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Research Article

Analytical tools for elucidating the biological role of melatonin in plants by LC-MS/MS

Melatonin (MT) presence in higher plants was recently discovered and the knowledge of its function in vivo is limited. Several studies have recently shown the occurrence of MT and related compounds in grapes and wines. The analysis of MT in plants and foods represents a highly challenging task due to its wide concentration range, the difficulty in the selection of the extraction solvents because of its amphipathic nature, and the fact that it reacts quickly with other matrix components. Thus, sample processing factors; preparation/cleanup procedures; and chromatographic/detection parameters, such as HILIC and reverse phase (C_8 and C_{18}) chromatographic modes, ESI, and atmospheric pressure chemical ionization (APCI) in both negative and positive modes were evaluated. Taken together, we have demonstrated that optimal conditions were quite different for each of the matrices under study. A sonication-mediated extraction step was necessary for grape skin (100% v/v methanol) and plant tissues (50% v/v methanol), while wine and must required a SPE preconcentration step. HILIC−(+) APCI ionization was better for MT standards, while C_8−(+) APCI was the best choice for grape skin and C_{18}−(+ESI) was suitable for wine. On the other hand, C_8−(+)ESI was the most appropriate for vegetal tissues of *Arabidopsis thaliana*. Proposed methods were validated and the LODs were in the low picogram levels range. The optimized approaches were applied to the determination of MT and its isomer in different vegetal/food samples; levels found within the range: 4.9–440 ng/g.

Keywords:
Chromatography / Melatonin / MS / Plant material

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1 Introduction

Melatonin (MT, *N*-acetyl-5-methoxytryptamine) is mostly known as the principal vertebrate pineal secretory product. Lerner et al. isolated it from the bovine pineal gland in 1958 [1, 2]. Thereafter, MT was considered exclusively as an animal hormone, specifically a neurohormone for nearly four decades until the discovery that a dinoflagellate also has the capacity to biosynthesize this indoleamine [3]. In 1995, Dubbels et al. [4] and Hattori and colleagues [5] demonstrated the presence of MT in plants. Since then, it has been found in more than 140 different plant species [6]. MT has also been found in numerous taxa, including fungi, bacteria, protozoans, and invertebrates [3, 7–9]. In mammalians, MT has multiple functions, influencing circadian rhythm, regulating bone metabolism, scavenging free radicals species, and stimulating the activity of antioxidative enzymes [10–12], and it also acts as a protective agent in ocular diseases [13]. In addition, MT can mitigate neurodegenerative diseases, such as Alzheimer´s and Parkinson´s diseases and could act as an anticancer agent [11].

Although it's role in plants is still unclear, it is thought to contribute to the antioxidative protection against free radicals and prooxidative substances, such as H_2O_2 or O^- [14, 15], which are generated during the photosynthetic process [16, 17]. A second possible function, homologous to MT's role in mammals, could represent the conveyance of photoperiodical information and signaling of darkness [18–20].

In higher plants, rhythmic MT with nocturnal maxima has only been described in *Chenopodium rubrum* [19, 21], and exogenously applied MT inhibited flowering in this plant under inductive light conditions [22]. New findings in *Lupinus albus* suggest a role as a plant growth regulator in roots similar to indole acetic acid [23, 24]. An elevation of MT in reproductive tissue, with declining MT level as the plant ages, was observed in *Pharbitis nil* [25]. The accumulation of MT in reproductive tissues, seeds and fruits, may be indicative of a mechanism for protection of the tissues from oxidative damage arising from drought, cold, heat, UV light, or environmental toxins [6, 26].

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Abbreviations: APCI, atmospheric pressure chemical ionization; **MeOH**, methanol; **MRM**, multiple reaction monitoring; **MT**, melatonin; **RP**, reverse phase

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Further evaluation of MT's occurrence and function in higher plants proceeds slowly because of problems related to extraction and reliable quantification of this hormone [25, 27, 28]. Chemical complexity of plant's extract can interfere with MT determinations, giving false positive results if methods from vertebrate's MT research are directly adopted, for example, because of coelution in LC or cross-reactivity with antibodies of immunological methods like RIA or ELISA [29, 30]. Van Tassel et al. [25, 28] observed that validation according to standard criteria, linear serial dilution and parallel inhibition for immunoassays, and coelution with authentic standard for HPLC are insufficient to ensure the identity of MT. Only GC or LC combined with MS detection was found to be trustworthy, but this could be cost and labor-intensive methods [25, 28, 31]. This might explain several contradictory results for plant material from the same species quantified with different methods [28]. For example, ginger tubers were reported to contain between 584 and 1423 pg/g (f.w.) [5, 32], whereas Van Tassel and O'Neill [28] found only trace amounts of MT in this plant organ. Differences in MT degradation and extraction efficiency could represent another source of variation.

