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

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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 derivatization with dansyl chloride followed by HPLC with fluorescence detection. Thirty-two compounds were identified in the wines analysed. High levels of AA were found, with proline being the most abundant with average levels of 1244 ± 398 and 1008 ± 281 mg/L in Cannonau and Vermentino wines, respectively. BA were detected at average concentrations <10 mg/L, except putrescine which reached 20.5 ± 10.2 mg/L in Cannonau wines. Histamine was never detected in any Vermentino wines. γ-Aminobutyric acid, 4-hydroxyproline, glycine, leucine + isoleucine and putrescine proved to be useful for differentiating Cannonau wines from Vermentino wines.

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 red Cannonau and white Vermentino grape varieties. Although some physical–chemical characterisation 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 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 yeast 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-amino 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 precursors of BA, basic nitrogenous compounds synthesised in all living organism by metabolic pathways that usually involve AA decarboxylation (Kusano, Berberich, Tateda, & Takahashi, 2008). BA, such as putrescine and cadaverine, have been reported in grapes (Agudelo-Romero, Bortolloti, Pais, 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) and during the fermentation processes from must to wine, microorganisms 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 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

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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) has released a document on risk assessment of BA in food (EFSA, 2011), confirming that histamine and tyramine are considered as most toxic and particularly relevant for food safety.

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 characterise monovarietal wines. However, AA and BA are difficult to analyse 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 when choosing an appropriate detector.

Several methods that allow simultaneous BA and AA analysis have been proposed. All of these methods use HPLC analysis and require derivatizing agents before or after chromatographic separation (Önal, Tekkeli, & Önal, 2013). The most frequently applied derivatization reagents are *o*-phthalaldehyde (OPA) (Kutlán & Molnár-Perl, 2003), 9-fluorenylmethyloxycarbonyl chloride (FMOC-Cl) (Bauza, Kelly, & Blaise, 2007; Molnár-Perl, 2011), dansyl chloride (DCl) (Mazzucco et al., 2010; Pineda, Carrasco, Pena-Farfal, Henríquez-Aedo, & Aranda, 2012), dabsyl chloride (Krause, Bockhardt, Neckermann, Henle, & Klostermeyer, 1995), diethyl ethoxymethylenemalonate (DEEMM) (Cejudo-Bastante et al., 2010; Gómez-Alonso, Hermosín-Gutiérrez, & García-Romero, 2007; Wang, Ye, Zhu, Wu, & Duan, 2014), *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 UV–Vis detection and is very selective. Dansyl chloride is one of the most widely used derivatization 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 derivatization reagents like OPA do not react with proline (Molnár-Perl, 2011), which is the most abundant AA in wine, while DEEMM can be unstable (Rebane et al., 2012).

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

2. Materials 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*), $Na_2B_4O_7{\cdot}10H_2O$, CH_3COONa, and dansyl chloride were purchased from Sigma–Aldrich (Milan, Italy). Primary reference standards of AA and BA (purity >99.9%) were obtained from Sigma–Aldrich, Merck and Carlo Erba (Milan, Italy). AA and BA stock standard solutions (*ca.* 1000 mg/L) were prepared by dilution with 0.1 M HCl/MeOH (1:1, *v*/*v*) and stored at 4 ± 1 °C until use. The derivatization agent solution was prepared dissolving 50 mg of dansyl chloride (DCl) in 10 mL of acetone, and was stored at 4 ± 1 °C until use (solution is stable for up to 3 months). Ultrapure water (18.0 MΩ cm, 25 °C) was obtained with a Milli-Q Advantage A10 System apparatus (Millipore, Milan, Italy).

2.2. Wine samples

Eight wine samples of "Cannonau di Sardegna DOC" and seven wine samples of "Vermentino di Sardegna DOC" produced in Sardinia (Italy) from the 2012 harvest were analysed. Samples were commercially available wines supplied directly by nine different wineries with certified origin and made using standard oenological procedures. Five wineries supplied one sample of both Cannonau and Vermentino wines, one winery supplied two samples of Vermentino wines obtained with grapes harvested in different areas, and the other three wineries gifted one sample of Cannonau wine each. These wineries together account for 90% of the production of Cannonau and Vermentino wines in Sardinia.

2.3. Amino acids and biogenic amines derivatization

Determination of AA and BA was carried out after derivatization 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 (derivatization agent) and 0.2 M $Na_2B_4O_7{\cdot}10H_2O$ (pH 9.3) solution up to a final volume of 1000 μL. The mixture was incubated for 30 min at 40 °C in an ultrasonic bath, and centrifuged at 12,000 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) fitted with a pump module 230, an autosampler module 410, and a Jasco 821-FP fluorimetric detector (Jasco Europe, Cremella, LC, Italy) with wavelengths set at 293 nm (Ex) and 492 nm (Em). Separation was obtained with a Phenomenex Gemini C18 110A column (150 × 4.60 mm, 3 μm; Chemtek Analitica, Anzola Emilia, Bologna, Italy) thermostated at 25 °C, using pH 4.1 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 phases at constant flow rate of 0.8 mL/min. The gradient (*v*/*v*) started with 95% solvent **A**, decreasing to 80% in 18 min, to 50% in 42 min, to 0% in 60 min and left at 0% till 64 min. Before each injection, the system was stabilised 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 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), 2005) by determining linearity, limits of detection (*LOD*), limits of quantification (*LOQ*), precision and accuracy. The linearity was evaluated by preparing standard mixtures (con-