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"Melatonin isomer" in wine is not an isomer of the melatonin but tryptophan-ethylester

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Abstract

Melatonin is a neurohormone, chronobiotic and antioxidant compound found in wine and deriving directly from grapes and/or synthesized by yeast during alcoholic fermentation. In addition, a melatonin isomer has been detected in different foods, wine among them. The special interest for melatonin isomer related to the fact that it was found in greater quantities than melatonin, and probably shares some of its biological properties. Despite this, its chemical structure has not yet been defined; although some researchers hypothesize it could be melatonin with the ethylacetamide group shifted into position N1. Thus, the aim of our study was to identify the structures of the melatonin isomer. For this purpose, melatonin and melatonin isomer in Syrah wine were separated chromatographically by a sub-2 μm particle column and detected by tandem mass spectrometry. The sample was then purified and concentrated by solid-phase extraction, hydrolyzed with alkali or esterase, and substrates and products quantified by UPLC-MS/MS. Moreover, melatonin, melatonin isomer and their product ions were evaluated by high-resolution mass spectrometry. The amount of melatonin isomer and melatonin in the wine was 84±4 and 3±0 ng/mL, respectively. In the solutions, containing diluted alkali or esterase, melatonin isomer was hydrolyzed in about 8 min. Correspondingly, tryptophan was detected and its amount increased and reached the maximum concentration in about 8 min. Melatonin concentration was not affected by diluted alkali or esterase. The fragmentation pattern of melatonin isomer was different from that of melatonin but comparable to that of tryptophan-ethylester. Finally, the so-called melatonin isomer identity was verified by co-chromatography with authentic standard of tryptophan-ethylester.

Introduction

Melatonin (N-acetyl-5-methoxytryptamine), produced mainly by the pineal gland in vertebrates, is synthesized from tryptophan via serotonin [1]. In animals, melatonin typically appears in the pineal gland but it was found also in other tissues [2], and it seems to be synthesized also in the intestine [3]. In humans, melatonin modulates physiological processes, such as circadian rhythms and reproductive functions, and possesses antioxidant properties as well [4]. The latter seems to be correlated with an increased longevity [5], the development of protective mechanisms against mutations [6], and it affects the immune system [7]. Moreover, the melatonin's protective actions have beneficial effects on memory processes [8] and in the treatment of several diseases such as Alzheimer's [9, 10] and amyotrophic lateral sclerosis [11].

Melatonin is found in a variety of foods [12] including edible plants [13-15], fruits [16-18], and beverages [19]. Melatonin is also detected in wine, and several researchers have related its presence to the activity of the yeast involved in the fermentation process [20-22]. Other authors have recently found melatonin in grapes and other tissues, suggesting that it was already present in the substrate [23, 24]. In both cases, the experimental evidences are still scarce. Thus, the relation among melatonin, its substrate and wine yeast necessitates further study, especially considering that reported studies were focused exclusively on melatonin production by *Saccharomyces* yeasts, ignoring any other non-*Saccharomyces* species.

Analyzing melatonin in wine by liquid chromatography-tandem mass spectrometry (LC-MS/MS) was first reported by Rodriguez-Naranjo et al. [25] the presence of a melatonin-like compound. They also

found it had the same molecular mass and shared several main product ions with melatonin such as the ions with *m/z* 174 and 216. On the other hand, in MS/MS this unknown compound gave also minor product ions not found in the melatonin fragmentation pattern, and they eluted earlier than melatonin. Thus, it was called a melatonin-isomer. Subsequently melatonin and the melatonin isomer were found in different wine varieties [18, 26] and foods such as beer, yogurt and bread [12, 27].

Yilmaz et al. [27] reported that *Saccharomyces cerevisiae* was ineffective in changing the melatonin concentration during bread dough fermentation, while the melatonin isomer amount increased. Gomez et al. [26] found the increase in melatonin isomer during a laboratory-scale winemaking process and in an aqueous medium containing yeast and nutrient, suggesting that the yeasts were responsible for the melatonin isomer formation. Moreover, their data also demonstrated that the amount of the melatonin isomer formed during wine fermentation was not related to the melatonin concentration [28]. This suggested that melatonin isomer probably did not derive from the biosynthetic pathway of melatonin, but rather it was produced by a different pathway.