Taken together, the development of reliable analytical methodologies for the extraction, purification, and determination of MT in vegetal tissues is of upmost importance. Indeed, it should be considered the additional challenges related to the vast variety in matrices as well as the extremely wide concentration range (ppm–ppq levels). Therefore, the main objective of the present manuscript is to present sensitive and reliable analytical tools to contribute for the elucidation of the biological role of MT in plants.

2 Materials and methods

2.1 Chemicals and reagents

MT was purchased from Sigma Chemical (St. Louis, MO). ACN, methanol (MeOH), and water Optima® LC-MS grade were purchased from Fisher Scientific (Fair Lawn, NJ). Formic acid, 98%, was obtained from Fisher Scientific (Loughborough, UK). Acetic acid (glacial, Trace Metal grade), HPLC grade ammonium acetate, and certified ammonium formate were obtained from Fisher Chemical (Fisher Scientific, Pittsburgh, PA). Ultrapure water (18 MW cm) was obtained from EASY pure (RF Barnstead, IA). Strata C_8 (500 mg/6 mL), Strata C_{18}-E (200 mg/6 mL), and Strata-X (100 mg/6 mL) cartridges were purchased from Phenomenex® (Torrance, CA).

Standard solutions were daily prepared by appropriate dilutions of a 26 mg/L methanolic stock solution under dim green light (2 μmol/m^{2}s) to prevent analyte degradation. Stability of the stock at low temperature was tested. After only a few days, we observed a significant degradation, concluding that stock solution must be stored at −18°C in a freezer to prevent MT degradation. Under these conditions, the stability was ensured for at least a week.

2.2 Mobile phase preparation

For hydrophilic interaction liquid chromatography (HILIC), the mobile phase was prepared by first dissolving a known amount of ammonium acetate or ammonium formate in water (stock solution), and then mixing the salt solution with the desired volume of ACN. The salt concentration in the text refers to the final concentration in the mobile phase. The mobile phases A and B for C_{18} and C_8 chromatography were prepared by adding 0.1% (v/v) of formic acid to both water and ACN.

2.3 Samples

2.3.1 Plant tissue and seeds

Arabidopsis wild type (Col-0) as seed and plant materials was used. Plan tissue was collected from 1-month-old plants grown under natural radiation and short day conditions (approximately 8-h light:16-h dark) at 22°C. Seeds were stratified for 4 days at 4°C before seeding.

2.3.2 Grapes

The grapes were obtained during 2012 in a commercial vineyard of *Vitis vinifera cv*. Malbec located in Gualtallary (1500 m a.s.l.; 69°77′W and 33°22′S), Tupungato, province of Mendoza, Argentina. Sampling was carried out at a commercial harvest during the morning when sugar concentration reached 24°Brix. Clusters were collected in black nylon bags and berries were processed immediately.

2.3.3 Wine

Four commercial wines Tannat, Merlot, Malbec, and Cabernet Sauvignon were purchased at a local market.

Besides, a 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 h, it was inoculated with 20 g h/L EC1118 (Lallemand, Montreal, Canada). The fermentation temperature was maintained at 25°C ± 1°C 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. The whole process was carried out in the dark to avoid MT degradation.

2.3.4 Sample pretreatment

MT extraction from leaves, seeds, and grape skin was carried out under dim green light (2 μmol/m^2 s) to prevent analyte

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degradation. Frozen tissues were dried under nitrogen gas, grounded, accurately weighed, and transferred to a 15 mL glass tubes. After that, 2 mL of 100% (v/v) MeOH and 50% (v/v) MeOH–water for grape skin and *Arabidopsis* tissues, respectively, were added to each sample and then tubes were vortexed during 30 s. Ultrasonication was employed to assist and accelerate the extraction of MT from vegetal tissues in an ultrasonic bath (200 W, 15°C; Cleanson 1106, Buenos Aires, Argentina) filled with cold water for 10 min. The supernatant was decanted and centrifuged for 5 min at 3500 rpm (1852.2 g). The resulting extract was filtered through a 0.22 μm syringe filter (Osmonics®) and stored in an amber vial suitable for LC-MS/MS analysis.

