPLC method allows the satisfactory determination of urinary HA and
345 MHA in less than 11 minutes. The use of MCX SPE cartridges was effective for the
346 selective purification and concentration of HA and MHA in human urine. Overall sample
347 treatment procedure achieved a final concentration of the analytes of fifty-fold in
348 relation to initial urine content. Online post-column derivatization of the analytes with
349 OPA permitted the sensitive detection of the analytes while minimizing sample
350 manipulation prior to UHPLC injection. Unequivocal identification of HA and MHA OPA
351 derivatives in standard and in urine samples has been accomplished through UHPLC-
352 ITD-FTMS. To our knowledge, this is the first UHPLC-FL method with OPA post-column
353 derivatization used to determine HA and MHA in human urine; thus becoming a
354 potential new approach for the routine diagnosis of histamine intolerant individuals.

355 Further studies involving more volunteers are needed to validate MHA as a biomarker
356 for the diagnosis of HIT.

357

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456

457 **Figure captions**

458 **Fig 1.** Chromatograms of HA and MHA standard solution of 10 mg/L (A) and a urine
459 sample (B).

460 **Fig 2.** Urinary content of HA and MHA ($\mu\text{mol/day}$) in twelve volunteers. Dashed lines
461 indicate normal levels of HA and MHA reported by literature.

462 **Fig 3.** Representative chromatogram, FTMS spectra and MS² spectra of OPA derivative
463 HA (A) and MHA (B) standards.

464 **Fig 4.** Representative chromatogram, FTMS spectra and MS² spectra of OPA derivative
465 HA (A) and MHA (B) in a urine sample.

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466 **Table 1.** Summary of validation results.

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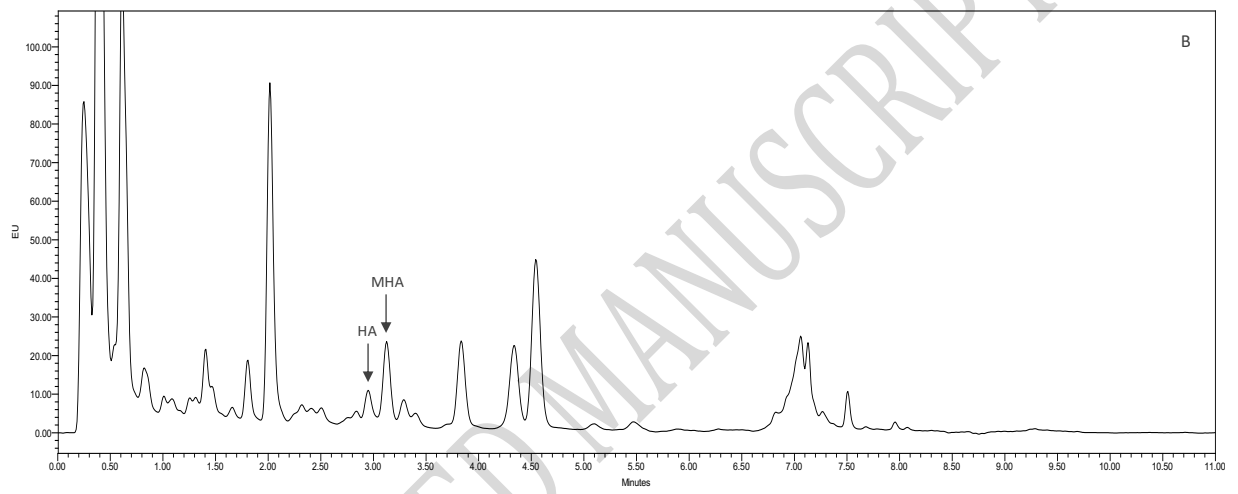
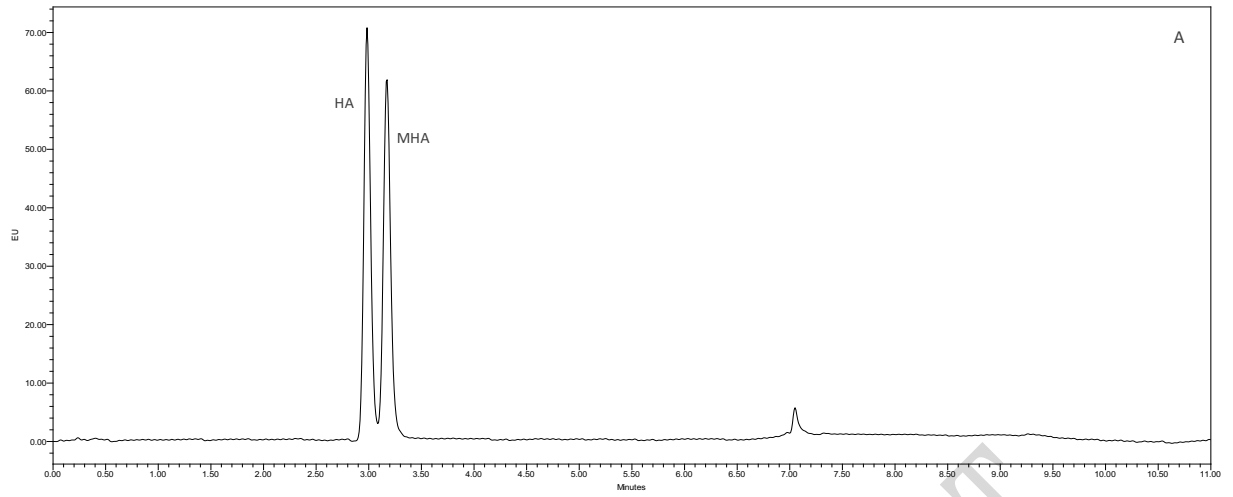
Sensitivity	Precision			Recovery ^c										
	LOD (mg/L)	LOQ (mg/L)	Urine	Addition level I 0.01 mg/L of HA and MHA	Addition level II 0.04 mg/L of HA and MHA	Addition level III 0.15 mg/L of HA and MHA								
			RSD (%) ^a	RSDH (%) ^b	RSD (%)	RSDH (%)	RSD (%)	RSDH (%)						
HA	0.025	0.035	2.15	8.12 - 10.83	2.98	7.60 - 10.13	5.24	6.84 - 9.11	4.32	5.81 - 7.75	100.58 (2.27)	97.91 (5.13)	99.24 (4.29)	0.53
MHA	0.028	0.045	2.77	6.03 - 8.04	1.09	5.96 - 7.95	1.24	5.80 - 7.73	0.32	5.63 - 7.50	99.95 (2.93)	99.65 (1.71)	102.41 (0.33)	0.74