The reported amount of melatonin in wine ranges from pg to ng/mL, and the quantitative differences could be attributed to different brands of wine, different strains of yeast or the employment of non-appropriate analytical methods. To evaluate the influence of the analytical method, Rodriguez-Naranjo et al. [25] estimated the melatonin levels in the same wine applying two of the most frequently used methods: the ELISA and LC-MS/MS. The reported results showed that melatonin levels measured by LC-MS/MS were higher than those obtained by ELISA up to the three orders of magnitude, and the ELISA method failed to identify melatonin isomer. Thus, the authors concluded that ELISA was not a reliable method for quantifying melatonin in wines. Regarding melatonin isomer, the highest amount detected in red wine was 170 ng/mL [18, 25-26]. Thus, melatonin isomer level in wine is generally higher than that of melatonin, and also for this reason MI has attracted much attention. Unfortunately, its identification is difficult due to different factors such as the lack of reference standard, low concentrations in the food samples that makes difficult its purification for the NMR analysis, and the large number of potential isomers. In this regard, Tan et al. [28] calculated that 42 combinations could be possible considering only the positions of the two side chains linked to the indole ring. The authors have proposed a nomenclature for the MI identification. To date, only N-acetyl-3-(2-aminoethyl)-6-methoxyindole is available as a standard. Rodriguez-Naranjo et al. [25] excluded it due to the different pattern of fragmentation and for its later chromatographic retention in comparison to melatonin isomer. Based on the data of Diamantini et al. [29], several authors [12, 26-28] identified melatonin isomer as 1-(2-alkanamidoethyl)-6-methoxyindole.

The aim of our study was to identify the main isomer of melatonin present in red wine. For this purpose, we applied the ultra-performance liquid chromatography coupled to high-resolution mass spectrometry (UPLC-HR-MS) and performed the fragmentation of analytes by HR-MS/MS. Subsequently, we also purified the wine sample by solid-phase extraction (SPE), hydrolyzed with alkali or esterase, detected and quantified the substrates as well as the products by UPLC-MS/MS. Finally, we verified the MI identity by co-chromatography with a commercially available reference standard.

Materials and methods

Chemicals

Melatonin, tryptophan-ethyl ester (tryptophan-EE), tryptophan and esterase from porcine liver (EC 3.1.1.1) were provided by Sigma-Aldrich (St. Louis, MO, USA). Methanol, boric and formic acids were from Merck (Darmstadt, Germany). Red wine (Syrah, Settesoli DOC 2012) was acquired in a local market, and ultrapure water was obtained from a MilliQ apparatus (Millipore, Milford, MA, USA).

Sample preparation for hydrolysis

Syrah wine aliquot (10 mL) was loaded on a 500 mg StrataX SPE column (Phenomenex, Torrence, CA, USA) pre-activated by 4 mL methanol followed by 8 mL of 0.1% formic acid in water. The column was washed with 5 mL of 0.1% formic acid and 5 mL of 70% methanol, dried under vacuum and eluted with 3 mL of methanol. The eluate was then dried under vacuum, the residue suspended in 1 mL of MilliQ-treated water, centrifuged at 1000 x g for 2 min, and the clear solution used for the hydrolysis tests.

Hydrolysis by alkali

Purified wine solution (0.2 mL) was incubated with 0.02 mL of 0.1 N NaOH and 0.1 mL of melatonin (1.0 µg/mL in water) at 30°C. Twenty µL of reaction mixture were collected at 0, 5, 10, 15, 20, 25, 30 min time points and diluted up to 40 µL with 0.1 N HCl. The solutions were subsequently diluted with methanol, centrifuged at 1000 x g for 1 min and the resulting supernatant analyzed by UPLC-MS/MS. The purified wine solution and melatonin were used as the controls.

Hydrolysis by esterase

One mL of the wine solution was incubated with 1 mL of esterase ($5x10^{-4}$, $1x10^{-3}$, $5x10^{-3}$ U) in 10 mM borate buffer (pH 7.5) and 100 µL of melatonin (5 µg/mL in water) at 25°C. At time zero, every 1 min for 10 min, and after 15, 20, 25, 30 min, 50 µL of reaction mixture were collected and diluted up to 0.1 mL with methanol. The resulting solutions were subsequently diluted with methanol and centrifuged at 1000 x g for 1 min. The resulting supernatants were analyzed by UPLC-MS/MS. Wine, melatonin and esterase borate solutions were used as the controls.

