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Development of the determination of melatonin in blueberries using LC-MS/MS

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ABSTRACT

Melatonin (MLT) is an indolamine that presents a functional activity with a broad spectrum of action, highlighting its antioxidant, anti-inflammatory and immunomodulatory and anti-apoptotic capacity. It is a widely distributed hormone, found in most of the foods that make up a normal diet. There are many studies that analyze the MLT content in different foods and using different techniques. However, very little research has focused on analyzing MLT concentration in blueberries. Therefore, the aim of this study was the development of a method for the extraction and determination of MLT in blueberries (*Vaccinium corymbosum*), using liquid chromatography-tandem mass spectrometry (LC-MS/MS), which has been shown to be one of the most reliable and precise techniques for this type of analysis. It was determined that using methanol as an extraction solvent obtained greater efficiency than using acetonitrile. Likewise, the highest recovery percentages were achieved with the BOND ELUT PLEXA solid phase extraction (SPE) cartridges. With the optimization of the extraction process and subsequent analysis, it was determined that the blueberries used in this study had an MLT concentration of 0.173 ± 0.028 ng/g.

1. Introduction

Melatonin (MLT) (N-acetyl-5-methoxytryptamine) is an indoleamine produced mainly by the pineal gland, being synthesized from tryptophan (Muñoz-Jurado et al., 2022; R. J. Reiter, 1991). In addition to the pineal level, MLT is also secreted by extrapineal sources such as cells of the immune system, brain, skin and gastrointestinal tract (Carrascal et al., 2018). MLT acts primarily by regulating sleep and helping to synchronize the circadian rhythm with the natural light and dark cycle (Patel et al., 2020). However, this hormone has a functional activity with a broad spectrum of action (Nikolaev et al., 2021), its antioxidant, anti-inflammatory, immunomodulatory and anti-apoptotic capacity being known, as well as its neuroprotective effect (Bahamonde et al., 2014; Esposito & Cuzzocrea, 2010; Muñoz-Jurado et al., 2022; R. Reiter et al., 2000; Rosales-Corral et al., 2012; Wang et al., 2020).

For decades, MLT was considered an animal neurohormone. However, in 1995, different studies confirmed the presence of MLT in higher plants (Talib et al., 2021), where it is called phytomelatonin (Chen & Arnao, 2022). It is now known that this indolamine is a widely distributed hormone that, in addition to being found in all mammals, is also present in plants, single-celled organisms, algae, insects and fungi (Meng et al., 2017; Slominski et al., 2012).

Due to the wide distribution of MLT, both in animals and plants, the consumption of most of the foods that make up a regular diet ensures the intake of MLT. Many studies have been carried out in which the levels of MLT in different foods are analyzed, using different techniques. However, more research is needed to develop more accurate methods to obtain reliable data (Feng et al., 2014), as there is a lot of diversity in the results reported by the various studies carried out, due to the difficulty of finding a suitable technique for MLT determination.

There are different techniques, developed in recent years, for the analysis of MLT, such as radioimmunoassay (RIA), enzyme-linked immunosorbent assay (ELISA) and chromatographic techniques. The latter offer high sensitivity and excellent detection specificity (Kolář & Macháčková, 2005; Yücel et al., 2018). Among them, high-performance liquid chromatography (HPLC) is more powerful and precise and does

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not require derivatization, as in the case of gas chromatography-mass spectroscopy (GC-MS) (Feng et al., 2014). Liquid chromatography-tandem mass spectrometry (LC-MS/MS) has been used in various studies to demonstrate the presence of MLT or to determine its levels in different matrices (Kolář et al., 1997; Wolf et al., 2001). This technique allows an increase in the validity of the results obtained, compared to immunological techniques (Feng et al., 2014). In addition, it allows the detection of MLT at very low limits (Huang & Mazza, 2011).

