A UHPLC method for the simultaneous analysis of biogenic amines, amino acids and ammonium ions in beer

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Running title: UHPLC detection of amino compounds in beer

35 ABSTRACT
36
37 This paper reports a novel UHPLC method for simultaneously quantifying nine biogenic
38 amines, 21 amino acids, and ammonium ions, in beer. Precision values of standard curves
39 slopes were lower than 3.4% and recovery was between 85% and 106%, indicating the absence
40 of matrix effect. Linear calibration curves were obtained for analyte concentrations between two
41 and four orders of magnitude (R^2>0.996). Repeatability tests returned mean variations of 3.2%
42 and 0.5% for beer and a standard solution, respectively. Sensitivity ranged between 0.03 mg/L
43 and 0.63 mg/L for the biogenic amines, and 0.05 mg/L and 5.19 mg/L for other compounds.
44 Original data on the habitual presence of ethanolamine in beers are presented. The method
45 allows for more samples to be assayed per unit time, it uses less solvent than other techniques
46 and therefore reduces costs and the associated waste. It could be a valuable tool for monitoring
47 the safety and quality of beers.

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51 *Keywords*: biogenic amines, amino acid, UHPLC, DEEMM, beer
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54 Chemical compounds studied in this article
55 Diethylethoxymethylenemalonate (PubChem CID: 6871); Ethanolamine (PubChem CID: 700);
56 Agmatine (PubChem CID: 199); Histamine (PubChem CID: 774); Tyramine (PubChem CID:
57 5610); Ethylamine (PubChem CID: 6341); Putrescine (PubChem CID: 1045); Cadaverine
58 (PubChem CID: 273); Tryptamine (PubChem CID: 1150); β-phenylethylamine (PubChem CID:
59 1001).
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63 **1. Introduction**

64 Beer is one of the most consumed of all fermented beverages. Its nutritional value, aroma
65 and flavour depend greatly on the proteolytic events that the raw materials (mainly barley and
66 hops) undergo during malting and brewing, resulting in each beer having a particular amino acid
67 composition (Gorinstein et al., 1999; Bokulich & Bamforth, 2013). The amino acid content of a
68 beer also reveals whether wheat adjuncts were present during brewing (Dale, Young & Brewer,
69 1989).

70 Certain amino acids can be decarboxylated by microbial enzymes, producing amino-
71 containing compounds known as biogenic amines (BA) (reviewed in Kalac & Krízek, 2003).
72 For instance, the decarboxylation of histidine renders histamine, while tyrosine, ornithine, lysine
73 and phenylalanine can be decarboxylated to produce tyramine, putrescine, cadaverine and β-
74 phenylethylamine, respectively (Lonvaud-Funel, 2001; Kalac & Krízek, 2003; Spano et al.,
75 2010; Linares, Martin, Ladero, Alvarez & Fernandez, 2011). High concentrations of BAs in
76 food are considered a biological hazard by international regulatory organizations (European
77 Food Safety Authority [EFSA], 2011; Food and Drug Administration of the United States of
78 America, 2011; Food & Agriculture Organization of the United Nations, 2014). Indeed, the
79 ingestion of large quantities of BAs can cause toxicological reactions leading to neurological
80 problems, headaches, hypo- or hypertension, nausea, heart palpitations and kidney poisoning
81 etc., with symptoms particularly severe in persons with suboptimal BA-detoxifying amine
82 oxidase capacity (Shalaby, 1996; Caston, Eaton, Gheorghui & Ware, 2002; Ladero, Calles-
83 Enríquez, Fernandez & Alvarez, 2010; EFSA, 2011). In addition, tyramine and histamine have
84 recently been shown to exert considerable cytotoxic effects *in vitro* (Linares, del Rio, Redruello,
85 Ladero, Fernandez, Martin & Alvarez, 2016). An extra concern regarding the consumption of
86 alcoholic beverages centres around a potential synergistic effect between ethanol/acetaldehyde
87 and some BAs causing the inhibition of detoxifying amino oxidases (Maynard & Schenker,
88 1962).

89 The type and concentration of BAs in beer are affected largely by the raw materials and
90 brewing techniques employed in the production process, plus the hygiene conditions maintained

91 (Halász, Baráth & Holzapfel, 1999; Kalac & Krízek, 2003). Different concentrations of
92 histamine, tyramine, putrescine, cadaverine, β-phenylethylamine and tryptamine have been
93 reported in different types of beer (Zee, Simard & Desmarais, 1981; Izquierdo-Pulido, Albalá-
94 Hurtado, Mariné-Font & Vidal-Carou, 1996; Kalac, Hlavatá & Krízek, 1997; Halász et al.,
95 1999; Slomkowska & Ambroziak, 2002; Bunka et al., 2012; Aflaki, Ghoulipour, Saemian &
96 Sheibani, 2014). However, the presence of ethanolamine and ethylamine, which are known to
97 appear in other alcoholic beverages (including wine; Galgano, Caruso, & Favati, (2009)), has
98 been little studied in beer.

99 The availability of rapid and validated methods for detecting BAs in foods is essential if their
100 concentrations are to be minimised (Kalac & Krízek, 2003; EFSA, 2011; Spano et al., 2010;
101 Alvarez & Moreno-Arribas, 2014). Currently, high-performance liquid chromatography (HPLC)
102 is the only reliable technique for monitoring BA concentrations in foods and beverages (EFSA,
103 2011). The use of chromatographic columns with particles under 2 μm in diameter (so-called
104 ultra-HPLC [UHPLC]) guarantees shorter elution times, greater sensitivity and improved peak
105 resolution.