For wine, C_8 cartridges were preconditioned with 2 mL of MeOH and 2 mL of ultrapure water. After the conditioning step, an aliquot of 6 mL of wine was loaded into the cartridge. The retained analyte was eluted with 2 mL of MeOH. This eluate was evaporated to dryness under a gentle stream of nitrogen (to prevent analyte degradation) and the residue was reconstituted in 300 μL water:ACN (50:50 v/v). Then this extract was filtered through a 0.22 μm PTFE syringe filter before injecting 10 μL into the ultra high performance LC system (UHPLC) MS/MS.

2.4 Chromatographic conditions

An Acquity™ UHPLC (Waters, Milford, NH) 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 ACN and water. The separation was performed by injecting 10 μL of sample onto ACQUITY UHPLC® BEH HILIC, C_{18} and C_8 columns (Waters) with 2.1 mm internal diameter × 50 mm length, and 1.7 μm particle size. The described chromatographic conditions were selected after testing several parameters and conditions as indicated below in Section 3.

2.4.1 HILIC conditions

For HILIC chromatography, the mobile phase A was 7.5 mM ammonium formate in water and the mobile phase B was 7.5 mM ammonium formate in ACN. HILIC chromatography was carried out with a flow rate of 0.2 mL/min. The gradient was started at an initial composition of 0% A and 100% B, then 3.0 min linear gradient to 10% A. A return to the initial conditions was accomplished by a 0.5 min gradient to 0% A, where it was held for 0.5 min. Thus, the total chromatographic run time was 4.0 min. The column was maintained at a temperature of 35°C.

2.4.2 C_{18} conditions

For C_{18} LC, the binary mobile phases consisted of water with 0.1% (v/v) of formic acid (A) and ACN with 0.1% (v/v) of formic acid (B) delivered at 0.3 mL/min. The gradient was started at an initial composition of 90% A and 10% B, then 2.4 min linear gradient to 65% A, after that 0.6 min linear gradient to 50% A. A return to the initial conditions was accomplished by a 0.2 min gradient to 90% A, where it was held for 0.8 min. Thus, the total chromatographic run time was 4.0 min. The column was held at a temperature of 35°C.

2.4.3 C_8 conditions

For C_8 chromatography, a mobile phase gradient program with solvent A (formic acid, 0.1% (v/v)) and B (ACN, 0.1% (v/v) of formic acid) was applied at a flow rate of 0.25 mL/min. The gradient program started with 10% B, followed by a linear increase of B to 50% in 3.0 min. Then, the mobile phase B was reduced to the initial conditions within 0.2 min, where it was held for 0.8 min. Thus, the total chromatographic run time was 4.0 min. The column was kept at a 35°C temperature.

2.5 MS instrumentation and MS/MS conditions

MS analyses were performed in a Quattro Premier™ XE Micromass MS Technologies triple quadrupole mass spectrometer (MS/MS). ZSpray™ ESI source (Waters) 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 3.3 kV and the extractor voltage was set at 3.0 V. Ultrapure nitrogen was used as desolvation gas with a flow of 800 L/h. Argon was used as collision gas at a flow of 0.19 mL/min. On the other hand, the MS/MS detector was connected to the UHPLC system through an Taurus™ atmospheric pressure chemical ionization (APCI) probe (Waters) running in positive (API+) mode at 600°C with N_2 as the nebulizer and the source temperature was kept at 120°C. The corona discharge pin current was set at 4.5 μA and the extractor voltage at 2.0 kV. Ultrapure nitrogen was used as desolvation gas with a flow of 200 L/h. Argon was used as collision gas at a flow of 0.18 mL/min achieving and analyzer pressure of ca. 3×10^{-5} Torr.

After optimization, detection was performed in multiple reaction monitoring (MRM) mode of selected ions at the first (Q_1) and third quadrupole (Q_3). To choose the fragmentation patterns of m/z (Q_1) → m/z (Q_3) for the analyte in MRM mode, direct infusions (via syringe pump) into the MS of MT standard solution in MeOH was performed and the product ion scan mass spectra was recorded. Thus, the transitions: 233 > 174 and 233 > 216 were assessed. Quantification of MT was done by measuring the area under the specific peak using MassLinx Mass Spectrometry Software (Waters).

