Evaluation of biogenic amines in wine: determination by an improved HPLC-PDA method

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Abstract

An improved, simple and sensitive method to quantify the biogenic amines ethanolamine, methylamine, ethylamine, isoamylamine and the usually investigated β-phenylethylamine, putrescine, cadaverine, histamine, tyramine, spermidine, spermine has been developed and validated in red and white wines. The analytes were derivatised with dansyl chloride and separated by HPLC coupled with PDA detector. The calibration curves showed good linearity ($r>0.9990$) and biogenic amines recovery varied from 72 to 97%. The repeatability ranged from 1 to 8% for red wine and from 1 to 5% for white wine. The detection and quantification limits were from 0.02 to 0.10 mg/L and from 0.08 to 0.30 mg/L, respectively..

The method was successfully applied to detect and quantify biogenic amines in Italian red and white wines from Abruzzo Region. The proposed method is suitable for simultaneous detection and for accurate and precise quantification of eleven biogenic amines in wines.

Keywords: wine, food analysis, biogenic amines, HPLC-PDA, validation

31 **1. Introduction**

32

33 In wine biogenic amines (BAs) are formed during must fermentation by yeasts and lactic bacteria (Lehtonen, 1996;
34 Gardini et al., 2005). Their presence has been reported to be related with climatic and geological factors of the wine-
35 producing regions as well as to vinification practices (Cecchini and Morassut, 2010). At high concentrations or
36 when the normal pathways of BAs catabolism are inhibited or deficient, they may be responsible for undesirable
37 toxicological effects (headache, respiratory distress, heart palpitation, hypotension, hypertension, nausea) (EFSA,
38 2011). In wine the total BAs concentration can range from a few mg/L to about 50 mg/L (Lehtonen, 1996) and it
39 could be considered as an indicator of authenticity (Herbert et al., 2005) in addition to other chemical compounds
40 such as polyphenols (Galgano et al., 2011). Actually, a legislation dealing with limits for BAs contents in wines is
41 still lacking and regulatory limits have not yet been established; however, upper limits for histamine content in wine
42 (2-10 mg/L) are recommended in some European countries (Lehtonen, 1996).
43 Many analytical methods have been developed to quantify these compounds in wines, including gas-
44 chromatography (Fernandes and Ferreira, 2000), capillary electrophoresis (Herrero et al., 2010), enzymatic methods
45 and immunoassays (Lange and Wittmann, 2002). Due to its high sensitivity, resolution and versatility, HPLC with
46 fluorescence or ultraviolet detector is the most extensively used technique for the determination of BAs in wine,
47 including an appropriate derivatisation step by o-phthalaldeyde (OPA) (Vidal-Carou et al., 2003; Kelly et al., 2010;
48 Arrieta and Prats-Moya, 2012), dansyl chloride (Dns-Cl) (Zotou et al., 2003; Hernández-Borges et al., 2007;
49 Proestos et al., 2008), dabsyl chloride (Dabs-Cl) (Romero et al., 2000), benzoyl chloride (Bnz-Cl) (Özdestan and
50 Üren, 2009), fluorenylmethylchloroformate (FMOC) (Bauza et al., 1995), 1,2-naphthoquinone-4-sulphonate (NQS)
51 (Hlabangana et al., 2006), 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate (AQC) (Hernández-Orte et al., 2006).
52 Recent liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS) and ultra-performance liquid
53 chromatography coupled to a quadrupole-time of flight mass spectrometry (UPLC/Q-TOFMS) have been shown to
54 be very powerful techniques to increase the performance of BAs analysis (García-Villar et al., 2009; Jia et al.,
55 2012), also without derivatisation (Millán et al., 2007).
56 In wines the presence of β-phenylethylamine, putrescine, cadaverine, histamine, tyramine, spermidine and spermine
57 is well documented, while relatively few information are available for other BAs, like ethanolamine and
58 methylamine, in particular, but also ethylamine and isoamylamine, even if they have been found in wines (Busto et
59 al., 1995; Galgano et al., 2003; Zotou et al., 2003; Hlabangana et al., 2006; Arrieta and Prats-Moya, 2012). The
60 detailed knowledge about the presence in wines of all the above-mentioned molecules would allow to assess if it is
61 necessary to develop or modify the oenological and/or technological practices in order to produce wines with

