AMINO ACIDS AND BIOGENIC AMINES IN RED VARIETAL WINES: THE ROLE OF GRAPE VARIETY, MALOLACTIC FERMENTATION AND VINTAGE

O. Martínez-Pinilla, Z. Guadalupe, Z. Hernández[1], B. Ayestarán[*]

Instituto de Ciencias de la Vid y del Vino (Universidad de la Rioja, Gobierno de La Rioja y CSIC), C/ Madre de Dios 51, 26006 Logroño, La Rioja, España.

[1]Departamento Matemáticas y Computación, Universidad de la Rioja, C) Luis de Ulloa, s/n, 26004 Logroño, La Rioja, España.

[*]Corresponding author: Tel.: +34 941 299725; fax: +34 941 299721; *E-mail address*: belen.ayestaran@unirioja.es (B. Ayestarán)

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ABSTRACT

This work studies for the first time the amino acid and biogenic amine composition of Rioja red wines made with the red minority varieties *Vitis vinifera* cv. Monastel and Maturana Tinta de Navarrete, using Tempranillo as a reference variety. The role of malolactic fermentation and vintage on these compounds was also analyzed, and discriminate analyses were applied to achieve a possible differentiation of the wines. Amino acid composition allowed a differentiation of wines according to grape variety. Monastel was characterised by the highest value in ß-alanine and Maturana Tinta de Navarrete by its highest value in OH-proline. However, biogenic amines were no able to classify varietal wines. The malolactic fermentation had significant changes on the amino acid and biogenic amine content, and allowed distinguishing wines that underwent this process from wines without malolactic fermentation. No correlation was found between total amino acids and total biogenic amines after malolactic fermentation, suggesting that a higher initial concentration of amino acids in the medium did no affect the concentration of biogenic amines after malolactic fermentation. Vintage influenced the amino acid and biogenic amine pattern, obtaining a clear separation of wines by vintages. Monastel and Maturana Tinta de Navarrete wines showed a minor varietal character and were more influenced by the climatic conditions of each vintage than Tempranillo wines. All the wines showed histamine levels below the human physiological threshold and implemented regulations.

Keywords: red minority varietal wines, amino acid and biogenic amine composition, winemaking stage, vintage.

Introduction

Amino acids in wine have different origins. Besides those that are present in grapes and that can be partially or totally metabolized by yeasts and lactic acid bacteria during the course of fermentations, some are secreted by yeasts and bacteria at the end of fermentations, some are released by proteolysis during the autolysis of dead yeasts, and other are produced by enzymatic degradation of the grape proteins. Moreover, grape variety, climate, viticulture practices and winemaking techniques, such as maceration time or malolactic fermentation, can affect the amino acid content in wines [1, 2]. Biogenic amines are mainly produced as a consequence of the decarboxylation of amino acids, although they can also be present in grapes. In wine, biogenic amines can be found at variable levels depending on grape variety, vintage, levels of amine-precursor amino acids, assimilable nitrogen content and processing techniques as the occurrence of malolactic fermentation [3-6]. While high concentrations of biogenic amines can cause undesirable physiological effects in sensitive humans, amino acids are precursors for aroma compounds and directly contribute to wine's aroma, taste and appearance [7-9].

Despite the wide range of factors affecting the amino acid and biogenic amine composition in wine, some researchers have successfully employed the amino acid and biogenic amine pattern to differentiate wines according to variety, vintage and winemaking techniques [1, 3, 10, 11]. Hence the importance of using free amino acid and biogenic amine profile to characterize wines made with minority varieties in different regions, which would allow to characterise wines with their own personality and different from the rest that exist in the international market.

In this sense, La Rioja (Spain), an autonomous community with a large vitiviniculture tradition, has raised the need to preserve and characterize its minority

vine varieties in order to maintain the authenticity and quality of its wines. This community has different vine-growing zones with an important number of minority grape varieties, which are perfectly adapted to these zones. Previous studies of local and minority varieties from these areas [12, 13] highlighted for their oenological interest Monastel and Maturana Tinta de Navarrete that could be a good complement to the widespread and most representative variety of the area, Tempranillo comprises 85% of the surface of red grapes cultivated in La Rioja. Although there are some studies on the growing potential of these varieties [12, 13] and on the sensory profiling of the wines produced made from these varietals [14], information on the amino acid and biogenic amine content of these wines is lacking. On the one hand, this study would allow to identify the amino acids and biogenic amines of wines produced with these minority varieties, and on the other hand, to achieve a possible characterization of these wines by means of their amino acid and biogenic amine pattern.

Therefore, the aim of this work was to study the profile and content of amino acids and biogenic amines in red varietal wines made with Monastel and Maturana Tinta de Navarrete, before and after malolactic fermentation. For this purpose, wines were produced in an industrial wine cellar during two different vintages, and Tempranillo was also studied as a reference variety. Stepwise Discriminate Analysis (SDA) were applied in order to achieve a possible differentiation of the wines regarding variety, winemaking stage and vintage.