Thanks to the research carried out, today we know that plants contain higher amounts of MLT than animals (Wasserbauer, 2017), seeing that medicinal plants are the main source of MLT for humans (Arnao & Hernández-Ruiz, 2018). Fruits are also an important source of MLT, among them cherries, which contain approximately 15.050 ng/g of MLT (Mannino et al., 2021). The rest of the berries also have significant amounts of MLT. Nowadays, the consumption of these fruits has experienced a significant increase not only because of their high nutritional value, flavour and nutraceutical characteristics, but also because of their beneficial properties for health, being dietary sources of bioactive compounds (Cosme et al., 2022; Olas, 2018), including MLT. In this regard, red fruit intake has been associated with a lower incidence of reactive oxygen species (ROS)-induced disorders, including cardiovascular disorders, cancer and inflammatory processes (Gomes-Rochette et al., 2016). Among berries, blueberries draw special attention for their high antioxidant capacity and high concentration of anthocyanins and other phenolic compounds (Prior et al., 1998; Yang et al., 2022). Their consumption has been associated with an increase in *ex vivo* serum antioxidant status (Giovanelli & Buratti, 2009; Yang et al., 2022). Daily intake of even moderate amounts of blueberries and anthocyanins (<50 mg) is associated with a reduced risk of disease (Kalt et al., 2020). However, after carrying out an exhaustive bibliographic review, it has been found that there are very few studies in which the levels of MLT in blueberries are evaluated (*Vaccinium corymbosum*). Only two studies have been found, carried out by Verde et al., (2016) and by Brown et al. (2012), in which the content of this indolamine in these fruits is analyzed. In these studies, two different species of blueberries are also studied, *Vaccinium corymbosum*, Duke variety (blueberry) and *Vaccinium macrocarpon* (cranberry), respectively, obtaining different concentrations. In these studies, different chromatographic techniques are also used. Verde et al. (2016) use HPLC with fluorescence detection and Brown et al. (2012) use HPLC with ultraviolet detection, which may also be the reason for the different results reported. The lack of studies on this topic does not allow us to state exactly the amount of MLT that blueberries contain, nor what is the optimal technique to determine it.

Therefore, the aim of this study was to develop a possible method for the extraction and analysis of MLT in blueberries (*Vaccinium corymbosum*) by LC-MS/MS, in order to have a robust and accurate technique that allows the study and evaluation of MLT levels in these berries. For this purpose, different solid phase extraction cartridges as well as different diluents will be evaluated to determine with which of them a higher MLT recovery is obtained. The MLT content of the blueberries will also be determined.

2. Material and methods

In order to obtain optimal MLT recovery and to make the determination of MLT as accurate as possible, the efficiency of different solvents as well as different solid phase extraction (SPE) cartridges were tested during the MLT extraction process.

2.1. Chemicals and reagents

The solvents methanol (MeOH) and acetonitrile (AcN), both with LC-MS quality, were obtained from Honeywell and the ammonium solution (25% w/w) was supplied by Scharlab S.L. Melatonin standard (Purity 99.17%) was purchased from LGC Ltd. Ultrapure water was used during all experiments (Milli Q-System, Millipore). Nylon syringe filters (23 mm, 0.22 μm pore) were supplied by Phenomenex.

Table 1
Liquid chromatography pump program.

Time	A.Conc	B.Conc
0.5	95.0	5.0
6.00	20.0	80.0
6.10	0.0	100.0
9.00	0.0	100.0
9.10	95.0	5.0
12.00	95.0	5.0

2.2. Cartridges for solid phase extraction (SPE)

Three different cartridges were used, in order to evaluate which of them was most efficient for the recovery of MLT. The cartridges used were: SPE OASIS WCX (150 mg 6 ml) (Waters™), SPE BOND ELUT PLEXA (200 mg 6 ml) (Agilent Technologies) and SPE EBH (60 mg 3 ml) (Scharlab S.L.).

2.3. Preparation of stock and standard solutions

A standard MLT stock solution of 1500 μg/ml in AcN:H2O (1:9) was prepared. From this stock solution, a working standard solution of 15 μg/ml was prepared. This was diluted to six different concentrations from 0.02 ng/ml to 10 ng/ml to perform a calibration curve. The stock solution was stored at −20 °C until analysis, for a maximum of 8 weeks. The remaining dilutions were prepared prior to analysis.

2.4. Sample preparation and extraction

The blueberry (Vaccinium corymbosum) samples were acquired in the local market (Origin: Huelva). They were crushed with a crusher (Moulinex DP805GBP) and 2 g of each sample (20 samples in total) were weighed in a glass tube. In order to study the validity of the extraction, half of the samples were fortified with 50 ng/g, from one of the MLT working standard solutions. Subsequently, the samples were vortexed for 1 min.

