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Monitoring melatonin and its isomer in *Vitis vinifera* cv. Malbec by UHPLC-MS/MS from grape to bottle

Abstract: Several studies have shown the presence of melatonin and related compounds in grapes and wines. The latter provides evidence of the possibility to enhance the nutraceutical properties of premium wines. However, there are many external factors that can influence the levels of this indolamine in grape and wines. In this study, the monitoring of melatonin and its tentatively identified isomer was carried out during the entire winemaking process in *Vitis vinifera* cv. Malbec by ultra high-performance liquid chromatography-tandem mass spectrometry. Laboratory and pilot studies were carried out to elucidate the role of grape, yeasts, and tryptophan in the evolution of the indolamines during the fermentation process. Melatonin was detected in grape extract within the range 120–160 ng/g while its isomer was found in musts and finished wines. Our results demonstrate that *Saccaromyces cervisiae* plays a decisive role in contributing to the content of melatonin and its isomer in wine.

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Introduction

Melatonin (*N*-acetyl-5-methoxitryptamine) is a neurohormone discovered more than 50 yr ago in the mammalian pineal gland. This molecule is a biogenic indolamine, which performs an important role in the regulation of circadian and seasonal rhythms. It plays a significant role in regulation of physiological cycles, as its synthesis is highly dependent on the light/dark cycle in plants and animals [1, 2]. Furthermore, melatonin is a proven free radical scavenger and broad-spectrum antioxidant [3, 4]. This antioxidant mechanism seems to be different from that of 'classical' antioxidants such as vitamin E, vitamin C, or glutathione, which are electron donors. These compounds are regenerated by redox reactions, which can in turn promote the formation of other oxidized species. On the contrary, melatonin seems to interact with free radicals by addition reactions, whose products are stable and are themselves antioxidants [5, 6]. Thus, melatonin is a 'suicide' antioxidant, which is not regenerated and does not promote further oxidation reactions [7, 8]. Also, owing to its amphipathic nature, melatonin is able to permeate all tissues and subcellular compartments. It has been identified in membranes, mitochondria, nucleoli, and cytosol [9–12]. Melatonin is an effective protector for lipid membrane structure, proteins, and DNA against free radical oxidation [8, 13, 14].

In recent studies, melatonin has been found in many organs of higher plants [15–17], including leaves, fruits, and seeds, at concentrations from picograms to micrograms per gram of tissue. It has been speculated that melatonin protects plants from intrinsic and environmental oxidative stress and that could play a role controlling flowering, as well as their processes [18–20].

In 2006, melatonin was first detected in berry skin of Italian and French grape varieties [21]. Recently, Murch et al. [22] determined melatonin and serotonin levels in grapes at different stages of maturation while Boccalandro et al. [1] showed that melatonin levels fluctuate with the schedule of harvest. Vitalini et al. [23] measured this indolamine in all berry tissues. Several studies have confirmed the presence of melatonin in wines [7, 18, 24–

26]. At least one group has reported that its concentration in red wine is higher than in white wines [27]. Lamont et al. [28] demonstrated that melatonin is a superior antioxidant present in red wine and contributes to validate the hypothesis that red wine has health benefits. However, there are many external factors that can influence melatonin levels in grapes and wines, such as genetic variation, the organ of the grape under study, the growth stage, infection with pathogens and pesticide treatments, agro-meteorological conditions and environmental stresses, heavy metal stress, vintage, and winemaking procedures [22, 24, 29, 30].

In a previous work, we demonstrated that melatonin levels fluctuate during the day/night cycle in plants of *Vitis vinifera* grown under field conditions [1]. We proved melatonin's antioxidant role in grapes and determined that the diurnal decay of melatonin in berry skins is induced by sunlight. The aim of this study is to monitor melatonin during the whole winemaking process with the aim of providing evidence of the possibility to enhance the nutraceutical properties of premium wines and contribute to the knowledge of the biological function of melatonin in plants.

Materials and methods

Reagents and solutions

Melatonin and L-tryptophan were purchased from Sigma Chemical (St. Louis, MO, USA). Acetonitrile, methanol, and water Optima® LC-MS grade were purchased from Fisher Scientific (Fair Lawn, NJ, USA). Formic acid, 98%, was obtained from Fisher Scientific (Loughborough, UK). Hakaphos R™ was obtained from Compo (Barcelona, Spain). D-(+)-Sucrose was purchased from Biopack (Buenos Aires, Argentina). Ultrapure water (18 MΩ cm) was obtained from EASY pure (RF Barnstead, IA, USA).