106 The simultaneous analysis of amino acids and BAs is difficult given their different structures
107 and the absence of a specific chromophore; a pre- or post-column derivatization step is therefore
108 usually required. Among the derivatizing reagents used for the simultaneous analysis of these
109 compounds in food matrices diethyl ethoxymethylenemalonate (DEEMM) has many
110 advantages: its coupling with primary and secondary amino compounds, the good stability of
111 the aminoenone derivatives produced, simplicity of use, and the absence of post-reaction by-
112 products (Alaiz, Navarro, Girón & Vioque, 1992; Gómez-Alonso, Hermosín-Gutiérrez &
113 García-Romero, 2007; Redruello, Ladero, Cuesta, Alvarez-Buylla, Martin, Fernandez &
114 Alvarez, 2013; Wang, Ye, Zhu, Wu & Duan, 2014). Further, it is the official derivatizing agent
115 of the *Organisation International du Vin* (OIV, 2014).

116 A DEEMM derivatization procedure coupled to UHPLC-photodiode array detection (DAD)
117 has been reported reliable for the simultaneous analysis of amino acids and BAs in cheeses and
118 red wines (Redruello et al., 2013; Wang et al., 2014). However, no reports exist on the use of

119 this technique for analysing beers. The present work reports the development and validation of a
120 UHPLC-based method for the simultaneous analysis of the aminoenone derivatives of the BAs,
121 amino acids and ammonium ions present in beer.

122

123 **2. Materials and Methods**

124 *2.1. Reagents and beer samples*

125 HPLC-grade acetonitrile and sodium hydroxide were purchased from VWR (Barcelona,
126 Spain), methanol and hydrochloric acid from Merck (Darmstadt, Germany), boric acid from
127 USB (Cleveland, OH, USA), and ammonium acetate, sodium azide, DEEMM, L-2-aminoadipic
128 acid (internal standard), amino acids, BAs and ammonium chloride from Sigma-Aldrich
129 (Madrid, Spain). All solutions were made with Milli-Q water. Eleven beers (lager or ale) made
130 by different European brewers and produced using different techniques, were chosen for
131 analysis. Four of them (two lager and two ale) were used during the matrix effect analysis (see
132 2.4.1 section for samples description). All samples were purchased in retail stores in Spain.

133

134 *2.2. Derivatization reaction*

135 Containers of beer were left open for 2 h at room temperature to remove their carbon dioxide
136 content. Samples of the de-carbonated beers were then centrifuged at 8000 g for 5 min to
137 eliminate any particulate matter. DEEMM derivatization reactions were performed as described
138 in Redruello et al. (2013), using 100 µl samples (or of standard solution when constructing
139 calibration curves). When necessary, samples were diluted with 0.1 N HCl. After derivatization,
140 samples were filtered through 0.22 µm polytetrafluoroethylene (PTFE) membranes (VWR) into
141 conical vials (VWR) prior to injection into the UHPLC system.

142

143 *2.3. Equipment and chromatographic conditions*

144 The chromatographic column and the UHPLC equipment used were those described in
145 Redruello et al. (2013). The mobile phase consisted of 25 mM acetate buffer plus 0.02% sodium
146 azide (eluent A; pH 6.7), methanol 100% (eluent B) and acetonitrile 100% (eluent C). Samples

147 (1 μl) were applied to the column and eluted at a flow rate of 0.45 mL/min according to the
148 ternary gradient shown in Table 1. The column was then returned to the initial conditions within
149 1 min, and allowed to equilibrate for 4.5 min before the next injection. Data were acquired and
150 analysed using Empower 2 software (Waters). The target compounds were identified by their
151 retention times compared to standards, and quantified using the internal standard method.

152

153 *2.4. Method validation*

154 *2.4.1. Evaluation of matrix effect*

155 The precision value (RSD, relative standard deviation) of standard curves slopes in five
156 different lots of a biofluid was used to evaluate the existence of matrix effect (Matuszewski,
157 2006). This value should not exceed 4% for the method to be considered free from matrix effect
158 (Matuszewski, 2006). Additionally, the RSD of the peak areas measured at all the spiked
159 concentrations in the five lots of biofluids should not exceed 10-15% (Matuszewski, 2006). We
160 used as five different biofluids four beer samples of different matrix complexity (an alcohol-free
161 french lager, an artisan spanish lager, and two abbey-style dark belgian ale beers) and a 0.1 N
162 HCl solution acting as solvent matrix. Mixtures of analytes at five different concentrations (15,
163 30, 60, 120 and 240 μM) were added (spiked) to each of the five matrices. The calibration
164 curves obtained for each analyte in the five matrices were then calculated and their slopes
165 compared. The RSD of the standard curves slopes as well as the RSD of the peak areas
166 measured at all spiked concentration were then calculated.

167 Recovery of each analyte was calculated from the calibration data obtained as described
168 above as [(area measured in the spiked sample)–(area measured in the non-spiked sample)/(area
169 measured in the solvent 0.1 N HCl solution] x 100. Total recovery for each analyte was the
170 average of the individual recoveries calculated from all the spiked concentrations and all the
171 matrices.