For the extraction process, first, 8 ml of the solvent (MeOH or AcN) was added, the headspace of each tube was filled with N_2, vortexed for 20 s, and sonicated for 30 min. After this time, the samples were centrifuged for 15 min at 9000 rpm. The supernatant was recovered and evaporated under a stream of N_2 at 50 °C. Next, each sample was redissolved with 6 ml of 2% NH_4OH. The samples were vortexed for 1 min and sonicated for 10 min to achieve total redissolution. Subsequently, the solid phase extraction (SPE) process was carried out following the instructions of each manufacturer, after which 1 ml was filtered through a syringe filter (0.22 μm nylon) into an amber vial, previously to injection in the LC-MS/MS equipment. All assays were carried out in darkness to avoid possible degradation of the MLT due to the effect of light.

2.5. LC-MS/MS analysis of melatonin

Chromatographic analyses were performed on a liquid chromatograph (ExionLC TM, Sciex) coupled to a triple quadrupole mass spectrometry detector (Model Triple Quad 5500+; Sciex), equipped with an electrospray ionization (ESI) source. Chromatographic separations were performed on a C-18 column (Synergi™ 4 μm, Fusion-RP 80 Å; 50 × 2 mm; Phenomenex). Mobile phase A was composed of 1 mM oxalic acid and 0.02% formic acid in water and mobile phase B was 0.1% formic acid in acetonitrile. The gradient pump program (Table 1) was used with a flow rate of 0.3 ml/min, at 40 °C temperature and 5 μl injection volume as analytical condition.

The detector conditions were optimized and are summarized in

Table 2
Optimized source parameters.

Parameters	
Curtain Gas	30 psi
Collision Gas	9
IonSpray Voltage	5500 V
Temperature	400 °C
Ion Source Gas 1	60 psi
Ion Source Gas 2	60 psi

Tables 2 and 3. Multiple reaction monitoring (MRM) mode was used for melatonin quantification. Analyst® software was used for data acquisition and Sciex OS for data processing and treatment.

3. Results and discussion

3.1. MLT identification

The MLT stock solution in AcN:H2O was analyzed. The protonated molecular ions of MLT showed an m/z 233.2. The fragmentation mass spectra of the MLT ion are shown in Fig. 1. The ion scan spectra of indoleamine showed a higher abundance of fragment ions at m/z 174.4. These results agree with those obtained by Karunanithi et al. (2014), who report a greater abundance of the fragmented ion 174.1 (Karunanithi et al., 2014). Similarly, Kocadağli et al. (2014) also obtain a higher relative abundance of the fragmented ion 174.2 (Kocadağli et al., 2014).

3.2. SPE optimization

Chromatographic methods are the main choice for the analysis of organic molecules. In them, sample preparation usually takes 80% of the total analysis time and requires liquid-liquid extraction and solid phase extraction (Nováková & Vlčková, 2009). The SPE method offers several advantages over other extraction methods, including: high recovery, effective preconcentration, need for less organic solvent, absence of foam in the formation of emulsions, ease of operation and greater automation possibilities (Badawy et al., 2022; Kataoka, 2003). The choice of sorbent is the key factor in SPE, because it can control parameters such as selectivity, affinity and capacity (Nováková & Vlčková, 2009). In the present work, polymeric and copolymeric sorbents with a

Table 4
Extraction procedure for WCX, PLEXA and EBH.

	WCX 150 mg 6 ml	PLEXA 200 mg 6 ml	EBH 60 mg 3 ml
CONDITIONING	1 ml MeOH 1 ml H_2O	1.5 ml MeOH 2.5 ml H_2O	2 ml MeOH 2 ml H_2O
LOAD	1 ml MLT 5 ng/g H_2O	1 ml 5 ng/g NH_4OH 2%	1 ml 5 ng/g NH_4OH 2%
WASHED 1	1 ml NH_4OH 5%	1 ml MeOH 5%	1 ml MeOH 5%
WASHED 2	1 ml MeOH	–	–
WASHED 3	1 ml FA 2% (MeOH): H_2O 25:75	–	–
ELUTION 1	1 ml FA 2% (MeOH): H_2O 75:25	1 ml MeOH	1 ml MeOH
ELUTION 2	–	1 ml MeOH	1 ml MeOH

Table 3
Transitions and optimized chromatographic parameters for melatonin analysis.