Plant material

The samples were obtained during 2010, in a commercial vineyard of *V. vinifera* cv. Malbec of selected clones planted without rootstock, in sandy soil and drip irrigated plots located in Gualtallary (1.500 m a.s.l.; 69°77′W and 33°22′S), Tupungato, province of Mendoza, Argentina. The grapevine (11-yr-old plants) were trained on a vertical trellis and pruned as Guyot, arranged in north-south-oriented rows spaced 2 m apart, with a distance of 1.20 m between two consecutive plants of each row. The vineyards were protected by antihail nets (black polyethylene) that produced 17% of shade. Sampling was carried out at a commercial harvest date during the morning when sugar concentration reached 24 °Brix. Clusters were collected in black nylon bags and berries were processed immediately.

Winemaking process

Pilot scale winemaking was carried out according to the following procedure: 150 kg of Malbec grapes of three rows were randomized, sampled, destemmed, and crushed, and introduced into the fermentation stainless steel tanks. The must was sulfited (50 mg/L $K_2S_2O_5$) and after 24 hr, it was inoculated with 20 g hr/L EC1118 (Lallemand, Montreal, Canada). The fermentation temperature was maintained at 25°C ± 1 until the end of the fermentation process. Pumping over and basic controls were carried out daily. When the alcoholic fermentation was completed (10 days), wines were sulfited (50 mg/L) and filtered. Temperature was maintained at 7°C and bottled 3 months after its preparation. Table 1 shows the enological parameters of wine measured according to Official Methods (OIV, 2009) [31]. The whole process was carried out in the dark to avoid melatonin degradation.

Laboratory assays

A laboratory scale study was carried out from 10 kg of grapes following the same vinification procedure. Three replicates were performed and samples were taken at 16:00 hr every day to monitor the melatonin content during vinification.

On the other hand, another laboratory assay was carried out to elucidate the role of yeasts and tryptophan (a melatonin precursor) in melatonin evolution during the fermentation process. Thus, the above-mentioned process was performed but in the absence of must. Instead, EC1118 (20 g hr/L), sucrose (250 g/L), and nutrients (Hakaphos R™, 1 g/L) were added to 200 mL of distilled water. Three different treatments were carried out in triplicate: (i) assay C: sucrose and nutrients, (ii) assay T1: EC1118, sucrose, nutrients plus melatonin (100 ng/L), and (iii) assay T2: EC1118, sucrose, nutrients plus tryptophan (500 ng/L). Samples were taken every day at 16:00 hr to monitor the melatonin levels during fermentation. All processes were carried out in the dark to avoid the melatonin degradation.

Grape sample pretreatment

Extraction from grape skin was carried out under dim green light (2 μmol/[m^2 s]) to prevent analyte degradation. Frozen grapes were peeled using a scalpel, dried under nitrogen gas, grounded, accurately weighed about 0.1 g, and transferred to a 15-mL glass tubes. An aliquot (2.5 mL) of methanol was added to each sample, and then tubes were shaken for 30 s in a vortex. Ultrasonication was employed to assist and accelerate the extraction of melatonin from vegetal tissues in an ultrasonic bath (200 W, 15°C; Cleanson 1106, Buenos Aires, Argentina) filled with cold water for 14 min. The supernatant was decanted and centrifuged for 10 min at 3500 rpm (1852.2 g). The resulting extract

Table 1. Enological parameters of finished wines

Parameter	
Ethanol (%vol.)	14.21
pH	3.68
TA	5.65
TPI	42.7
CI	1.85

TA, Total Acidity expressed as g/L tartaric acid; TPI, Total Phenol Index; CI, Color Intensity.

was filtered through a 0.45-mm syringe filters (Waters™) and stored in amber vials suitable for UHPLC-MS analysis.

Chromatographic conditions

An Acquity™ Ultra High Performance LC system (Waters, Milford, MA, USA) equipped with autosampler injection and pump systems (Waters) was used. The autosampler vial tray was maintained at 15°C. The needle was washed with proper mixtures of acetonitrile and water. The separation was performed by injecting 10 μL of sample onto an ACQUITY UHPLC® BEH C_{18} analytical column (Waters) with 2.1 mm internal diameter × 50 mm length, and 1.7 μm particle size. The binary mobile phases consisted of water with 0.1% (v/v) of formic acid (A) and acetonitrile with 0.1% (v/v) of formic acid (B) delivered at 0.45 mL/min. The C_{18} gradient was started at an initial composition of 85% A and 15% B, then 3.5 min linear gradient to 70% A, held for 0.5 min. A return to the initial conditions was accomplished by a 0.2 min gradient to 85% A, where it was held for 1.8 min. Thus, the total chromatographic run time was 5.0 min. The column was held at a temperature of 30°C. Samples were filtered just before injecting into the LC system.