2.6 Assay validation

2.6.1 Linearity

The calibration plots were measured under the optimal experimental conditions for each type of sample (wine aqueous

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Table 1. Summary of the best experimental/operational parameters and analytical methods' performance

Sample type and conditions	Pretreatment	Column	Mobile phases	Ionization	Matrix effect (%)	LOD (pg)	LOQ (pg)	Sample throughput (samples/h)	Recovery (%)[a]
Standard	Methanolic solution	HILIC	A: 7.5 mM NH_4COOH/H_2O B: 7.5 mM NH_4COOH/ACN	(+)ESI	–	0.89	2.97	13.30	–
Grapes skins	MeOH and sonication filtration (PTFE, 0.22 μm)	C_8	A: 0.1% HCOOH in H_2O B: 0.1% HCOOH in ACN	(+)APCI	69.35	2.61	8.71	4.10	99.30
Must and wines	Preconcentration (SPE C_8) Filtration (PTFE, 0.22 μm)	C_{18}	A: 0.1% HCOOH in H_2O B: 0.1% HCOOH in ACN	(+)ESI	88.40	0.12	0.42	7.50	104.94
Plant tissue (seeds and leaves)	MeOH/H_2O (50:50, v/v) and sonication. Filtration (PTFE, 0.22 μm)	C_8	A: 0.1% HCOOH in H_2O B: 0.1% HCOOH in ACN	(+)ESI	20.70	1.71	5.72	2.90	98.70

a) Mean value from five determinations.

solutions after SPE, 50% (v/v) methanolic *Arabidopsis* leaves solutions, and methanolic grape skin extracts). Eight points of the calibration curve were determined (three technical replicates at each concentration level). The calibration equations were calculated by the least-squares linear regression method. Thus, linearity was evaluated from values closer to the LODs values up to approximately 500 ng/mL.

2.7 Statistical method

ANOVA was used to determine significant differences among data. Each statistical analysis was done using the software Statgraphics Centurion XV (v 15.2.06) and normality of the data was tested before applying the ANOVA approach.

3 Results and discussion

3.1 Sample preparation optimization

Although the technology related to chromatographic separations and MS techniques advances, sample clean-up is still one of the most important bottlenecks of the analytical process. Effective sample preparation is essential for achieving good analytical results because matrix-related compounds may also coextract and interfere in the analysis. The extraction conditions in the analysis of MT in vegetal material is a difficult task taking into account the amphipathic nature of MT, and the fact that it reacts quickly with other matrix components considering its antioxidant capabilities.

For this reasons, optimization of the analyte extraction was carried out selectively for grape skin, wine, must, seeds, and leaves samples. In order to enhance the extraction, several approaches were considered for different liquid systems; including SPE, liquid–liquid extraction, and ultrasound assisted extraction. The following extractant solutions were tested: water, ethanol, MeOH, MeOH/water (50:50, v/v), ACN, isopropyl alcohol, and chloroform. Solvents chosen for each matrix can be observed in Table 1.

Off-line SPE was evaluated as a preconcentration step; C_{18}, C_8, and Strata X cartridges were tested. Important sensitivity enhancement for wine was obtained using the C_8 cartridges as has been described in the "sample pretreatment" section. Nevertheless, the procedure involves large-sample volumes followed by solvent evaporation, which resulted in a time-consuming and error-prone approach. It has to be pointed out that a preconcentration step is necessary for wine samples and not required in the case of grape skin and *Arabidopsis* tissues, MT concentrations in these samples are compatible with the MS/MS detection capability without SPE need.

The best results concerning to extraction efficiency, sample throughput, and sensitivity are shown in Table 1.

3.2 Chromatographic procedure

Taking into account that the LC-MS/MS is not a single technique, operational variables in sample preparation, chromatography, and ionization/fragmentation/detection should each be considered. In addition, HILIC is becoming a popular alternative to both normal and reversed-phase (RP) chromatography for the analysis of polar and ionic compounds.

Thus, in order to compare their efficiency, method optimization was performed in both HILIC and RP (C_8 and C_{18}) chromatographic modes. Suitable LC-MS/MS conditions, which provided satisfactory retention of MT without affecting sensitivity, were evaluated. In all chromatographic approaches, mobile phase composition, gradient program, type, and a concentration of an additive, column temperature, and flow rate were exhaustively studied. Optimization was carried out by the one-at-a-time methodology. Mobile phases consisting of different compositions of ACN-H_2O and MeOH-H_2O were evaluated. Both organic solvents provided about the same sensitivity. However, ACN was finally chosen due to a better peak shape. Also, we tested different additives including formic acid (0.1–0.01% (v/v)), acetic acid (0.10–0.01% (v/v)), ammonium formate (5.00–10.00 mM), and

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ammonium acetate (5.00–10.00 mM). The optimal parameters are shown in Table 1.