62 reduced BAs content, thus decreasing their possible harmful effects in sensitive humans. Starting from our previous
63 work (Martuscelli et al., 2013), the present paper reports a more convenient, complete and sensitive analytical
64 method to detect simultaneously eleven molecules, including ethanolamine, methylamine, isoamylamine, the
65 content of which in wine has been rarely reported. The experimental analytical conditions in wine were optimised,
66 also focusing on the analytical use of activated carbon to remove phenolic compounds (Marchal and Jeandet 2009).
67 The method was validated in terms of linearity, accuracy, precision, detection and quantification limits (ICH, 1995;
68 AOAC, 2002). As a practical application of the proposed analytical method, the BAs content in twenty Italian wines
69 from Abruzzo Region (Italy) has been reported.
70
71 **2. Materials and methods**
72
73 *2.1 Chemicals*
74 Unless specified, all reagents used were of analytical grade. Dansyl chloride (Dns-Cl), ethanolamine, methylamine,
75 ethylamine, β-phenylethylamine, isoamylamine, putrescine, cadaverine, histamine, tyramine, spermidine and
76 spermine were purchased from Sigma (St. Louis, MO, USA). With the exception of isoamylamine, all BAs were
77 obtained as their hydrochloride salts and the concentrations of standard solutions were corrected on the basis of their
78 purity and referred to as free base. Hydrochloric acid (37%, w/w), ammonia solution (30%, w/w), potassium
79 hydroxide, sodium hydrogen carbonate, activated carbon, acetonitrile (HPLC grade) were supplied by Carlo Erba
80 (Milan, Italy). Ultrapure water was obtained from a reverse osmosis system (Teknolabo, Milan, Italy).
81
82 *2.2 Sample preparation*
83 A pooled red wine sample and a pooled white wine sample were prepared to check and to validate the proposed
84 analytical method. Fifteen red and five white wine samples, collected from wineries located in the Abruzzo Region
85 (Central Italy) and ready for market, were analysed. Samples were filtered through a nylon 0.45 μm filter. 0.5 mL
86 both standard solutions and wine samples were mixed with 150 μL of a saturated sodium hydrogen carbonate
87 solution and the pH was adjusted to 11.5 with 0.1M potassium hydroxide solution. Then, 2 mL of Dns-Cl 0.5% *w/v*
88 in acetone were added and the reaction mixtures were incubated for 45 min at 40°C in a water-bath under agitation
89 (75 strokes, 1024 Shaking Water Bath, Foss Tecator, Denmark). The residual Dns-Cl was removed by adding 200
90 μL of ammonia solution and, after agitation, the reaction mixtures were left in darkness for 30 min at room
91 temperature. Then, the volume was made up to 5 mL with acetonitrile, the solution mixed and filtered through nylon
92 0.45 μm filters. 10 μL of the resulting standards and samples were injected into the HPLC system. Blank samples

93 were prepared at each analytical round by the same procedure starting from 0.5 mL of 0.1M HCl to monitor the
94 presence of peaks coming from the derivatisation process.
95 In carbon treatment 0.1% activated carbon was added to two commercially available red samples and two white
96 wine samples, the solutions stirred for 10 min at room temperature and filtered through nylon 0.45 µm; 0.5 mL of
97 the filtrate were derivatised and analysed as described for samples not treated.
98
99 *2.3 Chromatography*
100 BAs were analysed using an HPLC system consisting of an "Alliance" (Waters, Milford, MA, USA), equipped with
101 a Waters 2695 separation module connected to a Waters 2996 photodiode array detector (PDA), set at 254 nm. A
102 Supelcosil LC-18 column (5 µm particle size, 250mm × 4.6mm i.d.) from Sigma was used. The system was
103 governed by Waters Empower personal computer software. Stock solutions of each BA were prepared at a
104 concentration of 1000 mg/L in 0.1M HCl, kept in the dark at 4°C. Standard curve solutions including all the eleven
105 analytes were prepared by diluting stock solutions with 0.1M HCl to obtain final concentrations in the range 0.5-50
106 mg/L. BAs were separated using a linear gradient obtained from acetonitrile (A) and ultrapure water (B) as follows:
107 0-25 min 40%-80% A, 25-30 min 80%-100% A and 30-35 min 100%-40% A, with a flow-rate of 1 ml/min,
108 achieving a complete separation in less than 35 min. The column temperature was 25°C. The autosampler
109 temperature was kept at 4°C. Identification of all BAs was based on their retention times, determined injecting the
110 reference standards individually and as a mixture. The calibration curves of each BA, i.e. peak area *versus* amine
111 concentration, were linear and data were fitted by the least-squares method. Each line of regression calculated has
112 been used to compute the amount of the correspondent analyte in samples by interpolation, using external standard
113 method.
114
115 *2.4 Validation*
116 The analytical performances of the proposed method were studied to verify its applicability in the routine analysis of
117 wine samples.
118 Linearity was assessed by least squares fitting of six independent seven-points calibration curves in the range 0.5-50
119 mg/L, coming from separately derivatised aliquots.
120 The accuracy of the method was evaluated by means of a spiking and recovery procedure on each pooled wine
121 sample at three BA levels (2-5-10 mg/L), performing five replicates for each concentration level, for a total of 15
122 analyses for each kind of wine sample, quantifying and subtracting the endogenous amine content. The identity of
123 each BA was verified by matching automatically spectra acquired (PDA) and confirmed modifying mobile phase

