Determination of dansylated amino acids and biogenic amines by HPLC-FLD in Cannonau and Vermentino wines

Running title: Nitrogen compounds in Cannonau and Vermentino wines

Carlo Ignazio Giovanni Tuberoso[a,*], Francesca Congiu[a], Gabriele Serreli[a], Stefano Mameli[a]

[a] *Department of Life and Environmental Sciences, University of Cagliari, Via Ospedale 72, 09124 Cagliari, Italy*

* Corresponding author. Tel.: +39 0706758644; fax: +39 0706758612.

E-mail address: tuberoso@unica.it (C.I.G. Tuberoso).

Abstract

Free amino acids (AA) and biogenic amines (BA) were quantified for the first time in Cannonau and Vermentino wines, the two most popular "Controlled Designation of Origin" wines from Sardinia (Italy). An analytical method for the simultaneous determination of AA and BA was developed, using selective derivatisation with dansyl chloride followed by HPLC with fluorescence detection. Thirty-two compounds were identified in the wines analyzed. High levels of AA were found, with proline being the most abundant with levels up to 1244.33 ± 398.48 and 1007.89 ± 280.96 mg/L in Cannonau and Vermentino wines, respectively. BA were detected at average concentrations < 10 mg/L except putrescine which reached 20.51 ± 10.17 mg/L in Cannonau wines. The results of this work show general good quality of Cannonau and Vermentino wines as demonstrated by the detection of low level BA production due to proper winemaking technique.

Keywords: wine, HPLC, fluorescence detection, dansyl chloride, amino acids, biogenic amines, validation

1. Introduction

Cannonau and Vermentino wines are the most popular wines of Sardinia, protected by the European Union "Controlled Designation of Origin" (E-Bacchus, 2007). They are obtained from a red and white grape variety which goes by the same name, respectively. Although some physical-chemical characterization studies have been reported, no quantitative analysis of free amino acids (AA) and biogenic amines (BA) content in these wines has been described. Free AA in wine have different origins (degradation of the grape proteins, yeasts and lactic acid bacteria metabolism) and their content can be affected by grape variety, climate, viticulture practices and winemaking techniques, mainly maceration time and yeasts and lactic acid bacteria strains (Ribéreau-Gayon, Dubourdieu, Donèche & Lonvaud, 2006). The composition of the AA fraction is important since these compounds are indispensable for the nitrogen intake and have important biological effects (WHO/FAO/UNU, 2007). The most studied AA are the so called essential AA (histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophan, and valine), but other AA have interesting properties. For instance, arginine acts as a precursor for the biosynthesis of nitric oxide (NO) and supports the immune response (Campbell et al., 2006). γ-Aminobutyric acid (GABA), a γ-ammino acid derived from glutamic acid decarboxylation and not used in protein formation, regulates blood pressure, has a role in neurotransmission, and has diuretic and anti-stress effects (Diana, Tres, Quílez, Llombart & Rafecas, 2014). AA are the precursor of BA, basic nitrogenous compounds synthesized in all living organism by metabolic pathways that usually involve AA decarboxylation (Kusano, Berberich, Tateda & Takahashi, 2008). Grapevine contains BA, such as putrescine, cadaverine in berries (Agudelo-Romero, Bortolloti, Tiburcio & Fortes, 2013; Vincenzini, Guerrini, Mangani & Granchi, 2009). Different agricultural practices, such as conventional, organic and biodynamic, and winemaking procedures can

greatly affect the final amount of BA in wines (Tassoni, Tango & Ferri, 2013; Yañez, Saavedra, Martínez, Córdova & Ganga, 2012; Yildirim, Üren & Yücel, 2007) because during the fermentation processes from must to wine, microorganism can produce histamine and tyramine (Beneduce et al., 2010; Herbert, Cabrita, Ratola, Laureano & Alves, 2006). Thus, the presence of BA in wine can be a consequence of yeasts primary fermentation and bacteria malolactic fermentation metabolism (García-Marino, Trigueros & Escribano-Bailón, 2010) BA in food and beverages are of toxicological interest because they can have direct or indirect effects on the human vascular and nervous systems. At high a concentrations, they may induce headaches, respiratory distress, heart palpitation, hyper- or hypotension. Recent studies have demonstrated that the interaction between ethanol (a monoamine oxidase inhibitor) and amines seems to be synergistic. This is important for wine consumers that are sensitive to such compounds (Ladero, Calles-Enríquez, Fernández & Alvarez, 2010; Smit, du Toit & du Toit, 2008). Due to these issues, some European countries have identified upper limits for histamine in wine varying from 2 to 10 mg/L, but most of them are waiting for the EU to provide a regulatory framework for biogenic amines. To this end, the European Food Safety Authority (EFSA) is currently collecting data on biogenic amines in food (EFSA, 2010).