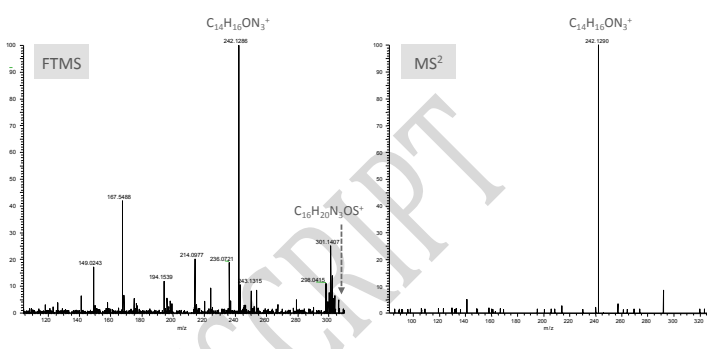
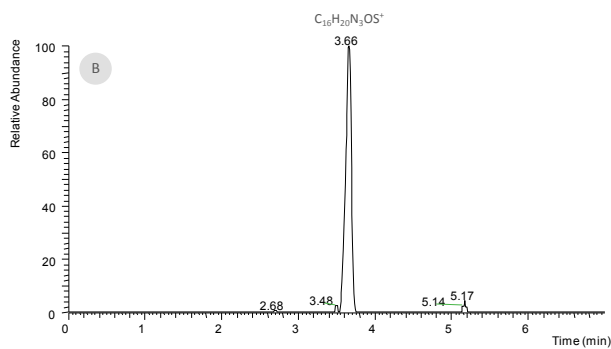
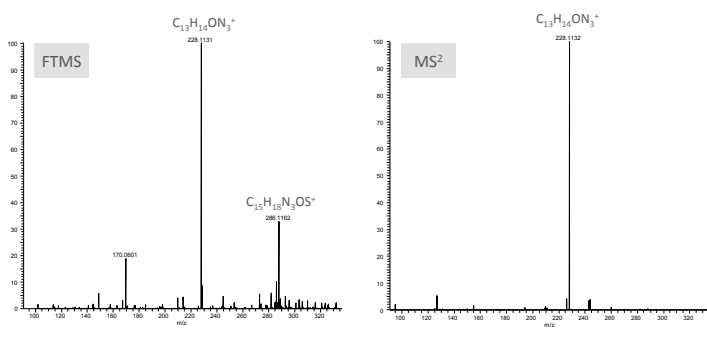
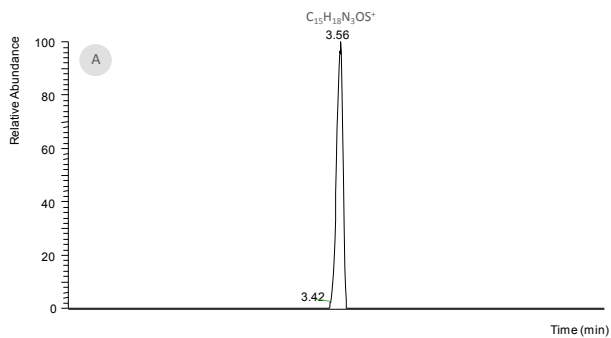
^a Relative standard deviation (RSD) for seven determinations.

^b Acceptable range for relative standard deviations according to Horwitz's formula for intra-laboratory studies (1/2 - 2/3 of the interlaboratory study calculate by the formula).