The effect of the mobile phase flow rate on the separation/retention of MT was evaluated using van Deemter plots. A total of 10 μL of the standard sample was injected onto the different chromatographic approaches at varying flow rates from 0.1 to 1.0 mL/min with isocratic separation. Thus, the optimal flows rates were as follows: 0.25 mL/min (C_8), 0.30 mL/min (C_{18}), and 0.20 mL/min (HILIC), as described in Sections 2.1–2.3. Under the optimal flow rates, we were able to isolate MT not only from matrix components but also from its isomers. The latter results crucial for studies involving the characterization of MT isomers [33–36].

The Van't Hoff plots for MT on the different columns over the temperature interval from 20 to 60°C were evaluated. MT retention's behavior decreased when increasing the column temperature. The optimal retention conditions were obtained when the temperature was fixed at 35°C for the three column systems. This temperature was selected for further experiments. Under the optimal mentioned conditions, the analyte was eluted at 1.08 min for HILIC, 2.32 min for C_{18}, and 2.34 min for C_8 within 4.0 min total run cycle for each approach.

The high sensitivity and selectivity achieved by coupling UHPLC with ESI-MS/MS is subject to some limitations when RP-HPLC is used: (i) decrease the ESI efficiency and stability as a consequence of polar compound elution in a highly aqueous mobile phase [37], and (ii) ion suppression due to poor retention and coelution of polar compounds [38]. In this study, retention efficiency on both HILIC and RP-HPLC columns was evaluated. In general, the concentration of ACN in the mobile phase resulted in a greater than three times increase in the MS signal when compared to the both RP phases.

As illustrated by McCalley and coworkers [39], when compounds are analyzed with HILIC, the buffer is an essential component in the mobile phase since peak shapes are affected if only weak acids are used as mobile phase additives. It is therefore important to employ a buffer compatible with the ACN-rich mobile phase. The type (ammonium formate or acetate) and concentration of buffer were evaluated for the HILIC approach. The use of a 7.5 mM ammonium formate concentration led to improved peak shape and retention time compared to ammonium acetate. The use of either buffer gave improved retention time, peak shape, and sensitivity compared to no buffer. Thus ammonium formate was chosen as suitable buffer in this study, which has the added benefit of being MS suitable. Table 1 show the column selected as optimal for each type of sample.

3.3 Optimization of ionizations and MS parameters

ESI and APCI are currently the most widely used API modes. Thus, these two techniques were tested for MT ionization. The APCI and ESI methods were optimized in respect of dominant conditions, such as capillary voltage, corona discharge current, source temperature, probe temperature, drying gas

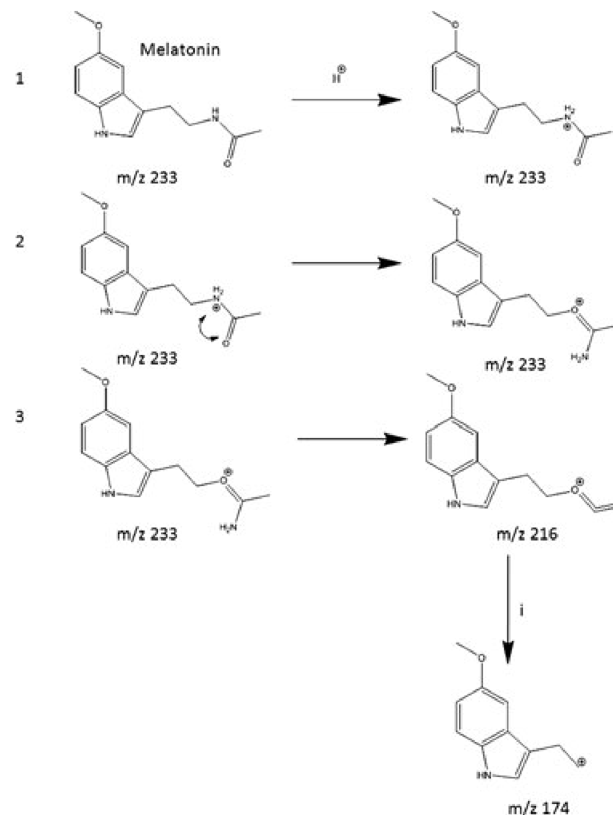

Figure 1. Possible melatonin (MT) ionization and fragmentation pathway. (1) Ionization step; (2) rearrangement; (3) losses of NH_3 and amide groups to generate the *m/z* 216 and 174 fragments, respectively.

flow, drying gas temperature (optimal parameters were mentioned in the "MS instrumentation and MS/MS conditions" section).