Experimental

Wine samples

Thirty six red wine samples were analyzed in this study (Table 1). All samples belonged to three red grape varieties cultivated in the autonomous community of La Rioja (Spain): 12 of them corresponded to Tempranillo, used as a reference variety, and

the others corresponded to the red minority varieties Monastel (12 samples) and Maturana Tinta de Navarrete (12 samples). All the samples were collected in two different vintages (2009 and 2010), and among them, half were collected from different deposits after the alcoholic fermentation while the other half were taken after the malolactic fermentation. Grapes were grown in the same vineyard under similar conditions to eliminate possible variations due to different soils, climatic conditions and viticulture procedures. All grapes were vinified under the same controlled procedures to avoid variations during the winemaking. Grapes were destemmed, sulphited with 3 g/HL SO_2 and inoculated with 25 g/HL *S. cerevisiae* yeast strain (Uvaferm VRB, Lallemand Inc. Spain)**.** The fermentation-maceration process was carried out at a maximum temperature of 28 ± 2 ºC and lasted 10 days. Wines were then run off and they were maintained at controlled wine cellar temperature for undergoing spontaneous malolactic fermentation. Malic acid content was 3 g/L ± 0.2 and 4 g/L ± 0.5 in vintage 2009 and 2010, respectively. After malolactic fermentation, malic acid was below 0.1 g/L and the lactic acid content was 2 g/L ± 0.2 in vintage 2009 and 2.6 g/L ± 0.4 in vintage 2010.

Chemical analysis

L-malic and L-lactic acid were analyzed by a multiparametric enzymatic autoanalyzer LISA 200 (Analytical Methodology LISA 200, Biocode Hyad, Le Rhem, France).

Amino acid and biogenic amine analysis

Amino acid and biogenic amine content were determined simultaneously using the method described by Gómez-Alonso et al. [15]. High performance liquid chromatography (HPLC) was performed using a modular 1100 Agilent liquid chromatograph (Agilent Technologies, Waldbronn, Germany) equipped with one

100 G1311A quaternary pump, an on-line G1379A degasser, a G1316A column oven, a
101 G1313A automatic injector, and a G1315B photodiode-array detector (DAD) controlled
102 by the Chemstation Agilent software. Twenty four amino acids and nine biogenic
103 amines were identified on the basis of the aminoenone derivative retention times of the
104 corresponding patterns (Sigma-Aldrich Chemie, Steinhein, Germany), and quantified
105 using the internal pattern method. All analyses were performed in triplicate in each wine
106 sample.

107 *Statistical analysis*

108 Significant differences between analytical determinations were analyzed by a factorial
109 analysis of variance (ANOVA) taking in account variety, winemaking stage and
110 vintage. For comparison of wines, a two-tailed Pearson correlation test was carried out.
111 Stepwise discriminate analysis (SDA) following the forward method was used to select
112 the variables most useful for differentiating wines according to grape variety,
113 winemaking stage and vintage. The F-statistical function was used as the criterion for
114 variable selection. ANOVA evaluations were performed using the Statistica 8.0
115 program for Microsoft Windows (Statsoft Inc., Tulsa, Oklahoma). Discriminate analysis
116 and two-tailed Pearson correlation test were carried out using the IBM SPSS Statistic
117 16.0 program (New York, USA).

118 **Results and discussion**

119 *Amino acids and biogenic amines composition of wines by grape variety, winemaking*
120 *stage and vintage*

121 Table 2 and Table 3 show the concentration (mg/L) of amino acids and biogenic
122 amines and the significance of the ANOVA analysis by variety, winemaking stage and
123 vintage, respectively. Data in the tables has been arranged by amino acids and biogenic
124 amines compounds and listed according to their order elution. Total amino acids and

125 total biogenic amines were calculated as the sum of the concentration of the individual
126 compounds.

127 From the 36 wines analysed, 12 corresponded to each studied variety (Table 1).
128 Regarding amino acids, wines made from Monastel and Maturana Tinta de Navarrete
129 showed the highest concentrations of total amino acids while Tempranillo wines
130 showed the lowest (Table 2). Tempranillo showed similar values to those obtained in
131 other studies for this variety [2, 15]. Regarding differences among varieties in the
132 concentration of individual amino acids (Table 2), several aspects should be
133 highlighted. Firstly, proline was by far the major amino acid in all the wines, and was
134 significantly higher in Maturana Tinta de Navarrete and Monastel than in Tempranillo
135 wines (96%, 90% and 83%, respectively). Some studies have observed that proline is
136 not consumed under anaerobic conditions during alcoholic fermentation [16], and it can
137 even be released during this stage [9]. These facts could explain that the resulting wines
138 after fermentation had a higher amount of proline than the initial musts. Secondly, it is
139 worth highlighting that, with the exception of proline, OH-proline and ß-alanine, the
140 concentration of all the remaining amino acids was higher in Tempranillo than in
141 Monastel and Maturana Tinta de Navarrete. Thus, Monastel wines showed the highest
142 value in ß-alanine. Despite the low content observed in individual amino acids in
143 Maturana Tinta de Navarrete wines, they could be differentiated from the rest of the
144 wines by its highest content in OH-proline. It is also worth mentioning that all the
145 amino acids analyzed varied significantly among samples with respect to the grape
146 variety factor (V) when the significance of the ANOVA analysis was made (Table 3).
147 These results suggested that distinctive amino acids for each grape variety could be
148 found.