UPLC-MS/MS analysis

The UPLC-MS/MS analysis was carried out on an Acquity UPLC separation module (Waters, Milford, MA, USA) coupled with a triple quadrupole mass spectrometer Quattromicro (Waters). A 1.7 µm BEH C_{18} column (100x2.1 mm, Waters) was used for separation. The eluents were 0.1% formic acid in MilliQ-treated water (solvent A) and methanol (solvent B). Ten µL of the sample were separated by

the UPLC using the following elution gradient: 5–60% B in 6 min and then 60–100% B in 3 min at a flow-rate of 0.55 mL/min. The column and samples were kept at 40 and 20°C, respectively. The capillary voltage was set to 3.0 kV, the cone voltage was 14 V, the source temperature was 130°C, the desolvating temperature was 350°C, the collision energy was 16 V, and argon was used at $2.1x10^{-3}$ mbar to improve fragmentation in the collision cell. Melatonin, melatonin isomer, tryptophan, and tryptophan-EE were determined in multiple reaction monitoring (MRM) mode, and the data were acquired by MassLynx 4.0 software (Waters). The mass spectrometer was operated in the electrospray ionization (ESI) positive mode and the fragmentation transitions were $(m/z)^+$ 233→174 for melatonin, tryptophan-EE and melatonin isomer, and $(m/z)^+$ 205→188, 146, 118 for tryptophan, with a dwell time of 0.15 sec. To keep the cone clear, the eluate from the first 1.1 min analysis was discarded by the divert valve. Calibration curves were obtained from melatonin, tryptophan, and tryptophan-EE stock solutions prepared by dissolving 10 mg of standard powder in 10 mL methanol, and their quantitative determination performed in the range 0.2–20 ng/mL.

UPLC-HR-MS analysis

The UPLC-HR-MS analysis was carried out on an Acquity UPLC separation module (Waters) coupled with a Q-Exactive hybrid quadrupole-Orbitrap MS through an HESI-II probe for electrospray ionization (Thermo Scientific, San Jose, CA, USA). The ion source and interface conditions were: spray voltage 3.5 kV, sheath gas flow rate 35, auxiliary gas flow rate 15 and temperature 250 °C, capillary temperature 350 °C. Two µL of each sample were separated on an ACQUITY UPLC HSS C_{18} column (100×2.1 mm, 1.8 µm) (Waters) kept at 50 °C. The eluents were 0.1% formic acid in MilliQ-treated water (solvent A) and methanol (solvent B). The UPLC separation was performed by using a linear elution gradient (5% to 100% of solvent B in 6.9 min) at a flow rate of 0.55 mL/min. The UPLC eluate was analyzed by MS using Full MS (<5 ppm mass tolerance) and targeted MS^2 analysis (t-MS^2). The resolution was set at 70K and 17.5K for Full MS and t-MS^2 scan types, respectively. The AGC target was 5E5 for Full MS and 2E5 for t-MS^2 scans. The maximum ion injection time was 200 and 100 ms for Full MS and t-MS^2 scans, respectively. The collision energy was 10, 20, 30, 40, 50 or 60 V for t-MS^2. The MS data were processed using Xcalibur software (Thermo Scientific).

Results and Discussion

To the best of our knowledge, this is the first paper reporting the characterization of the unknown compound found in wine and different foods, called melatonin isomer.

In a previous work [18] in different wines we found unknown compounds tentatively identified as melatonin isomers according to the tandem mass spectrometry data. Among these, the one characterized by the earliest retention (melatonin isomer) was the most abundant, and its quantity was up to 72 ng/mL in a sample of Syrah wine. For this reason, Syrah wine was used in this study.

A good separation of the tryptophan (RT 1.8 min), “melatonin isomer” (RT 2.8 min), tryptophan-EE (RT 2.8 min) and melatonin (RT 3.4 min) was achieved with a BEH C_{18} sub-2 µm particle column and under gradient mode elution. The amount of tryptophan, melatonin isomer and melatonin in the red

wine was 152±9, 84±4 and 3±0 ng/mL, respectively. The percentage recovery values of the SPE extraction for the melatonin isomer and melatonin from red wine samples were 70±3 and 90±4%, respectively. We used the lower melatonin isomer recovery elution conditions with the aim to reduce the amount of tryptophan present in the red wine. Thus, the amount of tryptophan, melatonin and melatonin isomer in the purified wine sample was 12±2, 27±2 and 588±25 ng/ml, respectively.