Ideally, the levels of AA and BA in wine should be analysed simultaneously. This is important to be able to study AA and BA evolution during must fermentation, to compare wines obtained with different winemaking procedures and to characterize monovarietal wines. However, AA and BA are difficult to analyze simultaneously because of their structural diversity (aliphatic, aromatic and heterocyclic skeletons, presence of groups with different pK). Such chemical heterogeneity complicates the chromatographic separation and is a challenge for the choice of the proper detector. Therefore, several methods that allow simultaneous BA and AA analysis have been proposed. All of these methods use derivatising

agents before HPLC analysis. The most frequently applied derivatisation reagents are *o*-phtalaldehyde (OPA) (Kutlán & Molnár-Perl, 2003), 9-fluorenylmethyloxycarbonyl chloride (FMOC-Cl) (Bauza et al., 2007); Molnár-Perl, 2011), dansyl chloride (DCl) (Mazzucco, Gosetti, Bobba, Marengo, Robotti & Gennaro, 2010; Pineda, Carrasco, Pena-Farfal, Henríquez-Aedo & Aranda, 2012), dabsyl chloride (Krause, Bockhardt, Neckermann, Henle & Klostermeyer, 1995), diethyl ethoxymethylenemalonate (DEEMM) (Gómez-Alonso, Hermosín-Gutiérrez & García-Romero, 2007; Cejudo-Bastante, Sonni, Chinnici, Versari, Perez-Coello & Riponi, 2010), *p*-*N*,*N*,*N*-trimethylammonioanilyl *N*'-hydroxysuccinimidyl carbamate iodide (TAHS), and 2,5-dioxopyrrolidin-1-yl *N*-tri(pyrrolidino)phosphoranylideneamino carbamate (FOSF) (Rebane, Oldekop & Herodes, 2012). Among the detectors, fluorescence and UV-VIS detection are the most widely used, along with the occasional use of MS. The fluorescence detector has a higher sensitivity than the UV-VIS detection and is very selective. Dansyl chloride is one of the most widely used derivatisation reagents because of its ability to form stable compounds with primary and secondary amines (Jia, Kang, Park, Lee & Kwon, 2011). This is important since some derivatisation reagents like OPA do not react with proline (Molnár-Perl, 2011) which is the most abundant AA in wine and also DEEMM can be unstable (Rebane, Oldekop & Herodes, 2012).

In view of the current situation, the aims of this work were i) to improve a HPLC-FLD analytical method to simultaneously analyse 22 amino acids, 13 biogenic amines, and the ammonium ion ii) to validate our approach in wine, and iii) to apply it to quantify free AA and BA in Cannonau and Vermentino wines.

2. Material and methods

2.1. Chemicals and reagents

All the chemicals used in this study were of analytical grade. Acetone, acetonitrile, methanol, acetic acid, hydrochloric acid (37%, *w/w*), $Na_2B_4O_7 \cdot 10\ H_2O$, CH_3COONa, and dansyl chloride were purchased from Sigma-Aldrich (Milan, Italy). Primary reference standard of AA and BA (purity > 99.9%) were obtained from Sigma-Aldrich, Merk and Carlo Erba (Milan, Italy). AA and BA stock of standard solutions (ca. 1000 mg/L) were prepared by dilution with 0.1 M HCl/MeOH (1:1, *v/v*) and stored at +4 C° until use. The derivatisation agent solution was prepared dissolving 50 mg of dansyl chloride (DCl) in 10 mL of acetone, and was stored at +4 C° until use (solution is stable up to 3 months). Ultrapure water (18.0 MΩcm, 25°C) was obtained with a Milli-Q Advantage A10 System apparatus (Millipore, Milan, Italy).

2.2. Wine samples

Eight wine samples "Cannonau di Sardegna DOC" and seven wine samples "Vermentino di Sardegna DOC" produced in Sardinia (Italy) from the 2012 harvest were analyzed. Samples were commercially available wines supplied directly by different wineries with certified origin and made using standard oenological procedures.

2.3 Amino acids and biogenic amines derivatisation

Determination of AA and BA was carried out after derivatisation with DCl. The reaction mixture was prepared in 1.5 mL Eppendorf Safe Lock Tubes™, and consisted of 50-100 μL of sample (wine or standards), 10 μL of 100 mg/L norvaline (internal standard, IS), 100 μL of dansyl chloride solution (derivatisation agent) and 0.2 M $Na_2B_4O_7 \cdot 10\ H_2O$ (pH 9.3) solution up to a final volume of 1000 μL. The mixture was incubated for 30 min at 40°C in an

ultrasound bath, and centrifuged at 12000 rpm for 10 min with an Eppendorf® MiniSpin centrifuge (Eppendorf, Milan, Italy). The supernatant was recovered and diluted with MeOH (1:1, *v/v*) for HPLC-FLD analysis.