149 Malolactic fermentation (ML) is part of the traditional winemaking techniques
150 for red wines and it is necessary to elaborate high quality wines that could be aged. In
151 the present study, we investigated if this winemaking stage had a marked influence on
152 the amino acid and biogenic amine content.

153 The malolactic fermentation caused significant changes in the concentrations of
154 individual amino acids (Table 2). Thus, malolactic fermentation produced increases in
155 nearly all the amino acids studied, and mainly in valine, methionine, isoleucine,
156 phenylalanine, asparagine, serine, glycine, threonine, leucine and lysine. The increase in
157 these amino acids was attributed to their release from wine proteins or peptides by the
158 action of proteases from the lactic acid bacteria [17]. On the contrary, glutamine and
159 arginine were the only amino acids which showed reductions (around 60%), leading to
160 changes in the amino acid profile after this winemaking stage. There is no consensus on
161 the bibliography as regards the evolution of the different amino acids during malolactic
162 fermentation, with the exception of arginine, in which case all authors report a
163 considerable decrease [2], probably due to the fact that arginine is one of the nutritional
164 requirements for the lactic acid bacteria [18]. Hence, some authors found a decrease in
165 total amino acids after this winemaking stage [1, 19], while others found an increase [2,
166 16, 20, 21]. Results of the ANOVA (Table 3) showed that the effect of the winemaking
167 stage (S) was also important, due to significant differences were found in all studied
168 amino acids, except in GABA and ornithine.

169 From the 36 wine samples analysed, 12 were produced in vintage 2009 and 24 in
170 vintage 2010 (Table 1). Taking in account individual amino acids, the most pronounced
171 change observed was in GABA, which showed double quantity in wines of vintage
172 2009 (Table 2). Regarding to the vintage factor (VI), significant differences were found
173 in 18 of the 24 amino acids studied (Table 3).

It is to point out that the interaction variety x winemaking stage (V x S) was not significant for most of the amino acids (Table 3). However, when the interaction V x VI was studied, all the amino acids quantified varied significantly (Table 3). Tempranillo wines maintained its amino acid profile in both vintages, whereas different profiles were obtained for Monastel and Maturana Tinta de Navarrete (Table 2). Thus, for Tempranillo wines major amino acids after proline were glutamine, glutamic acid, α-alanine, arginine and GABA. Similar profile was described by López et al. [16] in other Tempranillo wines from La Rioja. Monastel and Maturana Tinta de Navarrete wines showed the same major amino acids GABA, glutamic acid, glutamine, glycine and arginine in vintage 2009. However, in vintage 2010, the major amino acids for Monastel wines were α-alanine, glutamine, glutamic acid, arginine and glycine, whereas glutamic acid, OH-Proline, α-alanine, glycine and glutamine were the major ones for Maturana Tinta de Navarrete wines. Remaining amino acids were present in amounts that did not exceed 20 mg/L in any variety. These results suggested that Monastel and Maturana Tinta de Navarrete amino acid profiles had a minor varietal character and were more influenced by the climatic conditions of each vintage when the interaction of both factors was studied. However, Tempranillo wines amino acid profiles showed similar patterns from the same location in different years, in agreement with the work of Huang and Ough [22].

The interaction S x VI was significant in 17 of the 24 individual amino acids studied (Table 3). An overall increase in their total content was observed after malolactic fermentation in vintage 2009, whereas an overall decrease was observed in vintage 2010.