Transitions	CE		DP (volts)		EP		CXP	
	Manual	*Automatic*	*Manual*	*Automatic*	*Manual*	*Automatic*	*Manual*	*Automatic*
233.2 > 174.4	21.5	25	75.6	80	–	10	18.6	20
233.2 > 159.3	38.8	39	84.7	80	–	10	7.8	18
233.2 > 143.0	41.8	43	82.4	80	–	10	12.95	18
233.2 > 131.3	48	47	85.1	80	–	10	15	14

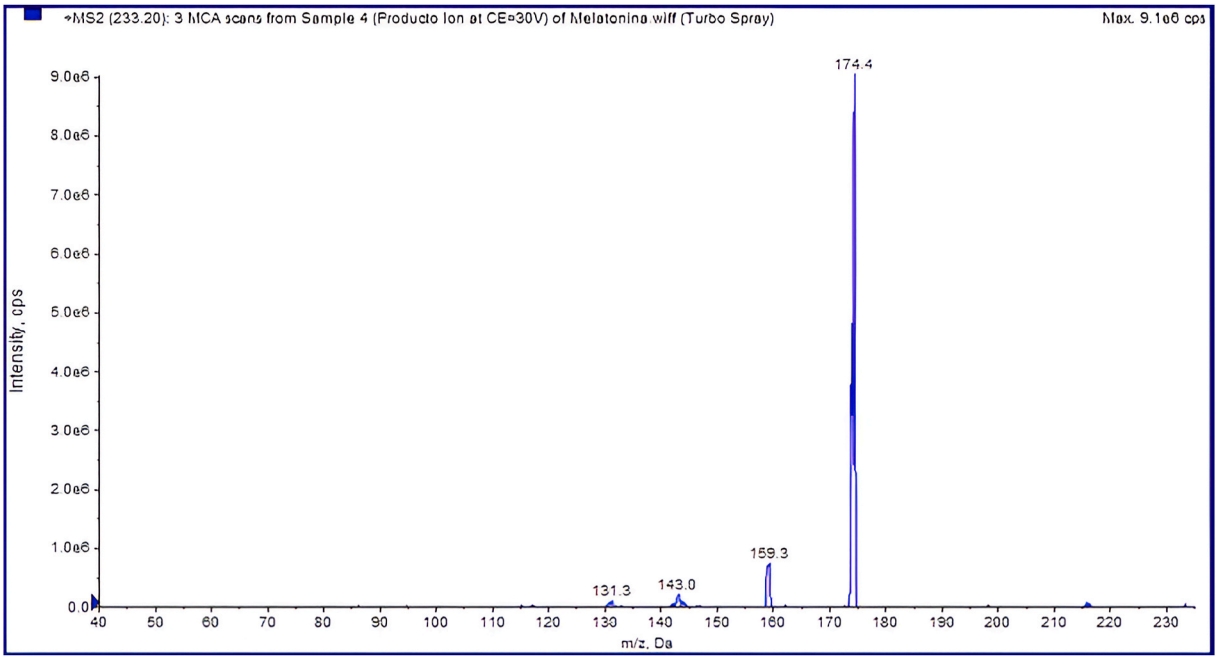

Fig. 1. Melatonin transitions.

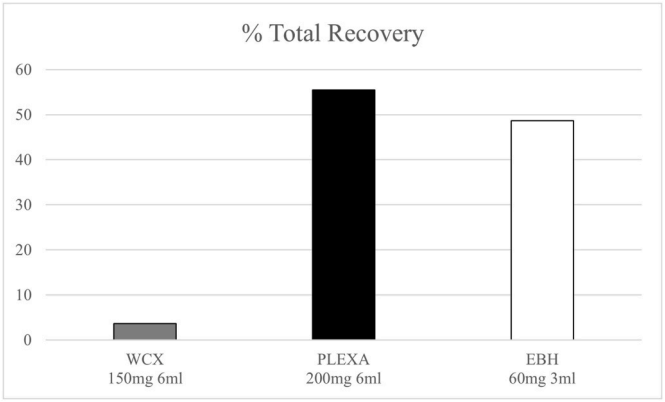

Fig. 2. Percentage of melatonin recovery, obtained with each cartridge in SPE.

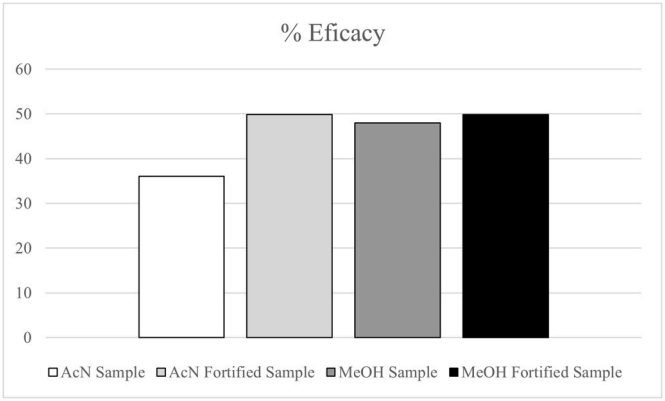

Fig. 3. Percentage of efficiency of the solvents used for the extraction of MLT in fortified and unfortified samples. AcN: Acetonitrile, MeOH: Methanol.

smaller particle size and thus a larger surface area were studied (Pavlović et al., 2010; Rosado et al., 2017).