As expected, hydrolysis reaction of the blank solutions demonstrated that melatonin and melatonin isomer amount did not change. Sodium hydroxide rapidly degraded melatonin isomer within the first 5 min of incubation, and melatonin isomer was no more detectable in the subsequent 5 min (data not shown). On the contrary, the concentration of melatonin was not affected by the action of the sodium hydroxide. The results obtained by nonspecific hydrolytic conditions, such as that with alkali, suggested that melatonin isomer, in contrary to melatonin, contained an easily hydrolysable bond, which could be the ester. To confirm/reject this hypothesis melatonin isomer was subsequently hydrolyzed using a specific enzyme such as esterase. We tested three different esterase concentrations (as indicated in Materials and Methods) and selected the optimal one to obtain a degradation kinetic (data not shown). Figure 1 shows the time-course of melatonin, melatonin isomer and tryptophan in a batch containing $5x10^{-4}$ U of esterase. As it was expected, melatonin and melatonin isomer amount did not change in the blank solutions in 30 min (data not shown). On the contrary, in the solutions containing esterase about 50% of melatonin isomer disappeared in 4 min, and after 8 min it was no longer detectable. Correspondingly, tryptophan increased slowly in the first two min and then reached the maximum concentration in about 8 min. Once formed, it was stable up to 30 min incubation with esterase (data not shown). Moreover, the tryptophan amounts detected in the hydrolysis batches were about 10% higher than those stoichiometrically produced by melatonin isomer, suggesting that melatonin isomer could be one of the main sources of tryptophan, but not the single one. Regarding melatonin, its concentration was not affected by the action of the esterase.

Melatonin, melatonin isomer and tryptophan-EE were analyzed by ESI-HR-MS in the positive ion mode as these conditions were more sensitive, and analytes gave a better fragmentation pattern during collision induced-dissociation (CID). High-resolution MS analysis showed that the most abundant ion of melatonin, melatonin isomer and tryptophan-EE had a *m/z* of 233.1285, corresponding to monoprotonated molecule ($[M+H]^+$). In the fragmentation spectra of melatonin, obtained in the collision energy range 10–60 V, the main product ion had *m/z* 174.0913 (**fa**), which could be justified by the cleavage of the CH_2-N bond with H rearrangement. As shown in figure 2, the ion **fa** could be generated by fragmentation of the ion **d** and **e** or directly by the protonated melatonin though the loss of CH_3CONH_2 moiety [29, 30]. The ions **d** and **e** were found only at lower collision energies and in a much smaller amount compared to the ion **fa**. At higher collision energies the ions **g**, **h** and **i** were also found (Fig. 2). The formation of these ions was probably due to an initial rearrangement of the ion **fa**, favored by the acquisition of aromaticity, giving the ion A, which easily lost a methyl group to give the ions **g**. The ion **ia**, a radical product, could also result from the homolytic cleavage of the ion **fa**. This behavior was probably dependent on the presence of the double bond conjugated to the indole ring system and the subsequent production of a stable indolic radical. Even in the fragmentation pattern of the tryptophan-EE and melatonin isomer homolytic

cleavages were present (Fig. 3), as in production of the ions **q** and **h**. It is important to underline that also in this case the C-C double bound conjugated to the indole system was broken. In contrary, the fragmentation of tryptophan-EE (Fig. 3) as the main product gave the ion **d**, which was generated by the cleavage of the NH_2 group and formation of a C-C double bound in the alkyl chain. At higher collision energies product ions different from those produced by melatonin appeared: the ion **l**, derived from the loss of the ethyl group by **d**, the ions **n**, **o**, **p**, **q**, **r**, **s** and **t**. It should be emphasized that both melatonin and tryptophan-EE during CID produced ions with *m/z* 174.0919 (**fa** and **fb**), which presumably corresponded to different structures depending on the parent ion (Fig. 2 and Fig. 3). The ions **fb**, produced by tryptophan-EE, underwent heterolytic rupture, unlike that produced by melatonin, and during CID the ions **p** were formed. The same product ions with the same relative intensities were found in the fragmentation pattern of the unknown compound found in the red wine and called "MI". As shown in table 1, melatonin and tryptophan-EE gave product ions with *m/z* 159.0684 (**g**) and 159.0922 (**n**), respectively. The difference of 23 mDa suggested that the two product ions were structurally different, and therefore tryptophan-EE and melatonin isomer had a fragmentation pattern partially different from that of melatonin. This difference was not detected using low-resolution MS, such as the triple quadrupole. The result highlights that HR-MS/MS should be the preferred technique in fragmentation studies.