Mass spectrometry instrumentation and MS/MS conditions

Mass spectrometry analyses were performed using a Quattro Premier™ XE Micromass MS Technologies triple quadrupole mass spectrometer with a ZSpray™ electrospray ionization source (Waters). The source was operated in a positive (ES+) mode at 350°C with N_2 as the nebulizer gas and the source temperature was kept at 150°C. The capillary voltage was maintained at 3.0 kV and the extractor voltage was set at 5.0 kV. Ultrapure nitrogen was used as desolvation gas with a flow of 800 L/hr. Argon was used as collision gas at a flow of 0.19 mL/min. Detection was performed in multiple reaction monitoring (MRM) mode of selected ions at the first (Q1) and third quadrupole (Q3). To choose the fragmentation patterns of m/z (Q1) $\rightarrow$ m/z (Q3) for the analytes in MRM mode, direct infusions (via syringe pump) into the MS of melatonin standard solution in methanol were performed and the product ion scan mass spectra were recorded. The data were acquired using MassLinx Mass Spectrometry Software (Waters).

Statistical analysis

T test, analysis of variance (ANOVA), and LSD Fisher test were performed to assess minimum differences between means, with a significance level of $P \leq 0.05$. The analysis was carried out with InfoStat/p 2008 version (http://www.infostat.com.ar).

Results and discussion

The calibration plot was measured under the optimal experimental conditions from methanolic standard solutions. Eight points of the calibration curve were determined, and three replicate injections of standard at each concentration level were performed. The calibration equations were calculated by the least-squares linear regression method, and unknown concentrations were calculated by interpolation. Thus, linearity was evaluated from values closer to the LOD up to approximately 500 μg/L. The linearity of the calibration curve was satisfactory with a determination coefficient (R^2) of 0.9998 and equation was $y = 1414x - 1126$.

The detection (LOD) and quantification (LOQ) limits were calculated as the analyte concentrations that gave rise to peak heights with signal-to-noise ratios of 3 and 10, respectively. LOD and LOQ were 30.16 and 85.10 ng/L, respectively.

A Certified Reference Material of wine with an informed value for melatonin does not exist. However, it is acceptable to assess the validity of the measurements through recovery of additions of known amounts of the analyte to a blank matrix [32]. Thus, to estimate the trueness, intra-day repeatability, and inter-day reproducibility, spiked samples were analyzed: five blank samples, three replicate measurements at 1, 10, 20, 50, 100, 200, 300, and 500 μg/L melatonin concentration levels, respectively. The same experiment was repeated on four other independent occasions with at least a 1-wk interval. The recovery studies showed satisfactory robustness leading recoveries higher than 95% and lower than 103%. Repeatability as intra-day variability was determined by calculating the relative standard deviation for the replicated measurements. The obtained values were better than 0.4% for the retention times and 5.0% for the peak areas for all the concentrations evaluated (Table 2). The overall within-laboratory reproducibility (Table 2) ranged from 1.6% to 10.7% at the tested concentration levels. In summary and taking into account the matrix complexity, the reported values for the method assessment parameters could be considered highly satisfactory.

Once the conditions for extraction, separation, and quantification were established, the method was applied to the determination of melatonin in grape skin, must, and wine. Melatonin content in *V. Vinifera* cv. Malbec grape skin was within the range: 120–160 ng/g dry weight. Surprisingly, melatonin was not detected in finished wines made from these grapes. Instead, a melatonin isomer was found. These results are in agreement with Rodriguez-Naranjo et al. [26], who reported an isomer not previously detected in wines.

Table 2. Robustness of the developed methodology

MEL concentration (μg/L)	Repeatability RSD (%)	Reproducibility RSD (%)
1	5.12	9.04
10	4.20	10.80
50	3.61	6.20
100	3.12	9.94
200	2.52	8.55
300	1.41	6.39
500	0.11	1.60

RSD, relative standard deviation.