2.4. HPLC-FLD Analysis

The HPLC determination of the AA and BA dansyl derivatives was performed with an HPLC-FLD Varian system ProStar (Varian Inc., Walnut Creek, CA, USA) fitted with a pump module 230, an autosampler module 410, and a Jasco 821-FP fluorimetric detector (Jasco Europe, Cremella, LC, Italy) with wavelengths were set at 293 nm (Ex) and 492 nm (Em). Separation was obtained with a Phenomenex Gemini C18 110A column (150 x 4.60 mm, 3 µm, Chemtek Analitica, Anzola Emilia, Bologna, Italy) thermostated at 25°C, using pH 4.10 buffer acetate/CH_3CN (solvent A; 6.25 mL CH_3COOH, 1.97 g CH_3COONa, 200 mL CH_3CN, water up 1 L) and acetonitrile (solvent B) as mobile phase at constant flow rate of 0.8 mL/min. The gradient (*v/v*) was generated 95% of solvent A, decreasing to 80 % in 18 min, to 50 % in 42 min, to 0 % in 60 min and let at 0 % till 64 min. Before each injection, the system was stabilized for 10 min with the initial A/B ratio (95:5, *v/v*). Injection volume was 20 µL. Chromatograms and data were acquired with a HP Hewlett Packard 3396 series II integrator (Hewlett Packard, Cernusco sul Naviglio, Milan, Italy). The quantitative analysis was performed using calibration graphs constructed according to the internal standard method, correlating the analyte/IS peak area ratios *vs*. the concentration. The HPLC-FLD chromatogram of the AA and BA standard solution is shown in Fig. 1.

2.5. Method Validation

The established method was validated in agreement with the International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human

Use (ICH) guidance note which describes validation of analytical methods (ICH Topic Q2 (R1), 1996) by determining linearity, limits of detection (LOD), limits of quantification (LOQ), precision and accuracy. The linearity was evaluated by preparing standard mixtures (containing the analytes and the IS) at six different concentrations, subjecting the mixtures to the derivatisation procedure and analysis by HPLC-FLD. The analyte/IS peak area ratios were plotted against the corresponding concentrations, and the calibration curves were constructed by means of the least-squares method. Table 1 reports the concentration range and the coefficient of determination (r^2) for each compound. The LODs and LOQs were calculated according to the equation $LOD = 3.3\sigma/S$ and $LOQ = 10\sigma/S$, respectively (where σ = standard deviation of the blank, and S = slope of the calibration curve). To evaluate precision, the intra- and inter-day repeatability were determined performing six injections of the same derivatised standard within one day and over three consecutive days, respectively. The relative standard deviation (RSD) for the peak area was determined as a measurement of precision. To evaluate the accuracy of the analytical method, the recovery study was measured by spiking known amounts of standards in Vermentino wine samples in triplicate at two concentrations (1 and 10 mg/L). The mixture was analyzed using the method described in previously. The matrix effect was evaluated comparing the response of a standards mix prepared at 10 mg/L both in Vermentino wine and in water. No statistical differences were observed ($p < 0.05$).

3. Results and discussion

The HPLC-FLD method we propose allowed the separation of 36 amines (22 AA, 13 BA, ammonium ion) and norvaline in ca. 60 min. It is important to note that this run time is shorter than the most commonly used methods for simultaneous determination of AA and BA,

which require from ca. 70 to 138 min (Herbert et al., 2006; Krause et al., 1995; Gómez-Alonso et al., 2007; Cejudo-Bastante et al., 2010). In complex mixtures containing AA and BA, only UPLC systems allow to obtain a good peak separation in less time (Jia et al., 2011). Also the number of separated AA and BA is relevant, the highest among the methods that use DCl derivatisation (Mazzucco et al., 2010; Jia et al., 2011). This fact allows to obtain a general chromatographic fingerprinting of the wines which gives lot of qualitative and quantitative information. Derivatisation using DCl reagent lead to the formation of a group of side products from 0 to ca. 6 min and the detection of two more peaks at 41.4 and 42.6 min (Fig. 1a). These peaks did not interfere with most of the AA and BA separation, therefore an accurate quantification of amines by HPLC-FLD could be achieved. We observed the co-elution of few amines (e.g. Phe+Trp and Leu+Iso), and some peaks present matrix interference (Ser, Met, His and Cad): nevertheless the experimental conditions we used allowed a very efficient separation even though a high number of compounds was detected (Fig. 1b). All methods that involve analysis of numerous AA and BA show critical separation of several compounds or matrix interference: for instance, Gln-His, Arg-Ala, HIS, PEA, TRA (Herbert et al., 2006); Thr, Ala-Arg, Val-Met, Trp-His, Phe-MTA, Lys-ETA, CAD, PUT (Kutlán & Molnár-Perl, 2003), GABA-Pro, Trp, AGM, Orn-Lys-His (Krause et al., 1995); Asp, Asn-Ser-Hyp, ammonium ion-AGM, Trp-Leu, SPD, TRA (Gómez-Alonso et al., 2007); Hyp, Gly-His-Thr, Arg-Ala, HIA, Trp, Leu-Phe, TYA, SPD, CAD (Cejudo-Bastante et al., 2010); Glu-Asp, Val-Met, Phe-Iso-Leu, Lys-TRA, HIS-Tyr (Jia, et al., 2011). We observed that to avoid loss in peak separation it is fundamental to control the pH of the mobile phase. Following optimization of this technique our protocol proved to be reproducible. Table 1 reports the main validation parameters calculated according to the ICH Topic Q2 (R1) guidance note on validation of analytical methods (ICH Topic Q2 (R1), 1996). The