Considering the data obtained by HR-MS, HR-MS/MS, co-chromatography and enzymatic hydrolysis, it could be presumed that the unknown compound, called melatonin isomer, is likely the tryptophan-EE. The presence of tryptophan-EE in wine is not surprising. In fact, some authors reported the presence of several ethyl esters of amino acids (AA-EEs) in sherries and wines, and in latters their concentrations were up to 58 mg/L [26, 27]. Thus, AA-EEs are an important group of compounds present in wines. AA-EEs are formed during the alcoholic fermentation by yeasts [32]. Their formation mainly occurs in the second half of the fermentation, when the ethanol content in the medium is higher. When the yeast remains in contact with the wine, as it happens at the end of the fermentation, the amount of AA-EEs increases. Some differences were found between the yeasts used for the wine production. Thus, in contrary to melatonin, AA-EEs such as tryptophan-EE derive solely from the yeast activity.

Gomez et al. [26] reported that during a laboratory-scale winemaking process the yeast *Saccharomyces* played a decisive role in the production of melatonin isomer but not in that of melatonin, and the amount of melatonin isomer was higher at the end of fermentation period. The authors also reported that, in an aqueous media containing yeasts and tryptophan, only the concentration of melatonin isomer increased, and it was time-dependent. Moreover, the presence of tryptophan in must stimulates the occurrence of the melatonin isomer. Yilmaz et al. [27] investigated the formation of melatonin and melatonin isomer as well as the changes in free AAs, especially tryptophan, during bread fermentation and baking. They found that melatonin amount did not change significantly during dough fermentation, whereas melatonin isomer content increased up to 17 ng/g. Moreover, at the end of the baking process tryptophan decreased by 58%. Overall, these data support our hypothesis that the main "melatonin isomer" described in literature is the tryptophan-EE.

Tryptophan ethyl ester dilates *in vitro* small mesenteric arteries [33] and was used by Jonas and Butler [34] to bypass defective gastrointestinal AA transport in a child with Hartnup disease. The authors reported that the normalization of the serum tryptophan concentration was reached by a sustained treatment with 20 mg/kg of TRP each 6 h, the amount clearly not achievable with the regular diet. Moreover, the authors stated that tryptophan-EE hydrolysis during absorption was apparently complete, since only tryptophan was detected in serum. In this regard, the authors reported that the lower limit of detection of the tryptophan-EE in serum was approximately 90 ng/mL. The latter is an higher value considering that melatonin levels are in the order of pg/mL. Thus, the presence of tryptophan-EE in plasma at concentrations lower than nM cannot be excluded. Finally, tryptophan-EE ingested with the diet provides a poor amount of tryptophan, which could be transformed into melatoninin the gastrointestinal tract, as reported by Konturek et al. [35]. Summing up, the identity of this molecule makes it less interesting than the supposed melatonin isomer from the nutritional point of view, mainly considering the small amounts present in food.

Author Contributions

Dr. Claudio Gardana - Designed the experimental plan, prepared the samples, performed hydrolysis tests and UPLC-MS/MS analyses, evaluated the obtained results and prepared the manuscript.

Prof. Paolo Simonetti - Provided funding for the study, designed the experimental plan and evaluated the data, critically revised the manuscript and approved its final version.

Dr. Milda Stuknytė - Set-up the HR-MS instrument, designed and performed the HR-MS/MS analysis, evaluated MS/MS data and commented on the manuscript.

Prof. Ivano De Noni - Designed the HR-MS/MS analysis, evaluated MS/MS data and commented on the manuscript.

References

1. REITER RJ. Pineal melatonin - Cell biology of its synthesis and of its physiological interactions. Endocrine Rev 1991; 12:151-180.

2. ACUNA-CASTROVIEJO D, ESCAMES G, VENEGAS C et al. Extrapineal melatonin; sources, regulation and potential functions. Cell Mol Life Sci 2014; 71:2997-3925.

3. BUBENIK GA. Gastrointestinal melatonin: localization, function, and clinical relevance. Dig Dis Sci 2002; 47:2336-2348.

4. REITER TJ, TAN DX, GALANO A. Melatonin: exceeding expectations. Physiology 2014; 29:325-333.

5. HARDELAND R. Melatonin and the theories of aging: a critical appraisal of melatonin's role in antiaging mechanisms. J Pineal Res 2013; 55:325-356.

6. KARBOWNIK M, REITER RJ, BURKHARDT S et al. Melatonin attenuates estradiol-induced oxidative damage to DNA: relevance for cancer prevention. Exp Biol Med 2001; 226:707-712.

7. MAURIZ JL, COLLADO PS, VENEROSO C et al. A review of the molecular aspects of melatonin's anti-inflammatory actions: recent insights and perspectives. J Pineal Res 2013; 54:1-14.

8. LARSON J, JESSEN RE, UZ T et al. "Impaired hippocampal long term potentiation in melatonin MT2 receptor-deficient mice. Neurosci Letters 2006; 393:23-26.