124 composition. The repeatability of the method was performed on the same spiked, that have been analysed also on
125 two other occasions by different operators to estimate intermediate precision. Repeatability and intermediate
126 precision were given as the relative standard deviations of the found concentrations. In addition, the intra-day
127 repeatability of retention times was calculated. The limits of detection (LODs) and the limit of quantification
128 (LOQs) were set on wine samples using signal-to-noise ratio (S/N) of 3 and 10, respectively.
129
130 **3. Results and Discussion**
131
132 The chromatograms shown in Fig.1 illustrate the efficiency of the proposed method. The resolution of all the eleven
133 BAs was mainly affected by the acetonitrile percentage and the column temperature. The best elution conditions
134 were observed using the linear gradient described. An initial 40% amount of acetonitrile allowed a good retention
135 and separation of less retained analytes (i.e. ethanolamine, methylamine and also ethylamine) among them and from
136 peaks produced during derivatisation, between β-phenylethylamine and isoamylamine in the central region of the
137 chromatogram and also between stronger retained tyramine and spermidine. The column temperature of 25°C and
138 the length of the column (25 cm) together with the adopted elution conditions allowed an excellent resolution of all
139 BA peaks, ensuring that there was no overlapping with additional peaks arising from wine matrix and from
140 derivatisation process.
141 Derivatisation is another critical step for BAs determination. The maximum detectability was achieved by using a
142 Dns-Cl concentration of 0.5% *w/v* in acetone, that was found suitable to obtain quantitative dansylation of all the
143 BAs investigated. A standard BAs solution, as well as the pooled red and white wine samples unspiked and spiked
144 with known amount of BAs were analysed three times at three different levels of Dns-Cl (0.5-0.75-1% w/v): no
145 significant differences in magnitude of peak areas of the BA derivatives were observed ($P<0.05$), while increasing
146 the concentration of the derivatising agent, the excess of the Dns-Cl interfered with the optimal resolution mainly of
147 ethanolamine. Best performance was achieved at 40°C: lower temperatures increased reaction time, while higher
148 ones enhanced interferences. The reaction occurs under alkaline conditions and for acid solutions like wines (pH 3-
149 5) satisfactory results were obtained with sodium hydrogen carbonate pH 11.5, being able to enhance the yield of
150 each BA, especially of spermidine and spermine. The BA derivatives stored at -20°C resulted to be stable for at least
151 1 week. After 72 h the mean area values of the solutions maintained into the autosampler at 4°C were not
152 significantly different ($P<0.05$).
153 About sample pre-treatment, some analytical methods in literature contemplate sample clean-up prior to
154 chromatographic analysis, including the addition of polyvinylpyrrolidone (PVP) for removing interferences of