172 *2.4.2. Linearity, repeatability and sensitivity*

173 Linearity, repeatability and sensitivity were validated according to Taverniers, de Loose &
174 van Bockstaele (2004). Linearity was tested by regression analysis from three independent

175 calibration curves that contained all the analytes (a mixture of amino acids, BAs and ammonium
176 chloride) dissolved in 0.1 N HCl as solvent matrix and that ranged from 0.0007 mM to 1.5 mM.
177 All the calibration mixtures included a fixed concentration (0.07 mM) of the internal standard
178 (IS). Calibration curves were calculated by plotting analyte peak area/IS peak area ratio against
179 the known concentrations of the analyte.

180 Intra-day repeatability (same analyst, apparatus and reagents) was assessed by injecting a
181 mixture containing all the analytes six times during the same chromatographic run. Inter-day
182 repeatability (same analyst and apparatus but different reagents) was assessed by injecting
183 individual samples of a standard mixture over eight days.

184 LOD and LOQ were calculated for each analyte as three times and 10 times the
185 baseline/noise ratio respectively. The 10 blank replicates used contained 0.1 N HCl instead of
186 the sample. The lowest concentration in the linear range was deemed to represent the LOQ.

187

188 **3. Results and Discussion**

189 *3.1. Optimisation of the UHPLC separation step*

190 Ethanolamine and ethylamine co-eluted with other amino compounds present in the beer
191 matrix: ethanolamine co-eluted with ammonium ions and tyrosine, while ethylamine co-eluted
192 with lysine. Modification of the pH of the ammonium acetate buffer did not improve the
193 separation achieved due to the very similar pKa values of the alpha ammonium ions of
194 ethanolamine (pKa 9.40) and tyrosine (pKa 9.11), and that of regular ammonium ions (pKa
195 9.26). Fine tuning of the solvent proportions (aqueous buffer/acetonitrile/methanol) providing
196 the ternary gradient was required until the profile described in section 2.4 was reached. The
197 final ternary gradient allowed the separation of the 21 amino acids, 9 BAs and ammonium ions
198 searched for within a 14 min elution time (Fig. 1A). The amino-compounds were identified in a
199 beer sample by comparison of their retention times against those recorded for the standard
200 mixture. No interfering peaks were observed (Fig. 1B).

201 The chromatographic run time was between 4 and 6 times faster than reported for previous
202 HPLC methods designed to simultaneously analyse amino acids and BAs in beer (Gómez-

203 Alonso et al. 2007; Hu et al., 2014). The achieved runtime highlights the proposed method as
204 one of the fastest liquid chromatography approaches for simultaneous amino acids, BAs and
205 ammonium ions analysis so far (the fastest UHPLC method was reported by Fiechter, Sivec &
206 Mayer in 2013 for acid-curd cheeses analysis). This would be an advantage for research and
207 company laboratories that deem speed an asset. Shorter analysis times also signify the use of
208 less solvent, reducing costs and the associated waste (and thus the environmental impact of the
209 method). The method might help also to rapidly evaluate technological aspects such as the
210 influence of substrate availability on amino acid metabolism and BA synthesis during brewing.
211 Also, questions regarding the influence of bacteria and yeasts on BA accumulation in beers, or
212 even the selection/application of degrading bacteria to eliminate BAs could be rapidly
213 answered. Many of these factors are being studied in the production process of wines (Ortega-
214 Heras et al., 2014; Tristezza et al., 2013; Capozzi et al., 2011; Arena et al., 2011; Landete,
215 Arena, Pardo, Manca de Nadra & Ferrer, 2008).
216
217 *3.2. Method validation*
218 *3.2.1. Absence of matrix effect*
219 We have calculated the RSD from five standard curve slopes constructed after spiking four
220 beers of different complexity plus a 0.1 N HCl solution as solvent matrix with five different
221 analytes' concentration. All the slopes RSD values were ≤ 3.4 % (Table 2); none of them
222 exceeded the cut-off RSD value (4%) to consider a bioanalytical method free from a matrix
223 effect (Matuszewski, 2006). Moreover, the RSD of the peak areas measured at all the spiked
224 concentrations and in the five matrices did not exceed 10 % in any case (Table 2). These high
225 precision values support the absence of matrix effect and the reliability of the present method to
226 quantify 31 aminoenone derivatives in beer samples, under the experimental conditions
227 employed. In a previous study involving beers and wines, no matrix effect was reported for any
228 of the aminoenones analysed therein (Gomez-Alonso et al., 2007). However, other authors have
229 reported a matrix effect for the aminoenones of proline and ethanolamine in wine in the early

230 stages of fermentation (Wang et al., 2014). This effect was attributed to the presence of glucose
231 concentrations of >50.0 g/L.

232 Table 2 also shows the total recovery of each analyte, after computing the recoveries
233 obtained in the five matrices and the five spiked concentrations of analyte. Recovery values
234 (from 85 % the lowest to 106 % the highest) are within the acceptable limits for the
235 concentrations added (Taverniers et al., 2004), indicating the accuracy of the proposed method.
236 The individual recoveries from each of the spiked concentrations can be found in Redruello,
237 Ladero, del Rio, Fernandez, Martin & Alvarez (2016).