Comparison was performed in both positive and negative polarity and the effect of the mobile phase on the analyte ionization was studied for both. The obtained results are detailed in Table 1. As can be seen, the application of a specific source for the ionization of MT depends principally on the sample to be analyzed. For all samples, positive polarities were used due to its better sensitivity for the analyte under study.

Preliminary experiments were conducted with the purpose of finding the best instrumental conditions that would allow identification of MT in wine, grape skins, and *Arabidopsis* samples at trace levels. MT standard solution (1 mg/L) in MeOH was introduced into the MS system at a flow rate of 20 μL/min via a syringe pump. Identification of the parent ion (*m/z* 233) was performed in the full-scan mode by recording mass spectra from *m/z* 100 to 400 in positive mode. To evaluate the transitions in the MRM mode, the precursor and the product ions (*m/z* 216 and 174) of MT were selected according to the analyte's fragmentation pattern (Fig. 1). The MRM conditions were further optimized for the analyte to obtain maximum sensitivity. Quantitative results including ion ratios were calculated using QuanLynx software bundled

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with MassLynx v4.1. As a result, the most sensitive transition (233 > 174) was selected for quantification.

3.4 Matrix effects

During the whole optimization process, a careful study of the effect of each experimental variable on matrix effects was performed. Once the optimal conditions for sample preparation, chromatographic and detection steps were established, ionization effects were evaluated.

As known, one downside of ESI-MS or ESI-MS/MS ionization/detection is that the ionization process is susceptible to matrix signal suppression or enhancement. The LC-MS response obtained from a standard can differ significantly from matrix samples. The origin and mechanism of matrix effects are not fully understood. Consequently, after selecting the proper chromatographic approach for each sample, the effect of the matrix was assessed by comparing the signal of MT in pure solvent to the signal in the sample matrices. Thus, calibration curves from spiked matrix and spiked pure solvent samples were created. The percentage of the quotient of the slopes (b) in the spiked and solvent samples was used as an indicator of the extent of the ion suppression or signal enhancement, which was calculated as shown in Eq. (1):

$$\text{Matrix effect } \% = 100 - \left[\left(\frac{b_{\text{spiked}}}{b_{\text{solvent}}}\right) \times 100\right] \quad (1)$$

No signal enhancement, but response reduction of approximately 90% due to wine matrix interference was observed. On the other hand, the ion enhancement/suppression on grape skin was also evaluated following the same procedure and a 75% signal decrease was obtained. In addition, *Arabidopsis* matrix effect was tested and a 20% drop change of the signal was observed. Calibration plots are shown in Fig. 2.

Consequently, quantification was carried out following the standard addition method.

3.5 Analytical performance

A Certified Reference Material of the studied matrices with an informed value for MT does not exist. However, it is acceptable to assess the trueness of the measurements through recovery of additions of known amounts of the analyte to a blank matrix. For this purpose, a pool of wine samples was used. Thus, with the aim to estimate the trueness, intraday repeatability, and interday reproducibility, spiked samples were analyzed: five blank samples, three replicate measurements at 1.00 ng/mL, 10.0 ng/mL, 20.0 ng/mL, 50.0 ng/mL, 100.0 ng/mL, 200.0 ng/mL, 300.0 ng/mL, and 500.0 ng/mL MT concentration levels, respectively. The same experiment was repeated on four other independent occasions with at least 1 week interval. The recovery studies showed satisfactory robustness leading recoveries higher than 95.00% and lower than 103.00%. Repeatability as intraday variability was determined by calculating the RSD (%) 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.