197 Finally, when the interaction V x S x VI was studied only the 29% of amino
198 acids varied significantly (Table 3) corresponding to OH-proline, glutamine, ß-alanine,
199 arginine, proline, cysteine and tryptophan.
200 In relation to the concentration of biogenic amines (Table 2), varietal wines
201 showed similar values, agreeing with those obtained in other studies for Tempranillo
202 wines [2, 4, 16] and in other red wines [4, 23-27]. Although no legal limit has been
203 established for total biogenic amines content in wines, some countries have formerly
204 defined limits for histamine, e.g. Switzerland (10 mg/L), Germany (2 mg/L), Belgium
205 (5-6 mg/L), and France (8 mg/L) [4, 24]. Taking into account the limit of 8 mg/L
206 proposed by Leitao et al. [28], all varietal wines did no represent a health risk. When the
207 significance of the ANOVA analysis by variety was studied, 7 of 9 biogenic amines and
208 total biogenic amines content showed significant differences (Table 3), suggesting that
209 these differences could be due to the different amino acid precursors and their
210 respective amounts in grape varieties, and/or the presence of the natural bacteria
211 microflora present on grapes [29].
212 Malolactic fermentation produced increases in six of the nine biogenic amines
213 analysed because latic acid bacteria are able to descarboxylate amino acids [30].
214 Consequently, changes in the biogenic amine pattern were produced, affecting mainly to
215 putrescine, tyramine, spermidine and isoamylamine. Total biogenic amine content was
216 between 2 and 3.7 fold in wines that underwent malolactic fermentation, in agreement
217 with other researches [2, 4, 29, 31-33]. Putrescine was the most abundant amine after
218 the malolactic fermentation, as was found in other studies [2, 4, 19, 34]. Putrescine can
219 be produced by decarboxylation of ornithine or by metabolism of arginine, through
220 being the agmatine intermediary. The Pearson correlation test revealed no correlation
221 between ornithine and putrescine, whereas a negative correlation was found between

222 arginine and putrescine (r = -0.38, α = 0.01) and a positive one between agmatine and
223 putrescine (r = 0.477, α = 0.01). This result supports the argument that the biosynthetic
224 pathway for putrescine is via arginine-agmatine rather than ornithine. The same result
225 was obtained by Bauza et al. [35] regarding polyamines and their precursors in
226 Grenache noir and Syrah grapes and wine of the Rhône Valley. It should be also
227 highlighted that phenylethylamine showed a negative correlation with its amino acid
228 precursor phenylalanine (r = -0.521, α = 0.01), while tyramine showed a positive
229 correlation with its precursor tyrosine (r = 0.415, α = 0.01). On the contrary, no
230 correlation between the rest of amine/amino acid precursor was found, in good
231 agreement with bibliography, where there is no consensus as regards the correlation
232 between amines and their precursors [2, 6, 19, 36]. It is important highlight that no
233 correlation was found between total amino acids and total biogenic amines, suggesting
234 that a higher initial concentration of amino acids in the medium did no affect the
235 concentration of biogenic amines after malolactic fermentation. However, this result
236 contradicts others studies [1, 2, 6]. Other factors such the type of bacteria performing
237 malolactic fermentation [33] and/or a lack of hygiene during the winemaking process or
238 associated with poor sanitary conditions of grapes could influence the final biogenic
239 amine content in wines [28]. It is worth mentioning that all biogenic amines varied
240 significantly among samples with respect to the winemaking stage factor, except
241 tryptamine and phenylethylamine (Table 3).

242 The content of biogenic amines also varied between vintages. Thus, wines of
243 vintage 2009 showed more total biogenic amine content than wines of vintage 2010,
244 leading to a change in their amine profile. These results were in good agreement with
245 other studies that reported that different amine content in wines from different years can
246 be due to the diversity of wine microorganism that are naturally differently selected

247 each year, probably due to climatic conditions [24, 29]. It is to point out that tryptamine
248 was no detected in vintage 2010 and histamine showed very low values. Within all the
249 biogenic amines studied, phenylethylamine was the only which did not show significant
250 differences when the vintage factor was studied (Table 3).

251 The interaction V x S showed significant differences in all amines, except
252 spermidine, tryptamine and isoamylamine (Table 3). Thus, agmatine showed an
253 increase of more than 190% in Tempranillo and Monastel wines and a decreased of 40%
254 in Maturana Tinta de Navarrete wines after malolactic fermentation. Furthermore,
255 Maturana Tinta de Navarrete wines showed the highest values in histamine after
256 malolactic fermentation. Nevertheless, histamine showed lower values than in other
257 studies [2] and below the limit of human health risk.

258 Tryptamine and phenylethylamine were no significant when the interactions V x
259 VI was studied. Besides the formers, histamine and agmatine were also no significant in
260 the interaction S x VI (Table 3).

261 Finally, when the interaction V x S x VI was analyzed, only the 33% of amines
262 varied significantly (Table 3) corresponding to histamine, putrescine and cadaverine.

263 *Discriminant analysis of wines according to their amino acids and biogenic amines*
264 *content*

265 Stepwise linear discriminate analysis (SDA) was applied as a supervised
266 classification technique in order to determine the amino acids and biogenic amines most
267 useful for differentiating wines according to grape variety, winemaking stage and
268 vintage.

269 Taking in account grape variety, the final model selected 12 amino acids: HO-
270 proline, ß-alanine, threonine, cysteine, histidine, asparagine, leucine, tyrosine, lysine, α-
271 alanine, proline and arginine. An accurate classification of wines by grape variety was

obtained, with a global classification of 100% of the wines (Fig. 1). This result suggests that wine amino acids may play an important role, as oenological compounds, to differentiate varietal wines. Besides, they contribute to the overall taste of wines. However, when biogenic amines were used to discriminate varietal wines, a clear differentiation could not be achieved (Fig. 2). The final model selected 5 biogenic amines: phenylethylamine, cadaverine, agmatine, isoamylamine and spermidine, with a global classification of only 83.3% of the wines.