Fig. 1 shows the chromatogram of a Malbec wine sample doped with 100 μg/L of melatonin as well as the mass spectrum of the MRM transitions of melatonin (the ratio between the two monitored transitions 233 > 174 and 233 > 216 was 9.56 ± 0.05) and the tentatively identified isomer (the ratio between 233 > 174 and 233 > 216 was 0.057 ± 0.002). Diamantini et al. [33] have studied the mass spectrometric behavior of the isomeric compounds of melatonin. They described their synthesis and biological evaluation, concluding that the pharmacophoric groups of melatonin can be shifted from C-5 and C-3 to the C-6 and N-1 indole positions without loss of potency [34]. They concluded that it should be possible to shift the pharmacophoric groups of the melatonin moiety to suitable positions on the indole nucleous while still remaining high affinity and efficacy.

As the authentic marker is not available to identify the isomer by MS fingerprint, it is appropriate to provide a tentative identification, the comparative abundance of minority fragments, and ions to elucidate position isomers. As shown in Fig. 1, melatonin is most stable abundant fragment is 174, while the most abundant fragment for the isomer found in wine is 216. According to Diamantini et al. [33], the only isomer which base peak is not 174 is the structure shown in Fig. 1. Mor et al. [35] studied the antioxidant activities and physicochemical properties of several melatonin isomers. They demonstrated that the isomer we identified in wine is even more potent than melatonin itself. Indeed, the relative lipophilicities of melatonin and the isomer explain the experimental chromatographic behavior found in our studies. Undoubtedly, further studies are needed to confirm the structure of melatonin isomer found in Malbec wines.

To evaluate the evolution of melatonin and its potentially identified isomer during the fermentative process, an experimental protocol was designed as described in the experimental section. Fig. 2 shows the evolution of melatonin and its isomer during fermentation. Melatonin isomer concentration in finished wines (lab and pilot scale) was within the following range: 18–24 ng/mL. It has to be pointed out that melatonin was not detected, while the concentration of the isomer increased as the alcoholic