9. POHANKA M. Alzheimer's disease and related neurodegenerative disorders: implication and counteracting of melatonin. J App Biomed 2011; 9: 185-196.

10. ROSALES-CORRAL SA, ACUNA-CASTROVIEJO D, COTO-MONTES A et al. Alzheimer's disease: pathological mechanisms and the beneficial role of melatonin. J Pineal Res 2012; 52:167-202.

11. JACOB S, POEGGELER B, WEISHAUPT JH. Melatonin as a candidate compound for neuroprotection in amyotrophic lateral sclerosis (ALS): high tolerability of daily oral melatonin administration in ALS patients. J Pineal Res 2002; 33:186-187.

12. KOCADAGLI T, YILMAZ C, GOKMEN V. Determination of melatonin and its isomer in foods by liquid chromatography tandem mass spectrometry. Food Chem 2014; 15:151-156.

13. MANCHESTER LC, TAN DX, REITER RJ et al. High levels of melatonin in the seeds of edible plants - Possible function in germ tissue protection. Life Sci 2000; 77:3023-3029.

14. SAE-TEAW M, JOHNS J, JOHNS NP et al. Serum melatonin levels and antioxidant capacities after consumption of pineapple, orange, or banana by healthy male volunteers. J Pineal Res 2013; 55:58-64.

15. PARK S, BYEON Y, KIM YS et al. Kinetic analysis of purified recombinant rice N-acetylserotonin methyltransferase and peak melatonin production in etiolated rice shoots. J Pineal Res 2013; 54:139-144.

16. STURTZ M, CEREZO AB, CANTOS-VILLAR E, GARCIA-PARILLA MC. Determination of the melatonin content of different varieties of tomatoes (*Lycopersicon esculentum*) and strawberries (*Fragaria ananassa*). Food Chem 2011; 127:1329-1334.

17. BURKHARDT S, TAN DX, MANCHESTER LC et al. Detection and quantitation of the antioxidant melatonin in Montmorency and Balaton tart cherries. J Agric Food Chem 2001; 49: 4898-4902.

18. VITALINI S, GARDANA C, SIMONETTI P et al. Melatonin, melatonin isomers and stilbenes in Italian traditional grape products and their antiradical capacity. J Pineal Res 2013; 54:322-333.

19. GARCIA-MORENO H, CALVO JR, MALDONADO MD. High levels of melatonin generated during the brewing process. J Pineal Res 2013; 55:26-30.

20. SPRENGER J, HARDELAND R, FUHRBERG B, HAN SZ. Melatonin and other 5-methoxylated indoles in yeast: presence in high concentrations and dependence on tryptophan availability. Cytologia 1999; 64:209-213.

21. RODRIGUEZ-NARANJO MI, TORIJA MJ, MAS A et al. Production of melatonin by *Saccharomyces* strains under growth and fermentation conditions. J Pineal Res 2012; 53:219-224.

22. AREVALO-VILLENA M, BARTOWSKY EJ, CAPONE D, SEFTON MA. Production of indole by wine-associated microorganisms under oenological conditions. Food Microbiol 2010; 27:685–690.

23. VITALINI S, GARDANA C, ZANZOTTO A et al. From vineyard to glass: agrochemicals enhance the melatonin and total polyphenol contents and antiradical activity of red wines. J Pineal Res 2011; 51:278-285.

24. MURCH SJ, HALL BA, LE CH, SAXENA PK. Changes in the levels of indoleamine phytochemicals during veraison and ripening of wine grapes. J Pineal Res 2010; 49:95-100.

25. RODRIGUEZ-NARANJO MI, GIL-IZQUIERDO A, TRONCOSO AM et al. Melatonin: a new bioactive compound in wine. J Food Compos Anal 2011; 24:603-608.

26. GOMEZ FJV, RABA J, CERUTTI S, SILVA MF. Monitoring melatonin and its isomer in Vitis vinifera cv. Malbec by UHPLC-MS/MS from grape to bottle. J Pineal Res 2012; 52:349-355.

27. YILMAZ C, KOCADAGLI T, GOKMEN V. Formation of melatonin and its isomer during bread dough fermentation and effect of baking. J Agric Food Chem 2014; 62:2900-2905.

28. TAN DX, HARDELAND R, MANCHESTER LC et al. Emergence of naturally occurring melatonin isomers and their proposed nomenclature. J Pineal Res 2012; 53:113-21.

29. DIAMANTINI G, TARZIA G, SPADONI G et al. Metastable ion studies in the characterization of melatonin isomers. Rapid Commun Mass Spectrom 1998; 12:1538-1542.