238

239 *3.2.2. Linearity, repeatability and sensitivity of the method*

240 Calibration curves were then constructed in 0.1 N HCl as solvent matrix solution. The
241 linearity of the results was verified by analysing the variance of the regression. An R^2 value of
242 >0.995 was returned for all the calibration curves (Table 3). The concentration range was linear
243 between two and four orders of magnitude, depending on the analyte.

244 Table 3 shows also the repeatability and sensitivity of the proposed method. The variation in
245 analyte retention time within the chromatographic column was <0.23% in intra-day tests
246 (repeatability) and <0.27% in inter-day tests (reproducibility). The variability of the peak area
247 for the analytes was between 0.06% and 1.66% in intra-day analysis, and between 0.33% and
248 1.58% in inter-day runs. These results indicate an acceptable repeatability and reproducibility of
249 the method (Taverniers et al., 2004).

250 Proline returned the highest LOQ of all the amino acids (5.19 mg/L), while serine returned
251 the lowest (0.05 mg/L). For the BAs, the LOQ ranged between 0.03 mg/L for putrescine and
252 0.63 mg/L for ethylamine. Ammonium ions returned an LOQ of 0.49 mg/L. The LOD was
253 below 0.13 mg/L for ethylamine, tyramine and histamine and ≤0.10 mg/L for ethanolamine,
254 agmatine, putrescine, tryptamine, cadaverine and phenylethylamine. The LOD range for the
255 amino acids and ammonium ions was between 1.04 mg/L for proline and 0.01 mg/L for serine.
256 Overall, the sensitivity results agree well with those provided by other UHPLC methods for
257 determining amino compounds in different foods (Latorre-Moratalla, Bosch-Fuste, Lavizzari,

258 Bover-Cid, Veciana-Nogues & Vidal-Carou, 2009; Mayer, Fiechter & Fischer, 2010; Redruello
259 et al., 2013; Wang et al., 2014) and even with that reported using quantitative nuclear magnetic
260 resonance (NMR) technique (Chatzimitakos, Exarchou, Ordoudi, Fiamegos & Stalikas, 2016).
261 Some analytical methods that employ mass detectors can reach sensitivities between 10 and
262 1000 times higher than those of HPLC-derived techniques (Daniel, Dos Santos, Vidal & do
263 Lago, 2015). However, HPLC-derived methods are amply sensitive to quantify the toxic
264 concentrations of BA (EFSA, 2011).
265

266 *3.3. Analysis of aminoenone derivatives in beer samples*

267 Under optimized experimental conditions, the method was used to determine the
268 aminoenone derivatives of amino acids, ammonium ions and BAs in different beer samples.
269 Wide variation was detected in the amino acid and BA concentrations of the different beers
270 (Table 4).

271 *3.3.1. Amino acid content*

272 The amino acid content of a beer determines its aroma and flavour. It depends directly on the
273 proteolytic events that take place during the transformation of the raw materials (barley, hops
274 and other cereals such as wheat or corn) during brewing (Gorinstein et al., 1999). Between the
275 initial and final stages of beer-making, the protein content decreases while the content of some
276 amino acids increase concomitantly (Gorinstein et al., 1999). The assimilation of the released
277 amino acids by microorganisms, mainly yeasts, during fermentation influences their final
278 concentrations in the beer (Jones & Pierce, 1964). In the present work, the most abundant amino
279 acid was always proline, with concentrations ranging from 238 to 688 mg/L (mean 421.33
280 mg/L). These findings are consistent with those of previous work (Gomez-Alonso et al., 2007).
281 Proline is also the most abundant amino acid in other fermented beverages, such as red wines,
282 although its concentration is some 4-6 times higher than in beers (Gomez-Alonso et al., 2007;
283 Kutlán & Molnár-Perl, 2003). A possible explanation for the abundance of proline in beer is its
284 poor assimilation by the yeasts present (Jones & Pierce, 1964). The next most abundant amino
285 acids detected were γ-aminobutyric acid (GABA) (mean concentration 57.79 mg/L), alanine

286 (49.21 mg/L), tyrosine (40.98 mg/L), phenylalanine (37.68 mg/L) and arginine (29.57 mg/L).
287 The mean concentrations of the remaining amino acids did not exceed 25 mg/L. The least
288 abundant amino acid was glutamine, the concentration of which never exceeded 3.5 mg/L,
289 followed by ornithine (mean concentration 2.62 mg/L), threonine (2.98 mg/L) and methionine
290 (4.73 mg/L). The few reports that exist regarding the amino acid content of beers generally
291 corroborate this concentration distribution (Gomez-Alonso et al., 2007; Hu et al., 2014; Jones &
292 Pierce, 1964; Dale et al., 1989). The only exception is GABA; some of these studies report it as
293 one of the most abundant amino acid, while others report it to be among the least abundant. This
294 might be explained by the analytical techniques employed. For instance, in those studies in
295 which DEEMM was the derivatizing agent (Gomez-Alonso et al., 2007; this work), GABA was
296 among the most abundant amino acids. However, where o-phthalaldehyde-β-mercaptoethanol
297 was used to derivatize the sample, only trace amounts of GABA were detected (Hu et al., 2014).
298 The amino acids and BAs profile of a beer has also been recently used to discriminate
299 between homofermentative and heterofermentative beer spoilage LAB (Geissler, Behr, von
300 Kamp & Vogel, 2016).
301
302 *3.3.2. BA content*
303 Ethanolamine (mean concentration 8.37 mg/L; range: 4.40-12.95 mg/L), putrescine (2.84
304 mg/L; 1.59-4.05 mg/L) and cadaverine (0.69 mg/L; detected but not quantified-0.92 mg/L) were
305 found in all the samples. Agmatine (4.46 mg/L; 0.00-12.50 mg/L) and ethylamine (0.50 mg/L;
306 0.00-1.35 mg/L) were recorded for eight of the 11 beer types. Histamine and β-
307 phenylethylamine were found in one sample only (and in trace amounts); tryptamine was
308 detected in no sample. Tyramine was found in 10 of the 11 beers. Its concentration was,
309 however, the most variable (relative standard deviation 258%); the majority of samples
310 contained less than 1 mg/L of tyramine, but one contained around 10 mg/L and another around
311 60 mg/L. Other authors report similar findings for beers of different geographical origin, and
312 propose this might be explained by the presence of lactic acid bacteria during fermentation
313 (Izquierdo-Pulido et al., 1996; Kalac & Krízek, 2003). Tyramine has traditionally been

