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Melatonin is formed during winemaking at safe levels of biogenic amines

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ABSTRACT

The European Food Safety Authority (EFSA) has accepted health claims for the food constituent melatonin because scientific evidence shows that it is effective at reducing sleep onset latency, and that it alleviates subjective feelings of jet lag. According to risk assessment data published by EFSA in 2011, histamine and tyramine are the most toxic biogenic amines and the ones that most affect food safety. The potential formation of biogenic amines is a concern in fermented foods because of the intense microbial activity. Conversely, *Saccharomyces cerevisiase* produces melatonin during fermentation in the winemaking process. This study aims to evaluate the production of potentially healthy melatonin and toxic biogenic amines during the winemaking process.

To this end, 11 biogenic amines (agmatine, cadaverine, histamine, methylamine, 2-phenylethylamine, putrescine, spermidine, spermine, tyramine, tryptamine and melatonin) have been monitored during the making of 5 monovarietal wines (*Merlot, Palomino Fino, Syrah, Tempranillo* and *Tintilla de Rota*). This paper shows that alcoholic and malolactic fermentation plays a crucial role in the formation of these compounds. Bioactive melatonin is formed at safe levels of the other biogenic amines.

1. Introduction

Biogenic amines (BAs) are low-molecular-weight organic bases with aliphatic, aromatic or heterocyclic structures which can be present in fermented foods (cheeses and sausages) and beverages (beer and wine) (Silla-Santos, 1996). They are formed mainly by the decarboxylation of amino acids or by the amination and transamination of aldehydes and ketones during the microbial, vegetable, and animal metabolism (Askar and Treptow, 1986). BAs are present in grapes and musts (Del Prete et al., 2009). The concentration of BAs in wine depends on such factors as the content of nitrogenous compounds in the grape, the grape's level of maturation and the nitrogenous fertilization of the soil (Ancín-Azpilicueta et al., 2008). They are formed by the action of yeasts during alcoholic fermentation (Goñi and Azpilicueta, 2001), by lactic acid bacteria during malolactic fermentation (Arena and Manca de Narda, 2001; Lonvaud-Funel, 2001), and/or by the presence of other microorganisms responsible for the spoilage of wine (Costantini et al., 2009).

The total level of amines in wines ranges from 0 to 130 mg L^{-1} (Soufleros et al., 1998). Histamine, tyramine, putrescine and cadaverine are the most frequent amines but nearly 25 have been described in wines (Önal, 2007). HPLC coupled with fluorimetric or spectrophotometric detection and mass spectrometry are the methods that have most commonly been used for analysing wines in recent years (Anlı and Bayram, 2008). However, the complexity of the matrix and the low concentrations of BAs are expected to complicate the analysis (Anlı and Bayram, 2008; García-Marino et al., 2010). Therefore, pre- or post-column derivatization methods are used with different reagents (dansyl chloride, *p*-phthalaldehyde, and 6-aminoquinolyl-*N*-hydroxysuccinimidyl carbamate) to increase the selectivity and sensitivity of the detection (Önal, 2007; García-Marino et al., 2010).

The intake of high concentrations of BAs can have such adverse effects as headaches, hypo- or hypertension, nausea, cardiac palpitation, renal intoxication, cerebral hemorrhage or even death depending on individual sensitivity and the capacity of detoxification (Silla-Santos, 1996; Shalaby, 1996). BA content, then, should be controlled for safety purposes. As well as their impact on health, they can have negative effects on aroma and flavour (Ancín-Azpilicueta et al., 2008) so they are a quality marker (Önal, 2007). In 2011, the European Food Safety Authority (EFSA) published a report on risk assessment based on the monitoring of biogenic amine formation in fermented foods (EFSA, 2011a). The report states that histamine and tyramine are the most toxic

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amines in foods. It reveals that further research is required if safe levels of BAs in foods is to be estimated as there is a lack of scientific data for a complete risk assessment.

The amino acid tryptophan is the precursor of all 5-methoxytryptamines such as tryptamine, serotonin and melatonin. Melatonin (*N*-acetyl-5-methoxytryptamine; Mel) is an indoleamine that is structurally related to tryptamine. It is synthesized mainly by the pineal gland and plays an important role in the synchronization of the sleep/wake cycle in humans. In contrast to other BAs, in 2010 EFSA accepted a health claim that it helps to alleviate subjective feelings of jet lag (EFSA, 2010). It has been concluded recently that the melatonin has been sufficiently characterised and the cause-effect relationship between the consumption of melatonin and the reduction in sleep onset latency has been accepted (EFSA, 2011b).

Melatonin has already been described in grapes and wines (Murch et al., 2010; Rodriguez-Naranjo et al., 2011a). It occurs in wines because of the metabolism of *Saccharomyces* during the alcoholic fermentation of the winemaking process (Rodriguez-Naranjo et al., 2011b). Its concentrations range from 0.16 $\mu g\,L^{-1}$ in Chardonnay (Stege et al., 2010) to 130 $\mu g\,L^{-1}$ in Tempranillo (Rodriguez-Naranjo et al., 2011a).

Scientific evidence shows that alcoholic fermentation is crucial for the formation of BAs and melatonin, structurally related substances with deleterious and healthy properties, respectively. Indeed, *Saccharomyces* produces bioactive melatonin but other deleterious BAs can be formed during fermentation. As far as we know, they have not been studied simultaneously during the winemaking process. A complete evaluation should consider the formation of melatonin and other BAs. This paper intends to assess both melatonin and BA formation from must to wine during red and white winemaking steps of five grape varieties to ensure that bioactive melatonin is formed at safe levels of BAs.