calibration curves for commercial standards were plotted covering the typical range of concentrations of the wines, with $r^2 > 0.995$ in almost all cases, indicating linearity of the derivatives' response. The limits of detection (LOD) for amines ranged from a minimum of 0.004 mg/L for MET to a maximum of 0.23 for asparagine Asp. The limits of quantification (LOQ) were below 1 mg/L for all compounds. The precision of our method was evaluated testing intra- and inter-day repeatability. The relative standard deviation (RSD) for the area under the peak was determined as a measure of precision. Six injections of the same standard containing all the AA and BA within one day and over three consecutive days, respectively were performed. All coefficients of variation were lower than 5 %, and therefore within acceptable limits. The accuracy of our method was evaluated using recovery rates. We added increasing amounts (1 mg/L and 10 mg/L) of each target compound to a Vermentino wine sample, and analysed each spiked sample in triplicate. Recovery rates were between 95.38 and 103.16 % (Tab. 1).

Figure 1c and 1d show AA and BA profiles in Cannonau and Vermentino wines, respectively. The chromatographic fingerprinting is qualitatively similar in both wines, but quantitative differences can be observed. Table 2 shows the concentration (mg/L) of AA and BA listed according to their order of elution. Each sample was analyzed in triplicate and thirty-two compounds were identified and dosed, showing a significant difference ($p < 0.05$) between the two wines. Among the AA, Pro and Arg were the most abundant both in Cannonau and Vermentino wines (1244.33 ± 398.48 and 1007.89 ± 280.96 mg/L for Pro; 82.41 ± 53.46 and 147.31± 42.83 mg/L for Arg). Both wines showed detectable levels of Glu, Ala and Asn. A higher concentration of GABA was found in Vermentino wines than in Cannonau wines (130.28 ± 44.80 and 43.14 ± 8.88 mg/L, respectively). Our findings indicate that there are higher levels of Tyr and Leu+Iso in Vermentino wines while Cannonau wines

have higher levels of His. Using our approach AGM was never detected (< LOD). The variability in the amount of some of the amines in these samples was probably due to differences in winemaking procedures. Low levels of BA were observed in both Cannonau and Vermentino samples. When compared to Vermentino wines, Cannonau wines had lower levels of BA (84.09 ± 9.03 and 144.84 ± 43.66 mg/L, respectively). In all Vermentino wines, the most prevalent BA were PUT (5.96 ± 2.62 mg/L), ETA (4.68 ± 1.24 mg/L), and CAD (2.06 ± 0.52 mg/L). BA such as HIA, SPM and DA were not detected and only traces of TYA and SPD were found. The most abundant BA found in all Cannonau wines were PUT (20.51 ± 10.17 mg/L), TYA (6.06 ± 2.86 mg/L), and ETA (7.55 ± 6.29 mg/L). HIA was only detected in five Cannonau samples with average levels of 8.11 ± 6.61 mg/L. No significant correlation ($p < 0.05$) was found between BA content and AA concentration. This was to be expected because their amount from grapes to wines is affected by different agronomic and oenological parameters (Cejudo-Bastante et al., 2010; Martínez-Pinilla, Guadalupe, Hernández & Ayestarán, 2013). Only specific experimental design that follows the AA and BA evolution by changing one variable at time (e.g. grape variety, geographical area, vintage, yeast and/or bacteria strains, and winemaking technology) can give reliable significant correlation.

Free AA and BA in Cannonau and Vermentino wines have not been studied. Mulas et al. (Mulas et al., 2011) analyzed several metabolites of Vermentino berries by NMR and quantified Ala, Arg, Pro, Gln, Iso, Leu, Val, and GABA in these grapes. Our findings are in line with what has been previously reported (Herbert et al., 2006; Kutlán & Molnár-Perl, 2003; Gómez-Alonso et al., 2007; Martínez-Pinilla et al., 2013), although caution is required when making this comparison due to differences in grape variety, geographical region, quality of raw material, winemaking processes, vintage, time, storage conditions and possible