30. COLLIN F, BONNEFONT-ROUSSELOT D, YOUS S et al. Online H/D exchange liquid chromatography as a support for the mass spectrometric identification of the oxidation products of melatonin. J Mass Spectrom 2009; 44:318-329.

31. HERRAIZ T, OUGH CG. Formation of ethyl esters of amino acids by yeasts during the alcoholic fermentation of grape juice. Am J Enol Vitic 1993;44: 41-48.

32. HERESZTYN T. Methyl and ethyl amino acid esters in wine. J Agric Food Chem 1984; 32:916-18.

33. JADHAV A, LIANG W, BALSEVICH J et al. L-Tryptophan ethyl ester dilates small mesenteric arteries by inhibition of voltage-operated calcium channels in smooth muscle. Br J Pharmacol 2012; 166: 232-242.

34. JONAS AJ, BUTLER IJ. Circumvention of defective neutral amino acid transport in hartnup disease using tryptophan ethyl ester. J Clin Invest 1989; 84:200-204.

35. KONTUREK SJ, KONTUREK PC, I. BRZOZOWSKA I et al. Localization and biological activities of melatonin in intact and diseased gastrointestinal tract (GIT). J Phys Pharm 2007; 58:381-405.

Figure Legends

Figure 1. Kinetics of Melatonin (□), Melatonin isomer (○) and Tryptophan (Δ) during hydrolysis by esterase of purified and concentrated red wine sample. Data are reported as means. The average initial concentration of Tryptophan was 0.03 nmol/mL.

Figure 2. Main fragmentation patterns of the protonated Melatonin. The protonated product ions were obtained by High Resolution CID in the range 10-60 V. See Table 1 for product ion identification.

Figure 3. Formation of protonated product ions after Tryptophan-EE or Melatonin isomer fragmentation. The fragmentations were obtained in HR-MS/MS with collision energy in the range 10-60 V. See Table 1 for product ion identification.

Table 1. Parent ion and fragments obtained by UPLC-HR-MS/MS of standard of melatonin, tryptophan-ethyl ester and melatonin isomer in purified red wine.

Compound	Calculated $[M+H]^+$	Found $[M+H]^+$	Error (PPM)	Sign	Fragments	Calculated	Error (PPM)	MF
Melatonin	233,1290	233,1285	2,1	d	216,1019	216,1024	2,3	$C_{13}H_{14}NO_2$
				e	191,1181	191,1184	1,6	$C_{11}H_{15}N_2O$
				f$_a$	174,0913	174,0919	3,4	$C_{11}H_{12}NO$
				g$_a$,g$_b$	159,0678	159,0684	3,8	$C_{10}H_8NO$
				h	143,0729	143,0735	4,2	$C_{10}H_9N$
				i$_a$, i$_b$	131,0730	131,0735	3,8	C_9H_9N
Melatonin isomer in wine	233,1290	233,1296	2,6	d	216,1020	216,1024	1,9	$C_{13}H_{14}NO_2$
				l	188,0707	188,0711	2,1	$C_{11}H_{10}NO_2$
				f$_b$	174,0914	174,0919	2,9	$C_{11}H_{12}NO$
				m	170,0600	170,0606	3,5	$C_{11}H_{12}NO$
				n	159,0918	159,0922	2,5	$C_{10}H_{11}N_2$
				o	144,0808	144,0813	3,5	$C_{10}H_9N$
				h	143,0730	143,0735	3,5	$C_{10}H_9N$
				p	132,0809	132,0813	3,0	$C_9H_{10}N$
				q	130,0652	130,0657	3,8	C_9H_8N
				r	118,0653	118,0657	3,4	C_8H_8N
				s	102,0554	102,0555	1,0	$C_4H_8NO_2$
				t	74,0244	74,0242	2,7	$C_2H_4NO_2$
Tryptophan-ethyl ester	233,1290	233,1285	2,1	d	216,1019	216,1024	2,3	$C_{13}H_{14}NO_2$
				l	188,0707	188,0711	2,1	$C_{11}H_{10}NO_2$
				f$_b$	174,0914	174,0919	2,9	$C_{11}H_{12}NO$
				m	170,0600	170,0606	3,5	$C_{11}H_8NO$
				n	159,0917	159,0922	3,1	$C_{10}H_{11}N_2$
				o	144,0808	144,0813	3,5	$C_{10}H_{10}N$
				h	143,0730	143,0735	3,5	$C_{10}H_9N$
				p	132,0809	132,0813	3,0	$C_9H_{10}N$
				q	130,0652	130,0657	3,8	C_9H_8N
				r	118,0653	118,0657	3,4	C_8H_8N
				s	102,0554	102,0555	1,0	$C_4H_8NO_2$
				t	74,0244	74,0242	2,7	$C_2H_4NO_2$

MF: protonated molecular formula.