314 considered responsible for the post-beer-consumption hypertension crises observed in patients
315 with depression and treated with monoamine oxidase inhibitors (Shulman, Tailor, Walker &
316 Gardner, 1997).

317 Histamine and cadaverine may also appear in beer due to the metabolism of contaminating
318 LAB (Kalac & Krícek, 2003). Indeed, the histamine content of beer has been proposed a good
319 indicator of brewing hygiene (Halász et al., 1999). In the present work, no histamine was
320 quantified in any sample, and the cadaverine concentration never exceeded 0.92 mg/L. This
321 would appear to indicate that proper hygiene conditions were followed during the production of
322 all the analysed beers.

323 Putrescine and agmatine are constituents of natural beer, originating in the malt (Kalac &
324 Krícek, 2003). The mean concentrations of agmatine and putrescine recorded in the present
325 work never exceeded 5.0 mg/L and 3.0 mg/L respectively; these values are similar to, or lower
326 than, those reported in the literature (Zee et al., 1981; Izquierdo-Pulido et al., 1996; Kalac et al.,
327 1997; Slomkowska & Ambroziak, 2002; Aflaki et al., 2014).

328 No tryptamine was found in any of the samples analysed in the present work. Only a few
329 reports exist regarding the content of this BA in beers, and in general it has been found at
330 concentrations of <2 mg/L (Izquierdo-Pulido et al., 1996; Aflaki et al., 2014).

331 Ethanolamine and ethylamine are BAs usually present in grapes and consequently found in
332 wines (reviewed in Galgano, Caruso & Favati, 2009). Until now, however, only one paper has
333 reported the presence of ethylamine in beer (Zotou, Loukou, Soufleros & Stratis, 2003), and to
334 the best of our knowledge the literature contains no report of ethanolamine in this beverage. In
335 the present work, the mean ethylamine concentration was 0.5 mg/L; the highest concentration
336 was 1.22 mg/L, quite similar to that recorded (1 mg/L) in the only other reported work to
337 analyse ethylamine in beer (Zotou et al., 2003). The mean ethanolamine concentration was
338 however among the highest for all the BAs analysed. The origin of ethylamine and
339 ethanolamine in beer remains unclear, although their regular presence in the samples examined
340 in the present work, which came from different countries and were produced following different
341 brewing methods, suggests they are a common constituent of beer.

342 In agreement with previous reports on the total BA concentration of beers (Kalac & Kricek, 343 2003; Loret, Deloyer & Dandrifosse, 2005), values of 7.03-78.90 mg/L (mean 23.66 mg/L) 344 were recorded in the present work. Thus, most of the analyzed samples represented no health 345 hazard. However, the beer with the highest content of tyramine (58.30 mg/L) might have posed 346 a risk, especially to persons with a suboptimal BA-detoxifying system.

347

348 **4. Conclusion**

349 The analytical approach presented here is applied for the first time in beer. DEEMM 350 derivatization followed by UHPLC separation provides a reliable means of simultaneously 351 determining the amino acid, BA and ammonium ion concentrations of beers. Amino acid 352 analysis can be used to ensure beer quality, and to detect counterfeit beers. BA determination is 353 also important in ensuring the safety of drinks and fermented foods.

354 In total, the technique separated nine BAs (histamine, tyramine, putrescine, cadaverine, β- 355 phenylethylamine, ethanolamine, ethylamine, tryptamine and agmatine), 21 amino acids, and 356 ammonium ions, in under 14 min of elution time. Not only does the proposed method allow for 357 more samples to be assayed per unit time, it uses less solvent than other techniques and 358 therefore reduces costs and the associated waste. Its high resolution, precision (repeatability and 359 reproducibility), accuracy, and sensitivity (limit of detection [LOD] and limit of quantification 360 [LOQ]) suggest it could be a very valuable tool for ensuring the food safety of beers.

361

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368

369 **Conflict of interest.** The authors declare that no conflict of interest exist.