155 polyphenols (Zotou et al. 2003; Proestos et al., 2008). Since PVP adsorbs not only phenolic compounds, but also
156 BAs, causing a considerable decrease in their levels (Özdestan and Üren, 2009), in the present work the use of
157 activated carbon has been investigated. Two commercially available red samples and two white wine samples were
158 tested three times with and without carbon pre-treatment, as reported in the Material and Methods. As shown in
159 Table 1, carbon application caused about 29% decrease of β-phenylethylamine in red wines up to its total
160 disappearance in white wines and, for tyramine, a percentage of decrease of about 30% and 40% for red and white
161 wines, respectively.
162 The calibration curve of each BA exhibited excellent linearity over the tested range ($r > 0.999$). In Table 2, the
163 equations for the calibration curves of all the BA, with 95% confidence interval of slope and intercept estimates, are
164 given.
165 Based on results for spiked samples, good recoveries were obtained for all the BAs, ranging from 90% for β-
166 phenylethylamine to 97% for isoamylamine, spermine values assessing between 72% and 75% in red and white
167 wine, respectively, and spermidine around 82%. Table 3 shows the homogeneity of the percentage of recoveries and
168 relative standard deviations for repeatability (RSD) data at the three levels of spiking for red and white wines. These
169 recovery values are in the same range than the ones obtained by LC-UV works after dansylation (Hernández-Borges
170 et al., 2007; Proestos et al., 2008) or better than those about the same molecules published using different
171 derivatising agents (Hlabangana et al., 2006; Özdestan and Üren, 2009; Bach et al., 2012). The repeatability of the
172 retention times was lower than 0.6% and the relative standard deviation for intermediate precision ranged from 2%
173 to 7% and from 2% to 5% for red and white wines, respectively, with an acceptable level of precision. LODs and
174 LOQs ranged from 0.02 to 0.10 mg/L and from 0.08 to 0.30 mg/L, respectively. A part from HPLC-MS/MS
175 methods, that provide very low LODs, in the order of few μg/L (García-Villar et al., 2009; Millán et al., 2007),
176 LODs and LOQs reported in this work were similar to those of Hernández-Borges et al. (2007) and Proestos et al.
177 (2008) and lower than those published earlier (Martuscelli et al., 2013) and those obtained with other derivatising
178 agent (Arrieta and Prats-Moya, 2012; Hlabangana et al., 2006; Özdestan and Üren, 2009). LOQs reported in the
179 present study were 10-fold lower than the minimum recommended limit for histamine in wines (2 mg/L), being
180 suitable for Bas determination in wine samples.
181 The proposed method was successfully applied to determine the BA concentrations in twenty commercial Abruzzo
182 wines. The biogenic mono-, di- and polyamine contents in red and white wine samples are reported in Table 4. The
183 variability in the BAs content could be associated to different vinification techniques, such as the must contact time
184 with grape skin, amino acids content at the initial and final phases of alcoholic fermentation and during malolactic
185 one (Vidal-Carou et al., 1990). Ethanolamine, putrescine, ethylamine were the most abundant amines in the analysed

wines, whereas isoamylamine was detected only in red wines. Ethanolamine, putrescine and ethylamine are generally present on grape skin; in particular, ethanolamine is a precursor of phosphatidylcholine, the most abundant phospholipid in the membranes of eukaryotic cells and its content increases during alcoholic fermentation because it is involved in the metabolism of phospholipids (Choi et al., 2004). Moreover, because of regulation phenomena, it is probably surrendered outside in the medium. This could explain its accumulation in red and white wines. Regarding putrescine, its concentration was much higher in red wines than in white ones. This data agrees with those obtained for Spanish wines (Bover-Cid et al., 2006). Other compounds, such as methylamine and cadaverine, were encountered at low concentrations; methylamine was found in 60% of red wines, cadaverine in 85% of all the samples, according to the fact that these substances, already present in grape must, are produced and degraded during vinification (Mafra et al., 1999). Tyramine was found only in 35% of all the samples, histamine in 15% and always below 1 mg/L. No BAs were found above the toxic levels reported in literature (EFSA, 2011).

4. Conclusions

The development of reliable methods able to detect BAs in wine is a powerful tool for critical analysis of the factors associated with their accumulation. The HPLC method proposed in this paper fulfills all the required analytical characteristics for the detection of BAs in wines, improving their analysis compared with earlier methods. The efficiency of the chromatographic separation combined with the high sensitivity and stability of the derivatisation reaction provided chromatograms of high resolution peaks, allowing a complete pattern of eleven amines, including ethanolamine, methylamine, isoamylamine, in a single run without interferences. No pre-analytical treatment of sample is required, avoiding time-consuming sample manipulations and reducing analytical variability. Validation results show that the method is simple for widespread and routine use, easy to automate, accurate and precise for BAs quantification in wines.

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Conflict of Interest

217

218 Authors declare that there are no conflicts of interest.

219

220 **References**

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