microbial contaminations (Vincenzini et al., 2009; Beneduce et al., 2010). Cannonau and Vermentino wines show similar levels of total AA, while higher amounts of AA are found in red wines (Gómez-Alonso et al., 2007) or white ones (Kutlán & Molnár-Perl, 2003). Previous studies indicate that Pro is by far the most abundant AA in all wine samples and its concentration shows huge variability according to wine type. Unfortunately, its amount is affected by different causes and thus it is not useful to discriminate monovarietal wines (Long et al., 2012). Our studies show that the levels of Pro in Sardinian wine samples was comparable with popular wines such as Chardonnay and Merlot (Long et al., 2012), or more local wines like Tempranillo (Martínez-Pinilla et al., 2013). In addition to Pro, GABA, Glu, Ala, Arg and Lys were found at high level. We detected high levels of GABA, Leu+Ile, particularly in Vermentino wines, and those AA discriminate Vermentino from Cannonau. The levels of BA we found in Vermentino and Cannonau wines were within ranges reported in the literature (Herbert et al., 2006; Martínez-Pinilla et al., 2013; Peña-Gallego, Hernández-Orte, Cacho & Ferreira, 2012). High levels of BA such as PUT, HIA, CAD, PEA, and TYA we found in Cannonau wine might be a product of malolactic fermentation, in agreement with other researches (Herbert et al., 2006; Martínez-Pinilla et al., 2013).

4. Conclusions

Here we show that the analytical method used in this study for the simultaneous determination of free AA and BA in Cannonau and Vermentino wines is accurate, precise and sensitive. Our findings indicate that the selective pre-column derivatisation using DCl reagent efficiently derivatises all amines, including proline. Furthermore, we have shown that the HLPC-FLD technique can be used to identify different types of wines looking at their AA and BA profile, evaluating common wine AA (such as proline), essential AA and AA precursors

of BA. Cannonau and Vermentino wines produced in Sardinia following to the "Controlled Designation of Origin" protocols have levels of BA within the average values suggesting that proper winemaking technique is used. Our studies suggest that further optimization of winemaking parameters (e.g. yeast strain, temperature, and maceration time) can be achieved and would improve the quality of wines making them safer for use by humans.

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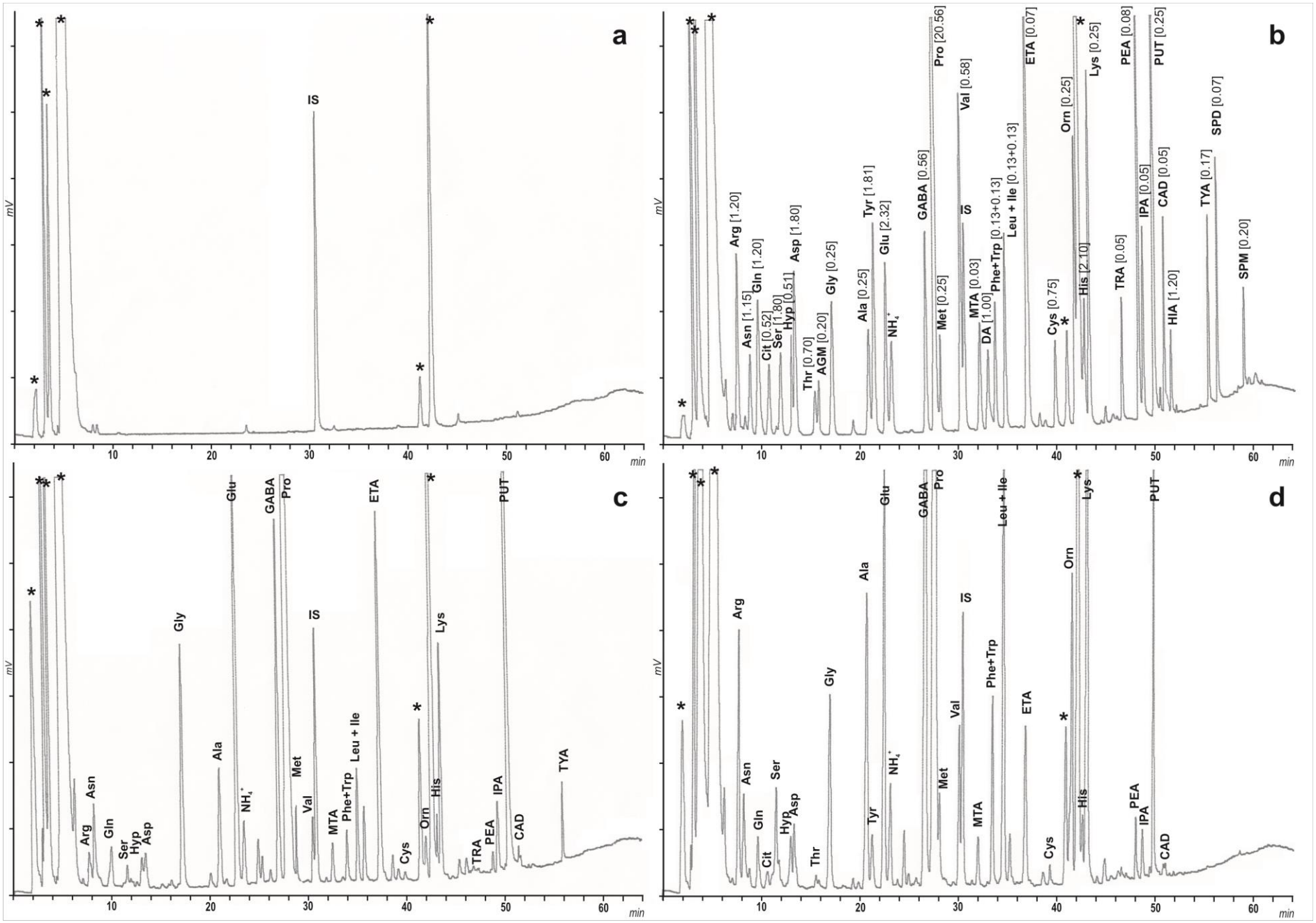
Figure Captions