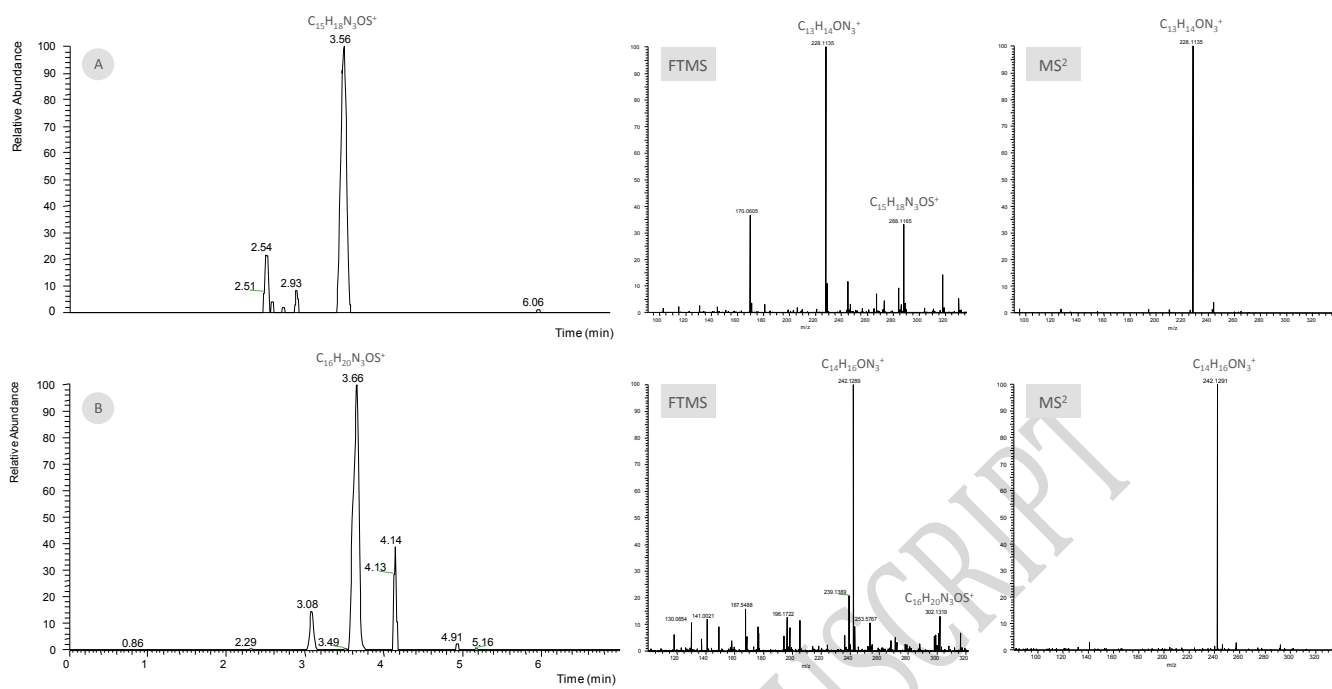
^c Mean recovery percentages and standard deviation in parentheses.

^d Variance outlier test Cochran C, C_{tab} (6,2,0.005)= 0.8534.

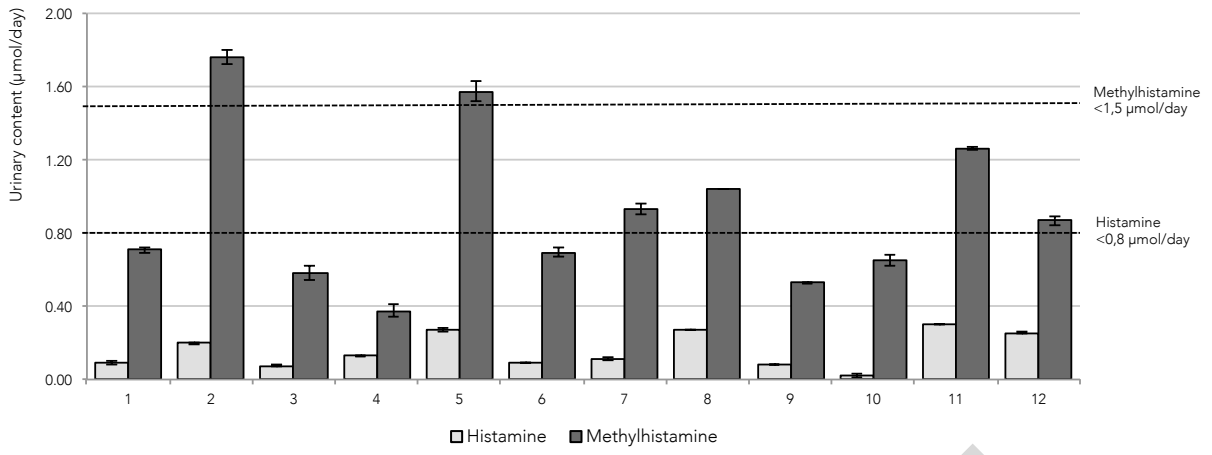




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