Figure 1

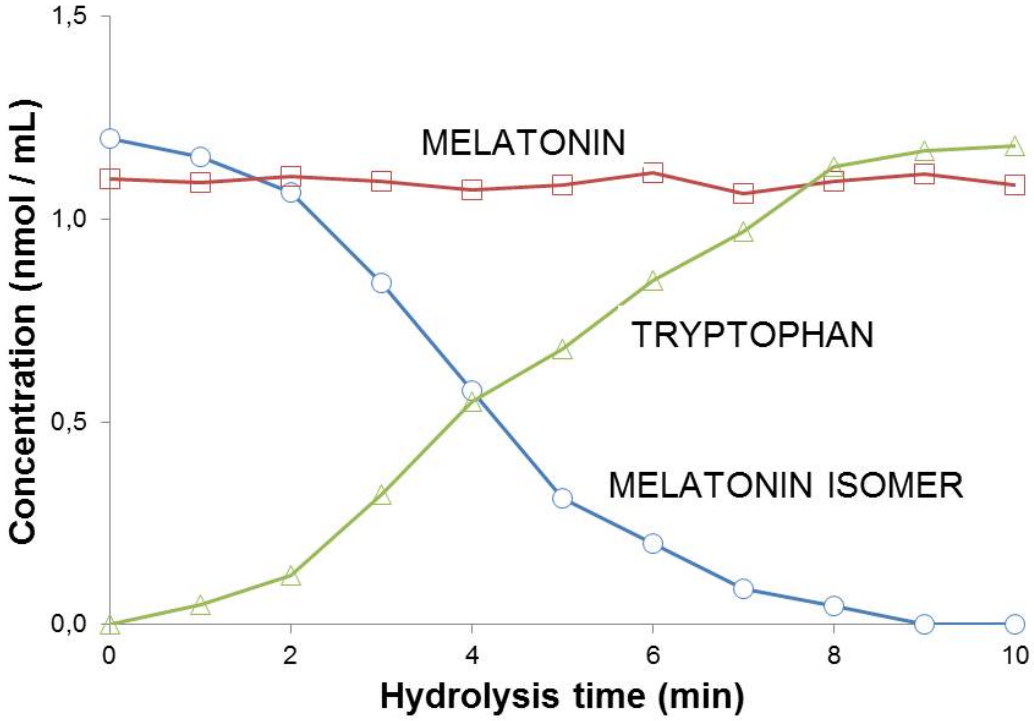

Figure 2

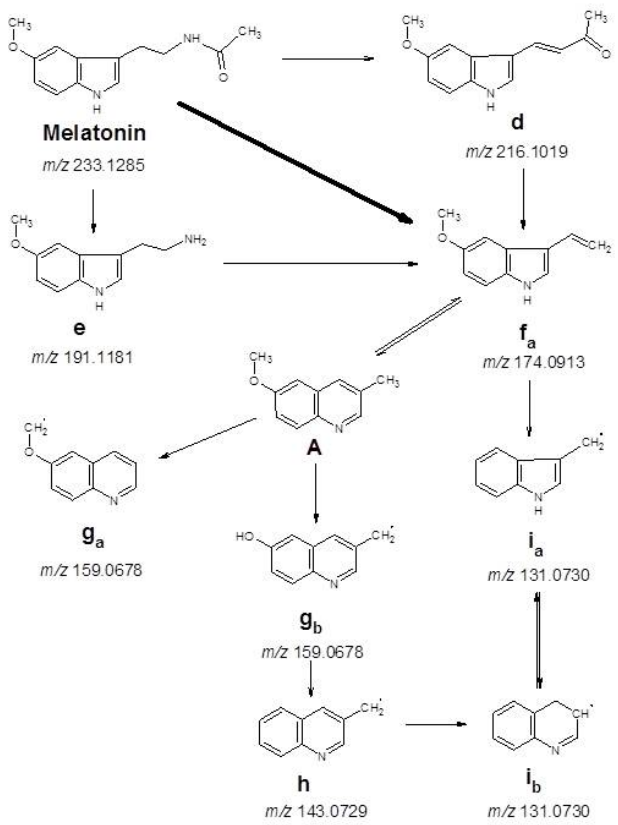

Figure 3