Fig. 1. Representative HPLC-FLD chromatograms of (**a**) blank with IS, (**b**) standard mix (concentrations mg/L are reported inside the square brackets), (**c**) Cannonau wine, and (**d**) Vermentino wine. Peaks marked with * are compounds produced by the derivatisation procedure. Chromatographic conditions are described in the text. For peak identification, see Table 1.


a
IS
mV
min
b
Arg [1.20]
Asn [1.15]
Gln [1.20]
Cit [0.52]
Ser [1.80]
Hyp [0.51]
Asp [1.80]
Thr [0.70]
AGM [0.20]
Gly [0.25]
Ala [0.25]
Tyr [1.81]
Glu [2.32]
NH4+
GABA [0.56]
Pro [20.56]
Met [0.25]
Val [0.58]
IS
MTA [0.03]
DA [1.00]
Phe+Trp [0.13+0.13]
Leu + Ile [0.13+0.13]
ETA [0.07]
Cys [0.75]
Orn [0.25]
His [2.10]
Lys [0.25]
TRA [0.05]
PEA [0.08]
IPA [0.05]
CAD [0.05]
HIA [1.20]
PUT [0.25]
TYA [0.17]
SPD [0.07]
SPM [0.20]
c
Arg
Asn
Gln
Ser
Hyp
Asp
Gly
Ala
Glu
NH4+
GABA
Pro
Met
Val
IS
MTA
Phe+Trp
Leu + Ile
ETA
Cys
Orn
His
Lys
TRA
PEA
IPA
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d
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Cit
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Thr
Gly
Ala
Tyr
Glu
NH4+
GABA
Pro
Met
Val
IS
MTA
Phe+Trp
Leu + Ile
ETA
Cys
Orn
His
Lys
PEA
IPA
CAD
PUT

Fig.1

Table 1
LC-FLD validation parameters of analytical procedure for amino acids and biogenic amines