Taking in account the winemaking stage, only with the three amino acids methionine, ornithine and cysteine were enough to discriminate wines. Four biogenic amines: isoamylamine, putrescine, spermidine and histamine were also able to differentiate wines that underwent malolactic fermentation. In other studies, putrescine and histamine, together with tyramine increased significantly after malolactic fermentation [2, 19, 31] and they could be used as chemical descriptors to distinguish malolactic fermented-wines. Both models showed a global classification of 97.2%.

When SDA was applied to discriminate wines by vintage, the final model selected 6 amino acids: GABA, ornithine, ß-alanine, glutamine, valine and isoleucine. GABA and alanine were discriminate variables by vintage in other studies [1]. Finally, tryptamine, histamine, cadaverine, spermidine and tyramine were able to group wines by vintage. Both models showed a satisfactory classification of 100%.

Conclusions

This work showed for the first time the amino acid and biogenic amine profile and content of wines made from the red varieties Monastel and Maturana Tinta de Navarrete. Grape variety, winemaking stage and vintage affected the amino acids and biogenic amines content in the two years of study. Therefore, Monastel was characterised by the highest value in ß-alanine, while Maturana Tinta de Navarrete

297 showed the greatest content in OH-proline. Both wines had a less varietal character and
298 were more influence by climatic conditions than Tempranillo wines in the two years of
299 study. All the wines showed histamine levels below the human physiological threshold,
300 fact of extreme importance from a health point of view. No correlation was found
301 between total amino acids and total biogenic amines after malolactic fermentation,
302 suggesting that a higher initial concentration of amino acids in the medium did no affect
303 the concentration of biogenic amines after malolactic fermentation during the study.
304 When the interactions of the factors variety, winemaking stage and vintage were studied
305 in this paper, only the 29% of amino acids and 33% of biogenic amines varied
306 significantly.

307 When discriminate analyses were applied, amino acids were able to differentiate
308 wines by variety, winemaking stage and vintage. On the contrary, amines were not able
309 to discriminate varietal wines. However, they could differentiate wines by winemaking
310 stage and vintage in the studied period. Consequently, the amino acid profile could be
311 used as a tool to differentiate wines according to grape variety, winemaking stage and
312 vintage, whereas amines might be used as descriptors of malolactic-fermented wines
313 and to discriminate wines from different vintages.

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FIGURE CAPTIONS

Fig. 1 Distribution of the wines in the plane defined by the two first discriminate functions by amino acids and grape variety. T: Tempranillo wine; O: Monastel wine; V: Maturana Tinta de Navarrete wine

Fig. 2 Distribution of the wines in the plane defined by the two first discriminate functions by biogenic amines and grape variety. T: Tempranillo wine; O: Monastel wine; V: Maturana Tinta de Navarrete wine

Table 1. Distribution of the wine samples according to variety, vintage and winemaking stage

Variety	Vintage 2009		Vintage 2010		
	OH[1]	ML[2]	OH[1]	ML[2]	*Total*
Tempranillo	2	2	4	4	12
Monastel	2	2	4	4	12
Maturana Tinta de Navarrete	2	2	4	4	12
Total	6	6	12	12	36

[1] OH: wines samples taken after alcoholic fermentation; [2] ML: wines samples taken after malolactic fermentation.

Table 2. Concentration (mg/L) of amino acids and biogenic amines by variety, winemaking stage and vintage