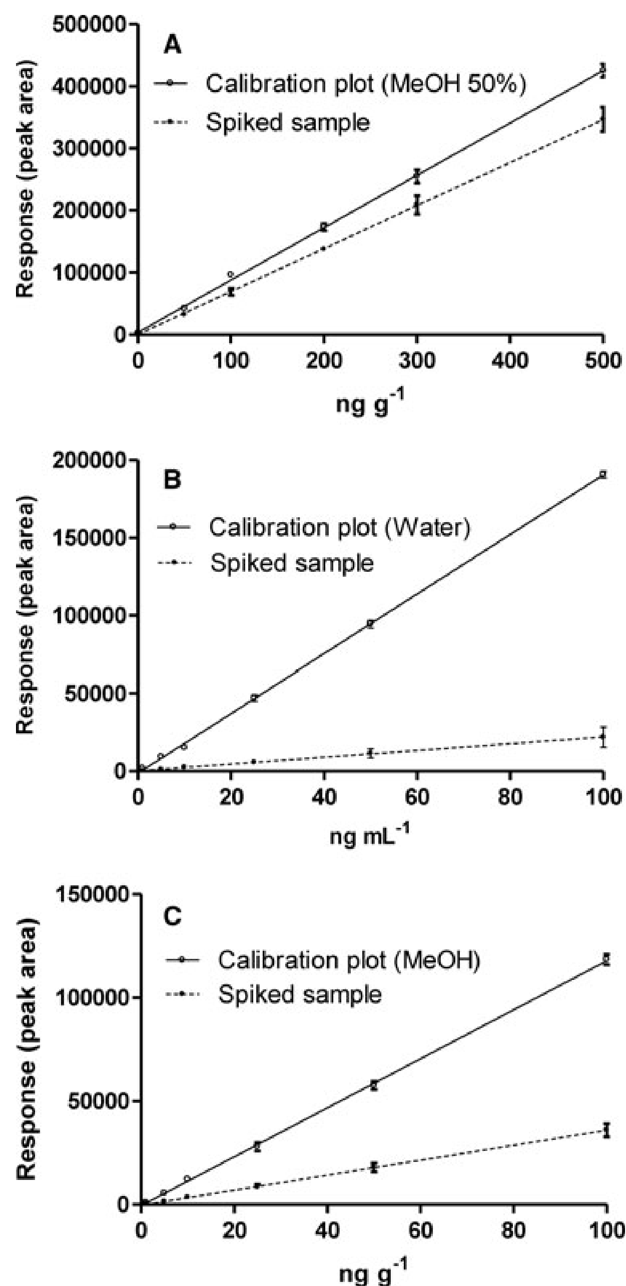

Figure 2. Calibration plots from spiked matrix and spiked pure solvent samples: (A) AT extract; (B) wine; and (C) grape skin extract.

The overall within-laboratory reproducibility 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. The LOD for MT was 0.89 pg (without preconcentration) and 0.12 pg (with SPE preconcentration) for a 10 μL sample volume.

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Table 2. Concentration found for each type of sample

Sample	Found concentration (ng/g and ng/mL)	
Wines	Melatonin (MT)	MT isomer[a)]
Tannat	N/D	151.74 ± 5.76
Merlot	N/D	211.28 ± 7.92
Cabernet	N/D	185.09 ± 5.25
Malbec 1	N/D	145.26 ± 2.82
Malbec 2[b)]	N/D	60.16 ± 4.87
Grapes		
Skins[c)]	440.02 ± 3.15	N/D
Musts	N/D	N/D
Arabidopsis		
Leaves[c)]	540.12 ± 2.99	N/D
Seeds	4.91 ± 3.27	N/D

Values are means ± SD ($n = 3$).
a) Isomer concentration was calculated from MT calibration plot.
b) Laboratory-made wine.
c) Expressed as dry weight.

3.6 Sample analysis

Once the optimal conditions were established for the different matrices under study, the developed methodologies were applied to the analysis of real samples. Commercial wines as well as laboratory-made wine, grape skin, different *Arabidopsis thaliana* tissues were analyzed. Table 2 shows the concentration found for each type of sample.

It has to be pointed out that MT was not detected in wines. Instead, an MT isomer was found in agreement with the reports of Gomez et al. and Rodriguez-Naranjo et al. [34, 35]. This isomer was previously described and it has been tentatively identified as MI A_1/M_5 [33]. In a previous work, we informed that *Saccaromyces* yeasts used during wine fermentation process play a decisive role in the production of MT and its isomer [34]. Figure 3 shows the mass spectrum of the MRM transitions of MT (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). As shown in the figure, MT's most stable abundant fragment is 174, while the most abundant fragment for the isomer found in wine is 216.