Compound		Rt (min)	LOD mg/L	LOQ mg/L	*Linearity* Concentration range mg/L	*Linearity* r^{2a}	*Linearity* Calibration equations	*Repeatability (intra-day)* Peak area (RSD[b], %)	*Intermediate precision (inter-day)* Peak area (RSD[b], %)	*Accuracy* Recovery ± SD[c] (%) 1 mg/L	*Accuracy* Recovery ± SD[c] (%) 10 mg/L
Arginine	Arg	7.7	0.18	0.55	0.61-12.23	0.9993	y = 0.3933x - 0.0348	1.16	1.02	98.44 ± 2.29	98.01 ± 2.29
Asparagine	Asn	9.0	0.23	0.69	0.74-14.83	0.9984	y = 0.2166x - 0.0093	0.84	1.14	98.21 ± 1.64	97.90 ± 2.63
Glutamine	Gln	10.0	0.07	0.21	0.27-5.35	0.9994	y = 0.1255x - 0.0175	1.14	0.99	97.37 ± 3.04	97.05 ± 3.37
Citrulline	Cit	10.9	0.04	0.11	0.11-2.23	0.9919	y = 0.4503x - 0.0532	0.79	1.12	96.57 ± 2.15	97.89 ± 2.65
Serine	Ser	12.0	0.16	0.49	0.51-10.20	0.9941	y = 0.34x + 0.0698	0.52	1.12	98.08 ± 2.88	97.67 ± 3.14
4-Hydroxyproline	Hyp	12.4	0.08	0.24	0.26-5.26	0.9985	y = 0.4369x - 0.0261	2.46	1.34	97.86 ± 3.29	102.24 ± 1.84
Aspartic acid	Asp	13.3	0.15	0.46	0.50-10.06	0.9995	y = 0.1704x + 0.1005	1.11	2.76	98.27 ± 1.89	97.62 ± 1.42
Threonine	Thr	15.9	0.14	0.41	0.49-9.84	0.9993	y = 0.1806x - 0.0111	1.10	0.54	98.84 ± 2.28	97.01 ± 3.41
Agmatine	AGM	15.8	0.05	0.16	0.18-3.64	0.9984	y = 1.6369x - 0.1827	1.05	2.33	97.40 ± 1.39	94.49 ± 3.16
Glycine	Gly	17.2	0.04	0.11	0.14-2.95	0.9959	y = 1.4641x - 0.0371	1.67	1.34	97.93 ± 3.20	98.00 ± 2.00
Alanine	Ala	21.0	0.08	0.24	0.25-5.03	0.9989	y = 0.2942x - 0.0476	0.71	1.03	98.77 ± 1.93	97.63 ± 2.75
Tyrosine	Tyr	21.6	0.08	0.23	0.25-5.02	0.9987	y = 0.2952x - 0.0029	1.17	1.36	97.78 ± 2.70	98.59 ± 2.33
Glutamic acid	Glu	23.4	0.15	0.45	0.55-10.91	0.9997	y = 0.1094x + 0.0175	2.22	1.60	97.44 ± 2.15	97.51 ± 2.95
γ-Aminobutyric acid	GABA	26.8	0.05	0.15	0.15-3.01	0.9964	y = 0.7252x - 0.0207	1.86	1.51	96.97 ± 1.72	97.89 ± 2.78
Proline	Pro	27.8	0.07	0.21	5.03-100.60	0.9980	y = 1.0995x - 0.0713	3.66	2.62	98.11 ± 2.20	98.02 ± 1.59
Methionine	Met	28.3	0.05	0.14	0.16-3.12	0.9989	y = 0.891x - 0.081	1.96	1.78	95.47 ± 2.18	97.82 ± 1.56
Valine	Val	30.4	0.06	0.17	0.18-3.58	0.9984	y = 1.5158x - 0.0354	0.51	1.41	102.10 ± 1.98	101.96 ± 2.06
Methylamine	MTA	32.3	0.00	0.01	0.01-0.21	0.9931	y = 3.9625x - 0.0289	1.20	0.49	97.59 ± 2.11	102.47 ± 2.11
Dopamine	DA	33.2	0.06	0.18	0.36-7.20	0.9980	y = 1.4115x + 0.228	2.62	2.02	97.71 ± 1.29	96.34 ± 4.30
Phenylalanine	Phe	33.8	0.06	0.17	0.21-4.22	0.9987	y = 1.2097x - 0.0849	1.64	1.88	98.04 ± 2.58	97.75 ± 2.12
Leucine	Leu	35.0	0.03	0.10	0.11-2.24	0.9991	y = 1.7401x - 0.0283	1.28	2.79	97.49 ± 2.25	96.10 ± 1.67
Ethylamine	ETA	37.2	0.01	0.04	0.06-1.11	0.9984	y = 5.0459x - 0.1335	0.57	0.96	98.82 ± 3.51	98.61 ± 1.78
Cysteine	Cys	39.8	0.06	0.18	0.21-4.23	0.9989	y = 0.3303x + 0.0771	2.86	2.71	97.63 ± 2.60	97.44 ± 2.12
Ornithine	Orn	42.2	0.04	0.13	0.26-5.24	0.9993	y = 1.9707x - 0.01	1.44	4.03	97.21 ± 2.09	98.09 ± 2.63
Histidine	His	43.0	0.15	0.46	0.50-9.99	0.9984	y = 0.1413x - 0.0413	2.70	3.04	95.38 ± 2.21	93.79 ± 3.51
Lysine	Lys	43.5	0.03	0.09	0.15-3.03	0.9985	y = 0.4385x - 0.0144	1.99	1.52	97.29 ± 1.87	96.48 ± 2.28
Tryptamine	TRA	47.0	0.01	0.02	0.02-0.46	0.9986	y = 5.8214x - 0.0363	0.47	1.32	97.94 ± 2.28	98.63 ± 3.66
Phenylethylamine	PEA	48.6	0.02	0.05	0.05-1.01	0.9987	y = 3.0694x - 0.1004	1.10	1.88	97.89 ± 1.97	98.66 ± 1.88
Isopentylamine	IPA	49.2	0.00	0.01	0.01-0.21	0.9987	y = 7.5805x - 0.0643	1.69	2.05	97.35 ± 1.35	97.83 ± 1.91
Putrescine	PUT	50.3	0.02	0.05	0.11-2.30	0.9949	y = 5.5167x - 0.0307	0.89	2.70	98.17 ± 3.56	97.68 ± 2.60
Cadaverine	CAD	51.3	0.00	0.01	0.01-0.21	0.9989	y = 7.8622x - 0.0388	2.67	1.41	97.90 ± 1.42	103.16 ± 3.76
Histamine	HIA	51.8	0.02	0.05	0.05-0.99	0.9986	y = 0.4859x - 0.0648	2.79	2.32	98.08 ± 1.78	101.57 ± 2.29
Tyramine	TYA	55.9	0.03	0.10	0.11-2.27	0.9985	y = 1.0466x - 0.0331	1.44	1.30	98.25 ± 2.18	96.97 ± 1.77
Spermidine	SPD	56.8	0.02	0.05	0.05-0.96	0.9989	y = 2.2977x - 0.0174	1.08	0.60	97.77 ± 1.54	97.80 ± 1.61
Spermine	SPM	59.2	0.02	0.05	0.05-1.08	0.9925	y = 0.409x - 0.0174	1.53	1.65	98.31 ± 2.91	97.68 ± 2.37