	Vintage 2009						Vintage 2010					
	Tempranillo		Monastel		Maturana Tinta de Navarrete		Tempranillo		Monastel		Maturana Tinta de Navarrete	
	OH	ML	OH	ML	OH	ML	OH	ML	OH	ML	OH	ML
Amino acids												
Aspartic acid	5.9 ± 1.9	12.7 ± 3.8	2.1 ± 0.9	5.6 ± 1.6	1.4 ± 0.4	4.8 ± 1.0	4.3 ± 0.1	4.5 ± 0.2	4.8 ± 0.1	4.9 ± 0.2	1.8 ± 0.0	1.9 ± 0.2
Glutamic acid	46.5 ± 13.5	59.1 ± 12.7	22.4 ± 9.1	35 ± 10	11.3 ± 1.4	20.0 ± 2.6	22.7 ± 0.6	21.7 ± 0.6	34.5 ± 1.0	28.9 ± 0.6	8.6 ± 0.3	10.4 ± 0.4
Asparagine	13.2 ± 4.4	17.9 ± 4.0	8.0 ± 2.9	15.2 ± 3.5	4.1 ± 0.9	9.2 ± 2.4	8.6 ± 0.5	11.8 ± 0.3	13.9 ± 0.4	18.4 ± 0.3	3.4 ± 0.1	4.2 ± 0.1
Serine	6.0 ± 1.9	8.5 ± 2.2	2.8 ± 1.2	5.6 ± 1.6	1.3 ± 0.4	3.5 ± 0.8	3.3 ± 0.1	3.8 ± 0.1	5.1 ± 0.2	5.5 ± 0.1	1.2 ± 0.1	1.7 ± 0.1
OH-Proline	1.9 ± 0.5	3.0 ± 0.1	2.9 ± 0.3	6.0 ± 1.1	5.6 ± 0.2	13.7 ± 0.5	3.1 ± 0.1	3.7 ± 0.3	4.1 ± 0.2	4.6 ± 0.3	7.9 ± 0.1	7.7 ± 0.2
Glutamine	48.8 ± 13.7	22.0 ± 2.1	18.6 ± 8.2	8.0 ± 2.9	6.7 ± 1.5	4.8 ± 0.7	26.8 ± 0.5	1.1 ± 0.1	36.8 ± 1.2	1.1 ± 0.0	6.2 ± 0.3	0.3 ± 0.0
Histidine	11.0 ± 2.9	16.8 ± 3.3	5.3 ± 1.7	7.7 ± 4.2	4.3 ± 0.7	3.0 ± 0.2	7.5 ± 0.2	8.8 ± 0.3	9.1 ± 0.3	9.9 ± 0.2	4.8 ± 0.1	4.9 ± 0.1
Glycine	17.6 ± 5.3	28.1 ± 4.2	9.3 ± 2.0	22.0 ± 3.6	6.1 ± 1.1	15.5 ± 1.6	11.8 ± 0.4	13.5 ± 0.4	18.0 ± 0.5	19.4 ± 0.2	6.9 ± 0.2	7.6 ± 0.1
Threonine	7.5 ± 2.1	13.2 ± 3.2	2.9 ± 1.2	7.9 ± 1.9	2.1 ± 0.5	6.4 ± 1.9	4.7 ± 0.1	6.3 ± 0.2	6.2 ± 0.2	7.2 ± 0.1	1.8 ± 0.1	3.7 ± 0.2
ß-Alanine	1.9 ± 0.4	2.9 ± 0.4	7.2 ± 0.8	14.1 ± 0.6	5.3 ± 0.7	10.9± 2.1	1.1 ± 0.1	1.2 ± 0.0	8.1 ± 0.2	7.7 ± 0.1	2.9 ± 0.1	3.0 ± 0.3
Arginine	28.1 ± 8.1	6.0 ± 1.0	8.4 ± 4.0	3.3 ± 0.9	6.1 ± 1.5	6.2 ± 1.7	15.5 ± 0.5	2.4 ± 0.2	19.3 ± 0.5	2.1 ± 0.0	6.0 ± 0.2	2.1 ± 0.4
α-Alanine	32.1 ± 7.8	61.4 ± 16.0	5.5 ± 1.3	37.6 ± 11.3	8.4 ± 2.8	21.1 ± 6	24.5 ± 1.2	26.8 ± 0.7	42.8 ± 1.3	42.0 ± 0.6	7.1 ± 0.2	8.3 ± 0.2
GABA	48.2 ± 14.0	44.9 ± 6.0	25.9 ± 5.2	11.1 ± 3.2	10.7 ± 0.7	15.1 ± 4.6	14.8 ± 0.9	16.1 ± 0.3	14.7 ± 0.4	15.1 ± 0.2	5.2 ± 0.2	5.8 ± 0.3
Proline	841 ± 150	1857 ± 358	1549 ± 467	3558 ± 553	1713 ± 173	4849 ± 145	1628 ± 88	1126 ± 358	3076 ± 30	1979 ± 41	2651 ± 89	1548 ± 64
Tyrosine	2.31 ± 0.2	6.3 ± 2.6	0.7 ± 0.3	2.3 ± 0.9	0.4 ± 0.2	1.9 ± 1.5	3.5 ± 0.1	3.1 ± 0.1	5.5 ± 0.3	4.6 ± 0.1	1.8 ± 0.2	1.8 ± 0.1
Valine	4.3 ± 1.4	7.8 ± 2.8	2.1 ± 1.0	3.7 ± 1.1	1.8 ± 0.4	4.0 ± 1.5	3.8 ± 0.3	7.0 ± 0.8	4.7 ± 0.2	9.3 ± 0.2	2.2 ± 0.2	6.1 ± 0.3
Methionine	2.7 ± 0.9	5.1 ± 2.5	1.4 ± 0.5	2.9 ± 0.4	1.3 ± 0.0	2.9 ± 1.0	1.9 ± 0.1	6.2 ± 1.0	2.8 ± 0.0	8.8 ± 0.1	1.3 ± 0.0	5.3 ± 0.1
Cysteine	1.3 ± 0.3	1.3 ± 0.1	1.0 ± 0.3	1.1 ± 0.1	0.7 ± 0.1	1.0 ± 0.6	1.4 ± 0.0	2.1 ± 0.5	1.7 ± 0.1	5.1 ± 0.0	1.2 ± 0.1	3.7 ± 0.1
Isoleucine	2.2 ± 0.7	4.6 ± 2.6	1.3 ± 0.4	2.5 ± 0.6	1.2 ± 0.1	2.4 ± 0.6	1.4 ± 0.1	3.3 ± 0.5	1.9 ± 0.2	4.7 ± 0.0	0.6 ± 0.0	2.8 ± 0.0
Tryptophan	3.0 ± 0.9	3.8 ± 0.8	1.9 ± 0.3	2.8 ± 0.8	1.6 ± 0.2	2.8 ± 0.3	2.0 ± 0.1	5.6 ± 1.0	3.4 ± 0.1	9.1 ± 0.1	1.1 ± 0.1	3.9 ± 0.0
Leucine	5.5 ± 2.7	9.6 ± 5.2	2.6 ± 1.0	4.6 ± 1.9	3.2 ± 0.6	7.2 ± 2.3	3.1 ± 0.2	5.8 ± 0.3	5.4 ± 2.7	8.2 ± 0.1	2.1 ± 0.1	3.4 ± 0.1