370

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560 **Figure caption**

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562 **Fig. 1.** Chromatograms of a 0.15 mM standard solution (A) and a beer sample (B) showing the
563 aminoenone derivatives of amino acids, ammonium ions and biogenic amines at 280 nm. Peak
564 assignments: 1, aspartic acid; 2, glutamic acid; 3, asparagine; 4, serine; 5, glutamine; 6,
565 histidine; 7, glycine; 8, threonine; 9, arginine; 10, GABA; 11, alanine; 12, proline; 13,
566 ammonium ion; 14, ethanolamine; 15, tyrosine; 16, agmatine; 17, histamine; 18, valine; 19,
567 methionine; 20, tryptophan; 21, isoleucine; 22, leucine; 23, phenylalanine; 24, ornithine; 25,
568 lysine; 26, ethylamine; 27, tyramine; 28, putrescine; 29, tryptamine; 30, cadaverine; 31, β-
569 phenylethylamine. IS, internal standard.

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588 **Table 1**
589 Ternary elution gradient for UHPLC determination of aminoenone derivatives of 31 amino compounds.
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Time (min)	0.00	1.20	1.90	2.00	6.52	7.53	7.82	9.20	10.00	13.50	14.00	14.50	16.50
% eluent A	90.0	92.0	92.0	90.0	90.0	83.0	83.0	71.0	75.0	40.0	18.0	-	-
% eluent B	2.0	1.6	1.6	2.0	2.0	3.4	3.4	5.8	-	-	-	20.0	20.0
% eluent C	8.0	6.4	6.4	8.0	8.0	13.6	13.6	23.2	25.0	60.0	82.0	80.0	80.0

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Table 2
Precision (relative standard deviation, RSD) and accuracy (recovery) of the UHPLC method for the determination of amino compounds. BAs are highlighted in grey colour.

Compound	Slopes RSD (%) [a]	Peak area total RSD (%) [b]	Total recovery (%) [c]
Aspartic acid	1.60	3.99	96.77
Glutamic acid	1.15	3.24	97.46
Asparagine	0.59	5.85	100.70
Serine	1.34	3.58	102.44
Glutamine	1.48	6.89	101.98
Histidine	2.44	5.69	99.23
Glycine	1.35	3.83	98.96
Threonine	1.05	6.85	100.56
Arginine	1.67	9.17	99.32
GABA	1.53	5.44	100.01
Alanine	2.37	5.00	105.75
Proline	3.40	4.28	95.98
Ammonium ion	2.55	4.43	85.00
Ethanolamine	2.63	4.01	99.89
Tyrosine	3.15	4.82	102.81
Agmatine	0.86	9.69	97.87
Histamine	2.49	4.39	102.51
Valine	0.96	4.88	99.59
Methionine	3.13	6.02	87.12
Tryptophan	2.18	2.73	99.10
Isoleucine	1.11	3.52	101.79
Leucine	1.10	5.67	100.62
Phenylalanine	1.06	5.12	99.36
Ornithine	0.61	2.59	101.78
Lysine	1.03	4.16	101.32
Ethylamine	0.84	2.75	98.99
Tyramine	0.94	3.20	103.43
Putrescine	1.85	3.59	102.74
Tryptamine	2.64	4.05	100.58
Cadaverine	1.50	3.78	105.90
Phenylethylamine	1.56	4.00	103.96

[a] Precision value of slopes of standard curves constructed in five different matrices
[b] Precision value of the peak areas of each compound taking into account all the spiked concentrations and all the matrices used
[c] Recovery value of each compound taking into account all the spiked concentrations and all the matrices used

672 **Table 3**
673 Repeatability, linearity (calibration) and sensitivity of the UHPLC method for the determination of amino
674 compounds (data derived from standards). The results for biogenic amines are highlighted in grey.

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Compound	Precision (%)				Calibration		Sensitivity		
	Intra-day[a]		Inter-day[b]				LOQ[d]	LOD[e]	
	Rt	area	Rt	area	R^2	Linear range[c] (mg/L)	μM	mg/L	μM
Aspartic acid	0.10	0.70	0.12	0.78	0.999	1.20 – 199.65	9.00	0.24	1.80
Glutamic acid	0.15	0.71	0.20	0.94	0.999	0.99 - 220.70	6.70	0.20	1.34
Asparagine	0.23	0.31	0.27	0.70	0.999	0.21 - 198.18	1.60	0.04	0.32
Serine	0.18	0.49	0.22	0.88	0.999	0.05 - 157.64	0.50	0.01	0.10
Glutamine	0.15	0.42	0.17	1.24	0.999	0.95 - 219.21	6.50	0.19	1.30
Histidine	0.13	0.32	0.16	0.80	0.999	0.25 - 232.73	1.60	0.05	0.32
Glycine	0.10	0.41	0.12	0.39	0.999	0.41 - 112.61	5.40	0.08	1.08
Threonine	0.11	0.51	0.14	0.58	0.999	0.35 - 178.68	2.90	0.07	0.58
Arginine	0.13	0.45	0.16	0.82	0.999	0.30 - 261.30	1.70	0.06	0.34
GABA	0.15	0.70	0.18	1.04	0.999	0.20 - 154.68	1.90	0.04	0.38
Alanine	0.20	0.61	0.21	1.02	0.998	0.50 - 133.64	5.60	0.10	1.12
Proline	0.07	0.27	0.08	0.48	0.996	5.19 - 575.65	45.10	1.04	9.02
Ammonium ion	0.05	0.27	0.06	1.25	0.998	0.49 - 27.08	27.40	0.10	5.48
Ethanolamine	0.04	0.38	0.05	1.58	1.000	0.23 - 91.62	3.70	0.05	0.74
Tyrosine	0.04	0.50	0.04	1.01	0.998	0.72 - 271.79	4.00	0.14	0.80
Agmatine	0.03	0.44	0.03	0.83	0.999	0.31 - 195.29	2.40	0.06	0.48
Histamine	0.03	0.41	0.03	0.47	0.999	0.58 - 166.73	5.20	0.12	1.04
Valine	0.02	0.43	0.02	0.43	0.996	1.15 - 175.73	9.80	0.23	1.96
Methionine	0.02	0.43	0.02	0.73	0.999	0.36 - 223.82	2.40	0.07	0.48
Tryptophan	0.03	0.52	0.03	0.71	0.999	1.04 - 306.35	5.10	0.21	1.02
Isoleucine	0.02	0.43	0.03	0.73	0.999	0.45 - 196.77	3.40	0.09	0.68
Leucine	0.02	0.59	0.02	1.20	0.999	0.26 - 196.77	2.00	0.05	0.40
Phenylalanine	0.02	0.69	0.02	1.16	0.999	0.13 - 247.79	0.80	0.03	0.16
Ornithine	0.02	0.45	0.02	0.81	0.999	0.61 - 198.24	4.60	0.12	0.92
Lysine	0.02	1.18	0.02	1.57	0.999	1.61 - 219.29	11.00	0.32	2.20
Ethylamine	0.02	1.66	0.02	1.45	0.998	0.63 - 67.62	14.00	0.13	2.80
Tyramine	0.02	0.16	0.02	0.72	0.999	0.62 - 205.77	4.50	0.12	0.90
Putrescine	0.01	0.09	0.01	1.03	0.999	0.03 - 132.23	0.30	0.01	0.06
Tryptamine	0.01	0.06	0.01	0.33	0.998	0.50 - 240.33	3.10	0.10	0.62
Cadaverine	0.01	0.42	0.01	0.83	0.999	0.50 - 153.27	4.90	0.10	0.98
Phenylethylamine	0.01	0.90	0.01	0.88	0.999	0.33 - 181.77	2.70	0.07	0.54