4 Concluding remarks

Our results clearly demonstrate that optimal conditions were quite different for the vegetal/food matrices under study. While $C_8-(+)$ APCI was the best choice for grape skin;

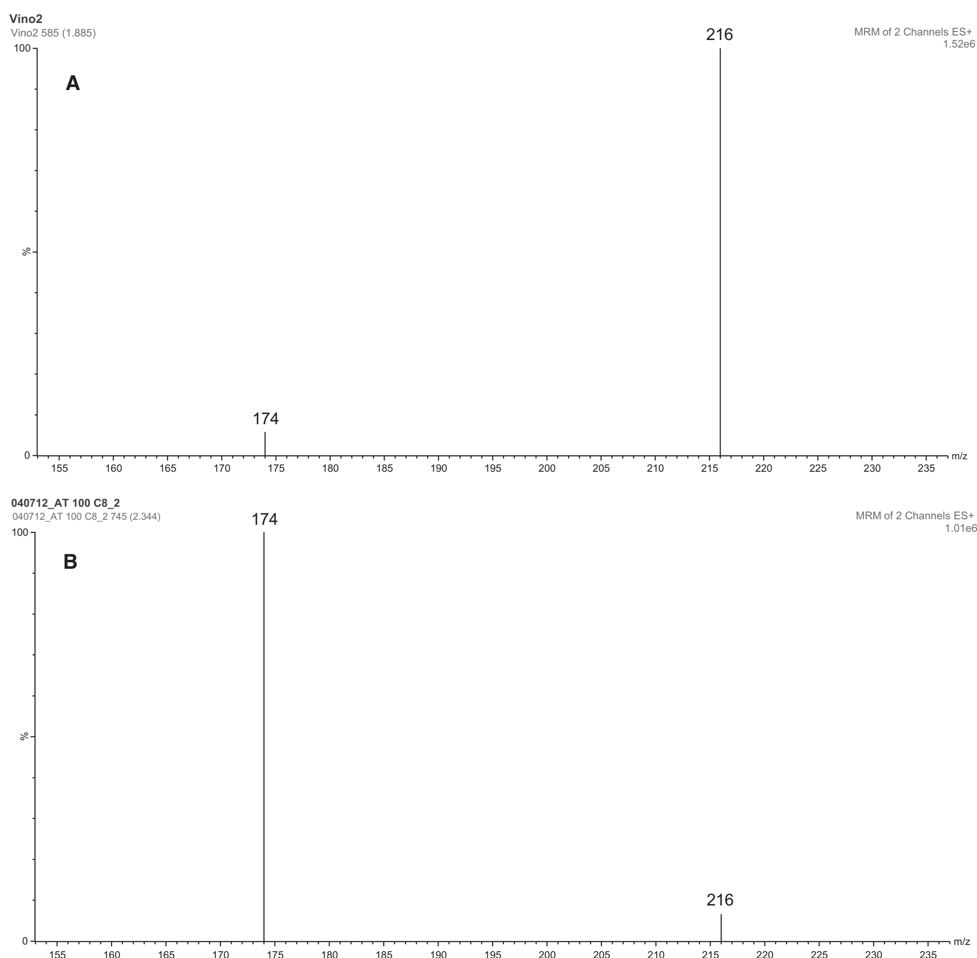

Figure 3. (A) Mass spectrum from MT isomer and (B) mass spectrum from MT. Conditions: ESI in positive mode associated to mass spectrometric detection in multiple reaction monitoring mode (selected transitions: 233 > 216 and 233 > 174) (experimental conditions as described in MS instrumentation and MS/MS conditions section).

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the optimal stationary phase/ionization system combination for wines and must was $C_{18}-(+)$ ESI. On the other hand, $C_8-(+)$ ESI was the most appropriate for vegetal tissues of *Arabidopsis*.

A novel HILIC–UHPLC–MS/MS method for the determination of MT was developed in this study. The HILIC approach was compared to a RP-UHPLC–MS/MS methodology based on the use of C_8 and C_{18} columns, with the results revealing slightly higher sensitivity for HILIC. Moreover, in HILIC mode, better linearity, and high accuracy in whole linear range was observed. Nevertheless, due to the observed matrix effects, HILIC stationary phase showed a proper performance only for standard solutions.

The matrix effects should be carefully assessed when vegetal tissues are involved; we found that the clean-up, chromatographic, and detection conditions have to be evaluated for each sample. Clean-up and preconcentration are usually necessary when low detection limits are required but sometimes the cleaning effect is not suitable for the complexity of the matrices.

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