(continued)

	Vintage 2009						Vintage 2010					
	Tempranillo		Monastel		Maturana Tinta de Navarrete		Tempranillo		Monastel		Maturana Tinta de Navarrete	
	OH	ML	OH	ML	OH	ML	OH	ML	OH	ML	OH	ML
Phenylalanine	4.0 ± 1.9	6.4 ± 3.3	2.2 ± 0.9	3.8 ± 1.2	1.9 ± 0.4	4.4 ± 1.4	2.5 ± 0.1	6.8 ± 1.0	4.2 ± 0.2	9.5 ± 0.1	1.5 ± 0.0	4.4 ± 0.1
Ornithine	16.9 ± 5.0	12.6 ± 0.5	2.9 ± 1.8	0.95 ± 0.2	0.8 ± 0.7	0.3 ± 0.1	9.0 ± 0.5	16.8 ± 0.1	7.2 ± 0.2	19.9 ± 0.5	1.4 ± 0.2	1.1 ± 0.0
Lysine	7.9 ± 3.2	16.7 ± 6.0	4.2 ± 1.9	10.6 ± 3.3	3.1 ± 0.6	8.8 ± 1.1	5.6 ± 0.3	8.4 ± 0.1	8.3 ± 0.3	11.7 ± 0.2	2.9 ± 0.1	3.6 ± 0.1
Total	1160 ± 74	2227 ± 454	1690 ± 424	3772 ± 502	1803 ± 162	5019 ± 502	1811 ± 93	1313 ± 47	3339 ± 33	2236 ± 43	2731 ± 89	1646 ± 64
Biogenic amines												
Histamine	3.3 ± 1	1.2 ± 0.1	1.9 ± 0.7	2.2 ± 1.9	1.1 ± 0.1	6.2 ± 1.6	n.d.	0.08 ± 0.01	n.d.	0.06 ± 0.01	n.d.	0.3 ± 0.0
Agmatine	5.3 ± 1.0	11.0 ± 0.1	8.7 ± 0.5	11.8 ± 0.4	4.6 ± 1.4	3.3 ± 0.3	1.88 ± 0.13	8.9 ± 0.2	2.4 ± 0.1	7.9 ± 0.2	2.3 ± 0.0	1.3 ± 0.3
Spermidine	1.16 ± 0.63	1.9 ± 1.2	1.11 ± 0.47	1.75 ± 0.16	1.2 ± 0.2	2.3 ± 0.2	0.73 ± 0.07	5.9 ± 1.9	1.6 ± 0.1	9.7 ± 0.4	0.39 ± 0.09	6.1 ± 0.1
Tyramine	0.16 ± 0.06	1.4 ± 0.7	0.10 ± 0.03	0.82 ± 0.1	0.5 ± 0.3	1.0 ± 0.0	0.12 ± 0.01	0.64 ± 0.04	0.17 ± 0.01	0.84 ± 0.02	0.11 ± 0.03	0.3 ± 0.1
Putrescine	2.4 ± 0.6	25.1 ± 0.5	2.8 ± 0.4	12.8 ± 3.0	5.7 ± 0.8	15.7 ± 2.9	3.1 ± 0.0	7.5 ± 0.2	5.2± 0.1	8.5 ± 0.2	5.6 ± 0.1	7.8 ± 0.2
Tryptamine	0.34 ± 0.04	0.10 ± 0.02	0.24 ± 0.04	0.37 ± 0.35	0.3 ± 0.1	0.13 ± 0.03	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Cadaverine	0.52 ± 0.09	0.6 ± 0.06	0.16 ± 0.10	0.27 ± 0.10	0.6 ± 0.1	1.7 ± 0.4	0.39 ± 0.01	0.43 ± 0.01	0.33 ± 0.01	0.36 ± 0.00	0.37 ± 0.01	0.4 ± 0.0
Phenylethylamine	0.34 ± 0.03	0.42 ± 0.04	0.19 ± 0.03	0.29 ± 0.01	0.11 ± 0.01	0.20 ± 0.02	0.37 ± 0.02	0.31 ± 0.01	0.25 ± 0.02	0.11 ± 0.01	0.54 ± 0.03	0.37 ± 0.01
Isoamylamine	1.07 ± 0.04	1.13 ± 0.09	0.12 ± 0.06	0.20 ± 0.10	0.09 ± 0.02	0.10 ± 0.02	0.29 ± 0.00	1.6 ± 0.3	0.18 ± 0.00	1.47 ± 0.06	0.22 ± 0.02	1.6 ± 0.1
Total	14.6 ± 2.1	42.8 ± 2.1	14.3 ± 2.3	30.5 ± 1.9	13.9 ± 0.4	30.6 ± 5.0	6.9 ± 0.2	25.4 ± 1.8	10.2 ± 0.3	28.9 ± 0.37	9.6 ± 0.2	18.2 ± 0.2