[a] Calibration equations built with the linear least-square method in triplicate.
[b] *RSD* Relative standard deviation obtained from five replicates
[c] *SD* Standard deviation. Average percent recovery obtained from triplicate measurements

Table 2

Amino acids and biogenic amines amounts (mg/L) in Cannonau and Vermentino wines

Compound		t_R (min)	Cannonau wine (n=8)				Vermentino wine (n=7)			
			min	max	mean	± SD	min	max	mean	± SD
Arginine	Arg	7.7	23.82	152.52	82.41	53.46	70.83	198.27	147.31	42.83
Asparagine	Asn	9.0	52.11	75.79	63.95	16.74	15.95	86.74	50.98	24.46
Glutamine	Gln	10.0	5.64	19.03	12.79	4.44	7.02	16.61	10.32	3.40
Citrulline	Cit	10.9	nd	26.49	17.97	5.33	nd	32.60	23.21	8.55
Serine	Ser	12.0	10.23	31.86	17.68	7.32	9.33	47.96	22.99	13.73
4-Hydroxyproline	Hyp	12.4	6.12	7.78	6.75	0.67	11.65	18.47	13.83	2.81
Aspartic acid	Asp	13.3	10.90	80.86	37.94	25.24	25.58	65.51	40.74	14.03
Threonine	Thr	15.9	11.42	19.28	14.41	4.25	5.84	14.71	11.17	3.07
Agmatine	AGM	15.8			nd				nd	
Glycine	Gly	17.2	34.91	45.30	39.40	3.43	17.79	35.15	24.23	6.98
Alanine	Ala	21.0	47.90	90.20	68.63	18.56	41.74	129.24	80.61	35.45
Tyrosine	Tyr	21.6	nd	tr	tr		nd	45.89	36.89	7.57
Glutamic acid	Glu	23.4	43.08	111.83	68.68	28.12	64.38	126.91	82.16	25.02
γ-Aminobutyric acid	GABA	26.8	29.80	52.52	43.14	8.88	70.56	191.93	130.28	44.80
Proline	Pro	27.8	862.77	1872.17	1244.33	398.48	711.38	1391.54	1007.89	280.96
Methionine	Met	28.3	23.85	33.17	28.51	6.59	18.98	37.30	27.31	6.15
Valine	Val	30.4	11.37	18.21	14.75	2.94	12.93	24.95	17.69	5.14
Methylamine	MTA	32.3	0.20	1.66	0.90	0.56	1.09	2.17	1.51	0.37
Dopamine	DA	33.2			nd				nd	
Phenylalanine*+Tryptophan	Phe*+Trp	33.8	17.85	18.53	18.19	0.48	22.00	34.41	27.36	5.20
Leucine*+Isoleucine	Leu*+Ile	35.0	14.32	28.07	20.94	2.55	34.53	87.91	59.10	22.45
Ethylamine	ETA	37.2	4.14	11.30	7.55	6.29	3.44	6.60	4.68	1.24
Cysteine	Cys	39.8	6.55	6.62	6.58	0.19	6.68	6.73	6.70	0.02
Ornithine	Orn	42.2	6.43	67.22	30.60	28.27	3.93	54.95	26.73	22.30
Histidine	His	43.0	17.65	75.16	30.62	21.97	7.56	15.03	11.24	3.08
Lysine	Lys	43.5	32.11	46.92	37.25	8.39	48.89	60.58	53.51	5.36
Tryptamine	TRA	47.0	tr	0.05	0.05		tr	0.06	0.06	
Phenylethylamine	PEA	48.6	nd	1.21	1.21		nd	1.78	1.78	
Isopentylamine	IPA	49.2	0.07	0.14	0.10	0.05	0.08	0.17	0.12	0.04
Putrescine	PUT	50.3	11.42	32.81	20.51	10.17	1.48	10.55	5.96	2.62
Cadaverine	CAD	51.3	1.00	2.42	2.13	0.56	1.13	2.48	2.06	0.52
Histamine	HIA	51.8	tr	8.11	6.61	1.54			nd	
Tyramine	TYA	55.9	5.08	11.52	9.06	2.86	nd	tr	tr	
Spermidine	SPD	56.8	nd	1.27	1.27		nd	tr	tr	
Spermine	SPM	59.2			nd				nd	

* amino acid used to quantify the peak (literature data on wines report this compound predominant to the other)

nd not detected (below the limit of detection)

tr traces (below the limit of quantification)