2. Materials and methods

2.1. Chemicals and reagents

The biogenic amines agmatine (Ag), cadaverine (Cad), histamine (His), methylamine (Met), 2-phenylethylamine (Phe), putrescine (Put), spermidine (Sne), spermine (Spe), tyramine (Tyr), tryptamine (Tne) and melatonin (Mel), and the internal standard 2-aminoheptanoic acid were purchased from Sigma–Aldrich (Steinheim, Germany). Sodium acetate trihydrate and triethylamine (TEA) were supplied by Fluka (Steinheim, Germany), orthophosphoric acid by BDH Prolabo (Barcelona, Spain), acetonitrile and methanol by Merck (Darmstadt, Germany) and formic acid by Panreac (Barcelona, Spain). All reagents were of analytical grade. A Milli-Q purification system (Millipore, Bedford, MA, USA) was used to obtain ultrapure water.

The AccQ Fluor reagent kit (containing ACQ reagent, acetonitrile and 0.2 mM sodium borate buffer pH 8.8) was supplied by Waters (Milford, MA, USA).

2.2. Samples

The grapes used for this study were grown under strictly controlled conditions in the experimental cultivars (latitude 36:45:29N, longitude 06:00:58W) of Rancho de la Merced Research Centre (Instituto de Investigación y Formación Agraria y Pesquera, IFAPA) in Jerez de la Frontera (Cádiz, Spain) under warm climate. The grapes were from the 2010 vintage and had been harvested at their optimum ripeness. The winemaking process was carried out in an experimental winery at pilot scale. Red wines were produced with the varieties *Merlot*, *Syrah*, *Tempranillo* and *Tintilla de Rota*. Red grape varieties were de-stemmed, crushed and placed in a 100 L steel vessel. Peptolytic enzymes (0.03 $g\,kg^{-1}$, Vinozym® Vintage FCE, Novozymes, Spain) and sulphur dioxide (50 $mg\,kg^{-1}$) were added to maximise polyphenol extraction and to protect the must. One day later, fermentation was started after yeasting (*Saccharomyces cerevisiae* yeast, Actiflore® F5, Laffort, Spain). Temperature was maintained at 27 °C ± 1 during alcoholic fermentation (AF). As soon as tumultuous AF had finished (density 999 $g\,L^{-1}$), the wine was pressed (Willmes, Germany). Lactic acid bacteria *Oenococcus oeni* (1 $g\,HL^{-1}$, Challenge Easy ML, Sepsa-Enartis, Spain) and nutrients (20 $g\,HL^{-1}$, Nutriferm ML, Sepsa-Enartis, Spain) were used for malolactic fermentation (MLF). When this step of the process had finished, the wine was racked and clarification (10 $g\,HL^{-1}$ egg white albumen, Laffort, Spain) was performed. Finally, the wines were bottled.

The white wine was produced with the *Palomino Fino* grape variety. Grapes were destemmed, crushed and pressed, and sulphur was added (80 $mg\,kg^{-1}$). After pressing, the must was dejuiced for 12 h at 4 °C, and AF was started after yeasting (Actiflore PM, Laffort, Spain). AF took place at controlled temperature (18 ± 1 °C). After AF, the wine was maintained under control conditions (T^a = 18 °C) until racked. Then, it was stored in a cold chamber (at 0 °C) until it was clarified with gelatine (15 $mL\,HL^{-1}$, Laffort, Spain) and bentonite (0.6 $g\,L^{-1}$, Laffort, Spain). Finally, the wine was filtered and bottled.

Table 1 shows grape varieties, the dates the grapes were sampled and the winemaking steps. The samples were frozen and stored at −20 °C for further analysis. Table 2 shows the main oenological parameters of the musts and wines obtained (OIV, 1990).

2.3. Standard solutions and sample preparation

A stock solution was prepared by dissolving BAs in methanol up to a concentration of 2 $g\,L^{-1}$. Six standard solutions ranging from 0.01 to 1 $mg\,L^{-1}$ were prepared by diluting the stock solutions in water to generate external calibration curves.

One millilitre of must or wine sample was centrifuged at 14 000 rpm for 3 min and filtered through PTFE membrane filters (0.45 μm). The solid-phase extraction procedure described by Peña-Gallego et al. (2009) was performed using Oasis MCX (30 mg) extraction cartridges from Waters (MA, USA).

Table 1
Varieties, winemaking steps and sampling dates of the grapes used to produce the wines in the study.

Variety	Code	Step number	Name of step	Description	Sampling date
Merlot	M1	Step 1	Harvest	Grape must	02/09/2010
Syrah	S1				02/09/2010
Tempranillo	T1				18/08/2010
Tintilla de Rota	TR1				09/09/2010
Palomino Fino	P1		Press		25/08/2010
Merlot	M2	Step 2	Density 1020	Tumultuous AF	08/09/2010
Syrah	S2				07/09/2010
Tempranillo	T2				23/08/2010
Tintilla de Rota	TR2				20/09/2010
Palomino Fino	P2		Dejuice	Before AF	26/08/2010
Merlot	M3	Step 3	Press	Before MLF	14/09/2010
Syrah	S3				13/09/2010
Tempranillo	T3				27/08/2010
Tintilla de Rota	TR3				27/09/2010
Palomino Fino	P3		Density 1020	Tumultuous AF	30/08/2010
Merlot	M4	Step 4	Racking	After MLF	22/10/2010
Syrah	S4				08/10/2010
Tempranillo	T4				15/09/2010
Tintilla de Rota	TR4				22/10/2010
Palomino Fino	P4			After AF	06/09/2010
Merlot	M5	Step 5	Stabilization	Before bottling	01/12/2010
Syrah	S5				01/12/2010
Tintilla de Rota	TR5				01/12/2010