OH: after alcoholic fermentation; ML: after malolactic fermentation; n.d.: no detectable.

Table 3. Significance of ANOVA for the factors variety (V), winemaking stage (S) and vintage (VI)

	Variety (V)	Winemaking stage (S)	Vintage (VI)	V x S	V x VI	S x VI	V x S x VI
Amino acids							
Aspartic acid	***	***	***	ns	***	***	ns
Glutamic acid	***	*	***	ns	***	**	ns
Asparagine	***	***	ns	ns	***	ns	ns
Serine	***	***	**	ns	***	**	ns
OH-Proline	***	***	*	***	***	***	***
Glutamine	***	***	***	***	***	**	**
Histidine	***	*	ns	*	***	ns	ns
Glycine	***	***	***	ns	***	***	ns
Threonine	***	***	***	ns	***	***	ns
ß-Alanine	***	***	***	***	***	***	***
Arginine	***	***	ns	***	***	ns	***
α-Alanine	***	***	ns	ns	***	***	ns
GABA	***	ns	***	ns	***	ns	ns
Proline	***	***	***	**	***	***	***
Tyrosine	***	**	**	ns	***	***	ns
Valine	***	***	***	ns	***	ns	ns
Methionine	**	***	***	ns	***	***	ns
Cysteine	***	***	***	***	***	***	***
Isoleucine	**	***	ns	ns	**	ns	ns
Tryptophan	***	***	***	*	***	***	**
Leucine	*	***	ns	ns	***	ns	ns
Phenylalanine	***	***	*	ns	***	*	ns
Ornithine	***	ns	*	ns	***	**	ns
Lysine	***	***	*	ns	***	**	ns
Total	***	***	***	***	***	***	***
Biogenic amines							
Histamine	*	*	***	***	*	ns	***
Agmatine	***	***	***	***	**	ns	ns
Spermidine	*	***	***	ns	**	***	ns
Tyramine	ns	***	***	*	*	*	ns
Putrescine	***	***	***	***	***	***	***
Tryptamine	ns	ns	***	ns	ns	ns	ns
Cadaverine	***	***	***	***	***	***	***
Phenylethylamine	**	ns	ns	*	ns	ns	ns
Isoamylamine	***	***	***	ns	***	***	ns
Total	**	***	***	***	**	*	*

*, **, *** indicate significance at $p < 0.05$, $p < 0.01$, $p < 0.001$, respectively. ns indicates no significant difference at $p < 0.05$.

Figure 1

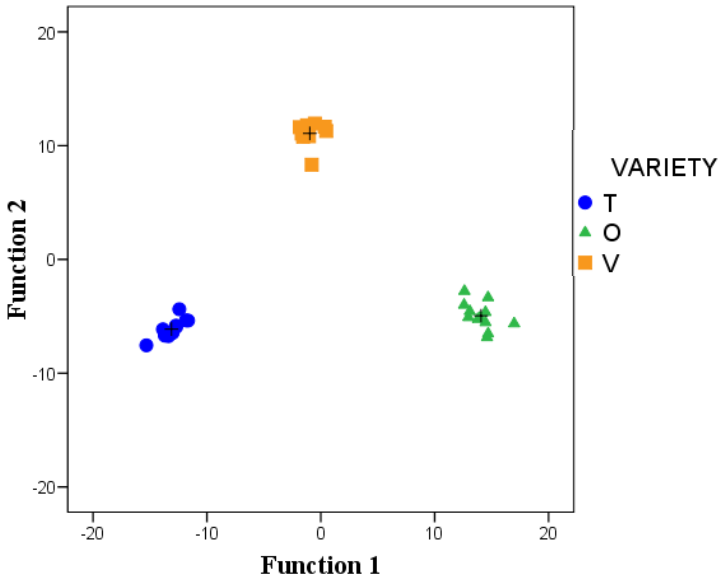

Figure 2