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690 [a] RSD (relative standard deviation) of peak retention time (Rt), and peak area, based on six runs of a standard mixture (187 μM) in
691 one day
692 [b] RSD of peak Rt, and peak area, based on eight runs of a standard mixture (187 μM) over eight independent days.
693 [c] Concentration range between the limit of quantification and the upper linear limit.
694 [d] Limit of quantification : signal/noise ratio = 10.
695 [e] Limit of detection: signal/noise ratio = 3.
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Table 4

Biogenic amines, amino acids and ammonium ions in different samples of beer (mg/L). Results for biogenic amines are highlighted in grey.

Brewing style Colour country Analytes	Lager Pale Spain	Lager Pale Spain	Lager Pale Spain	Lager Pale Spain	Lager Pale Spain	Lager Pale Portugal	Lager Dark Spain	Lager Dark Spain	Ale Pale Belgium	Ale Dark Belgium	Ale Dark Belgium	Mean ± SD	RSD [a]
Aspartic acid	10.65	11.31	22.89	10.65	6.66	15.17	31.81	27.42	11.18	41.26	11.45	18.22 ± 11.06	60.69
Glutamic acid	8.39	13.09	20.75	6.03	2.80	14.27	38.25	23.10	13.09	41.78	7.95	17.23 ± 12.78	74.17
Asparagine	3.17	4.49	22.46	3.17	1.06	4.49	7.27	10.70	2.77	10.31	3.17	6.64 ± 6.10	91.83
Serine	2.21	3.26	16.50	2.63	1.05	3.05	5.57	13.98	4.52	7.36	3.15	5.75 ± 5.02	87.28
Glutamine	0.73 [b]	3.22	1.90	nd	nd	0.29 [b]	0.88 [b]	nd	nd	1.75	nd	0.81 ± 1.06	130.63
Histidine	14.27	32.43	19.86	20.01	14.89	18.15	34.75	37.39	26.07	40.03	9.31	24.29 ± 10.42	42.92
Glycine	12.69	29.88	13.51	13.36	8.48	14.56	36.33	27.40	27.78	38.89	13.51	21.49 ± 10.75	50.03
Threonine	0.83	0.23 [b]	14.53	0.48	0.12 [b]	0.48	1.55	8.81	0.71	4.53	0.48	2.98 ± 4.65	156.13
Arginine	15.16	36.76	34.49	14.28	3.83	18.64	60.97	20.03	38.50	76.65	5.92	29.57 ± 22.87	77.36
GABA	54.24	76.62	27.22	68.68	48.36	42.38	55.99	51.35	80.95	64.76	65.17	57.79 ± 15.59	26.97
Alanine	26.73	67.71	31.98	27.97	10.96	37.42	114.57	67.00	72.52	60.58	23.88	49.21 ± 30.17	61.30
Proline	511.29	608.69	237.63	335.60	349.88	279.31	688.02	366.46	325.93	562.53	369.34	421.33 ± 146.78	34.84
Ammonium ion	9.55	2.92	8.50	9.39	2.96	5.58	31.46	18.14	15.38	3.21	2.92	10.00 ± 8.80	87.96