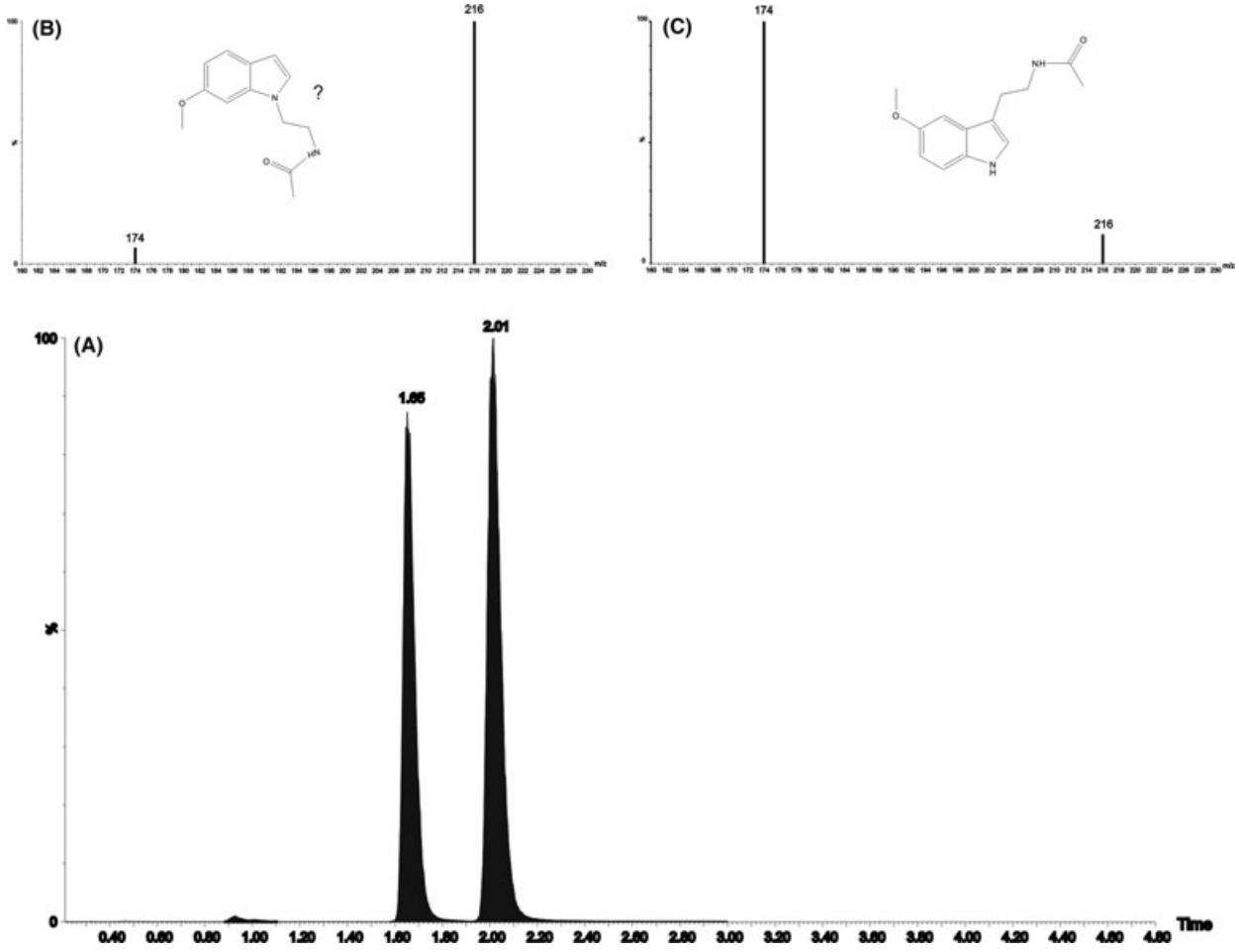

Fig. 1. (A) Chromatographic profile of a Malbec wine spiked with melatonin. Conditions: C18 column; mobile phase ACN:H_2O gradient containing 0.1% formic acid; flow rate: 0.45 mL/min; temperature: 30°C; injection volume: 25 μL. (B) Mass spectrum from melatonin hypothetic isomer fragment ions. (C) Mass spectrum from melatonin fragment ions. Conditions: Electrospray ionization in positive mode associated to mass spectrometric detection in multiple reaction monitoring mode (selected transitions: 233 > 216 and 233 > 174) (experimental conditions as described in Mass spectrometry instrumentation and MS/MS conditions section).

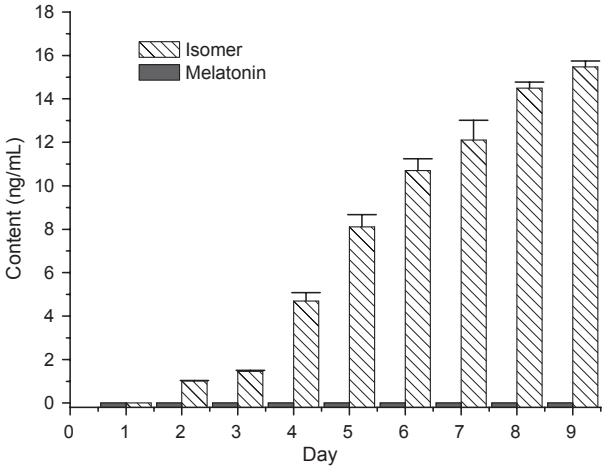

Fig. 2. Melatonin and its isomer evolution during laboratory scale winemaking. The vinification procedure was as described in Materials and methods section. Bars indicate standard errors of the means (n = 3).

fermentation develops. Such findings suggest that melatonin is not extracted from grape skin to must at detectable levels and, on the other hand, that *Saccharomyces cervisiae* plays an important role in the production of the melatonin isomer.

Sprenger et al. [36] demonstrated that melatonin and two structurally related compounds are formed in high concentrations in *S. cervisiae*. They also verified that melatonin can be synthesized via the most common pathway from tryptophan to *N*-acetylserotonin as a direct precursor. They found that the addition of exogenous tryptophan increases melatonin levels to a considerable extent. In view of our results, a laboratory assay in the absence of must was performed to elucidate the role of yeast and tryptophan (a melatonin precursor) in melatonin and its isomer evolution during the fermentation process. Fig. 3 shows the evolution of melatonin and its isomer during fermentation in aqueous media containing yeast and nutrients. Interestingly, from day 2, melatonin was already detected but its concentration remained constant during the fermentation process. On the other hand, the isomer was detected by day 4 and its concentration increased during alcoholic fermentation as observed in the presence of must. The indolamines were not detected in the control treatment. Such results suggest that in *S. cervisiae,* levels of melatonin and its isomer are influenced by growth conditions. Under the same conditions, the biosynthesis process of melatonin isomer is slower. Fig. 4 shows the evolution of melatonin and its isomer during fermentation in aqueous media containing yeast, nutrients, and tryptophan. Undetectable levels of melatonin were observed for the entire process, and the

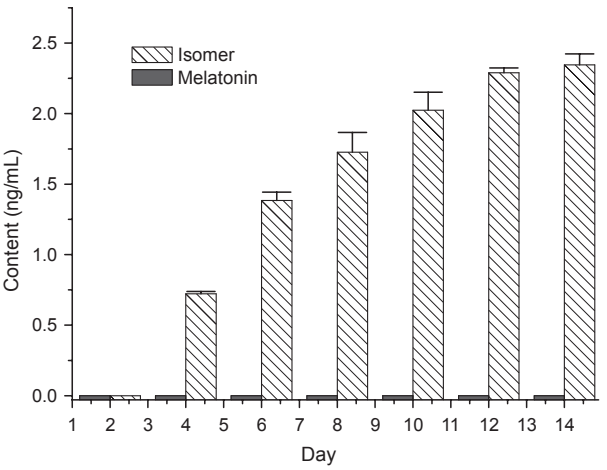

Fig. 4. Melatonin and its isomer evolution during laboratory scale fermentation in an aqueous media containing tryptophan. Conditions: EC1118 (20 g hr/L), sucrose (250 g/L), Hakaphos R™ (1 g/L), tryptophan (500 ng/mL), 200 mL distilled water. Bars indicate standard errors of the means (n = 3).

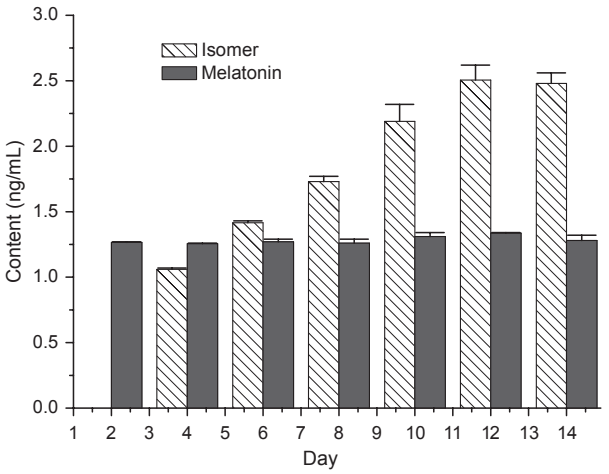

Fig. 3. Melatonin and its isomer evolution during laboratory scale fermentation in an aqueous media. Conditions: EC1118 (20 g hr/L), sucrose (250 g/L), Hakaphos R™ (1 g/L), 200 mL distilled water. Bars indicate standard errors of the means (n = 3).

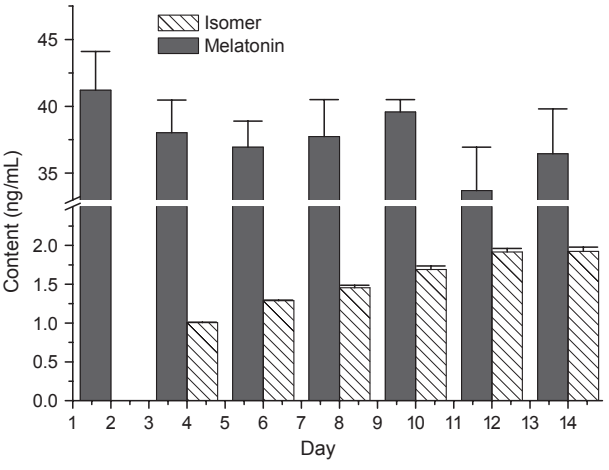

Fig. 5. Melatonin and its isomer evolution during laboratory scale fermentation in an aqueous media containing melatonin. Conditions: EC1118 (20 g hr/L), sucrose (250 g/L), Hakaphos R™ (1 g/L), melatonin (100 ng/mL), 200 mL distilled water. Bars indicate standard errors of the means (n = 3).

isomer evolution led to similar results as shown with the previous assays. The indolamines were not detected in the controls. Unexpectedly, enhancements were not observed with tryptophan supplementation. Fig. 5 shows the evolution of melatonin and its isomer in aqueous media containing yeast, nutrients, and melatonin. As can be seen, the addition of exogenous melatonin does not alter the behavior of the fermentation process.

Taken together, these results demonstrate that *Saccaromyces* play a decisive role in the production of melatonin and its isomer. Contrasting results were observed when the fermentation process was carried out in the presence of must, where biosynthetic pathway is directed toward the isomer production. Moreover, the same behavior was observed in the aqueous fermentation with tryptophan supplementation. These findings suggest that the presence of the amino acid in must stimulates the occurrence of the melatonin isomer in Malbec wines.

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