Ethanolamine	7.02	11.73	4.40	9.04	6.54	5.31	7.64	9.22	8.49	12.95	9.77	8.37 ± 2.57	30.73
Tyrosine	49.65	84.98	31.71	31.16	4.53	12.14	6.70	65.41	66.68	84.43	13.41	40.98 ± 30.72	74.95
Agmatine	7.03	12.11	nd	nd	nd	0.13 [b]	8.46	0.13 [b]	0.39	12.50	8.33	4.46 ± 5.24	117.41
Histamine	nd	nd	nd	nd	nd	nd	nd	nd	0.33 [b]	nd	nd	nd	-
Valine	13.36	28.70	15.46	7.73	1.64	7.61	41.82	30.58	27.06	24.13	3.05	18.29 ± 13.01	71.13
Methionine	2.83	5.67	8.80	3.88	3.73	4.33	8.65	5.22	3.28	3.88	1.79	4.73 ± 2.24	47.26
Tryptophan	17.36	28.39	16.34	6.13	11.85	11.85	23.89	25.32	21.24	19.61	8.99	17.36 ± 7.11	40.98
Isoleucine	3.94	13.91	17.05	3.67	1.18	5.12	32.80	18.23	10.10	13.12	2.36	11.04 ± 9.42	85.28
Leucine	5.38	24.27	34.11	5.64	2.10	8.53	40.53	42.90	20.46	31.75	4.46	20.01 ± 15.54	77.66
Phenylalanine	29.90	62.28	32.21	15.86	2.97	16.85	83.92	55.50	49.89	59.47	5.62	37.68 ± 26.30	69.80
Ornithine	1.72	2.38	0.53 [a]	2.11	1.45	2.78	4.63	6.61	2.11	2.64	1.85	2.62 ± 1.66	63.50
Lysine	1.75	3.36	21.49	3.22	0.88 [b]	5.85	9.36	7.46	3.22	24.56	2.34	7.59 ± 8.06	106.26
Ethylamine	0.31 [b]	nd	0.49 [b]	0.54 [b]	0.40 [b]	0.49 [b]	nd	1.22	0.68	nd	1.35	0.50 ± 0.45	90.92
Tyramine	0.96	0.96	0.14 [b]	0.55 [b]	0.96	10.15	**58.30**	nd	0.82	0.55 [b]	0.55 [b]	6.72 ± 17.35	258.08
Putrescine	2.29	3.44	1.59	3.35	2.29	1.85	3.53	4.05	2.38	3.88	2.64	2.84 ± 0.84	29.50
Tryptamine	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	-
Cadaverine	0.61	0.82	0.41 [b]	0.82	0.61	0.51	0.61	0.92	0.61	0.82	0.82	0.69 ± 0.16	23.12
Phenylethylamine	nd	nd	nd	nd	nd	nd	0.36	nd	nd	nd	nd	0.03 ± 0.11	331.66
BA total content	18.24	29.05	7.03	14.30	10.81	18.56	78.90	15.54	13.71	30.69	23.47	23.66 ± 19.70	83.24
Amino acids total content	786.23	1141.62	641.43	582.28	478.56	523.26	1328.27	910.87	808.07	1214.00	557.18	815.62 ± 298.07	36.55
Analytes total content	814.02	1173.59	656.96	605.96	492.33	547.40	1438.63	944.56	837.15	1247.91	583.57	849.28 ± 317.37	37.37

a, relative standard deviation for the samples analysed (as a percentage); b, value between the LOD and LOQ.

748 FIGURE 1

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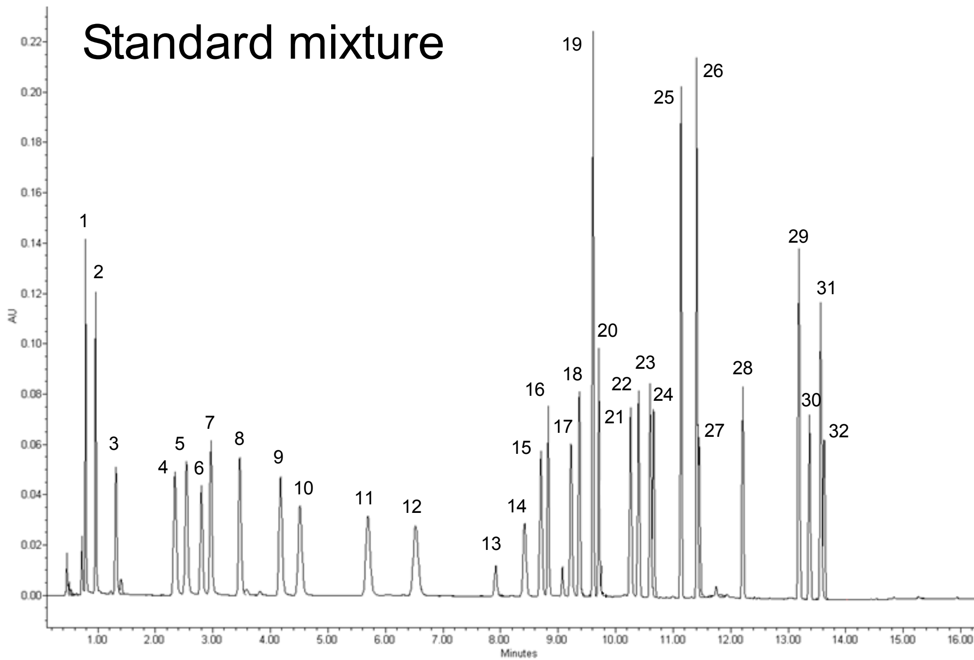

B