On the other hand, in this part of the process, the presence of interferences coming from matrices as complex as red fruits, in this case blueberries, must be taken into account, since this could affect the precision of the method (Rosado et al., 2017). Therefore, the existence of interference is a key factor to take into account when selecting the SPE cartridge.

The SPE cartridges used in this study were SPE OASIS WCX (150 mg 6 ml), SPE BOND ELUT PLEXA (200 mg 6 ml) and SPE EBH (60 mg 3 ml), following the manufacturer's instructions (Table 4). To carry out this test, dilutions of MLT of 5 ng/g in water or in 2% NH_4OH were prepared, according to the requirements of each cartridge.

The comparison of the recoveries obtained with each cartridge (Fig. 2) shows a slightly higher recovery of MLT, with the use of PLEXA cartridges, with which a recovery of 55.45% is obtained. Therefore, since these cartridges offered greater MLT recovery, as well as greater signal and less interference, they were selected for carrying out the SPE process.

3.3. Solvent optimization

The solvent used in the extraction has a significant impact on the performance of this (Setyaningsih et al., 2015). Because MLT exhibits amphipathic characteristics, an inappropriate choice of extraction solvent could lead to poor recovery of indoleamine (Verde et al., 2022). Therefore, an attempt was made to optimize the extraction of MLT using two different solvents, MeOH and AcN, in order to determine which of them gave the highest yield. A test was carried out with blueberry samples, fortified with 5 ng/g of MLT and without fortification. The SPE process was carried out with PLEXA cartridges.

Table 5
Amount of melatonin obtained in the different analyzes of blueberries.

Blueberries	Calculated Concentration (ng/g)
1	0,16
2	0,15
3	0,18
4	0,15
5	0,17
6	0,14
7	0,2
8	0,23

Table 6
Amount of melatonin represented in ng/g obtained in the different analyzes of blueberries fortified with 50 ng/g of MLT.

Fortified Blueberries	Calculated Concentration (ng/g)
1	21.29
2	20.97
3	20.04
4	20.24
5	20.01
6	19.22
7	25.69
8	19.78
9	25.4
10	24.12
11	24.25
12	23.99

The results obtained in this test show that the solvent with the highest efficiency was MeOH (Fig. 3). These results agree with those reported by Verde et al. (2022), who used solvents of different polarities, such as methanol, ethanol, ethyl acetate and isopropanol, obtaining the best results with methanol. Likewise, Oladi et al., (2014) in their study also evaluated the effect of the solvent on the extraction, using methanol, acetonitrile and chloroform. Again, methanol was the solvent selected for obtaining the best results.

3.4. Melatonin levels

Analysis of MLT in foods is a difficult task since it is found at low levels in most of them (Kocadağli et al., 2014). As mentioned, chromatographic techniques, specifically LC-MS/MS chromatography, allow greater precision in the analysis of molecules. However, in this technique special attention must be paid to the extraction procedure, since it is influenced by the type of solvent, the volume of solvent, the temperature, the sonication time and the pH (Nawaz et al., 2016; Oladi et al., 2014). In particular, in the case of MLT extraction, several difficulties arise due to its powerful antioxidant activity, which often leads to a rapid reaction with environmental or other matrix components (Hamid et al., 2010; Setyaningsih et al., 2015). This is known as matrix effect and refers to changes (usually reduction) in the ionization efficiency (and consequently in the peak area) of an analyte, which are caused by co-eluting compounds of biological material and/or chemicals or laboratory material used for sample preparation (Kolář & Macháčková, 2005). Taking the above into account and having optimized the cartridge and the solvent to be used, the blueberry samples were analyzed.

Eight different analyzes of blueberry samples were carried out, obtaining an average of 0.173 ± 0.028 ng/g. Likewise, to evaluate the validity of the extraction, 12 blueberry samples fortified with 50 ng/g were analyzed, obtaining an average of 22,083 ± 2035 ng/g, which represents a recovery percentage of 49.91%. The results obtained in

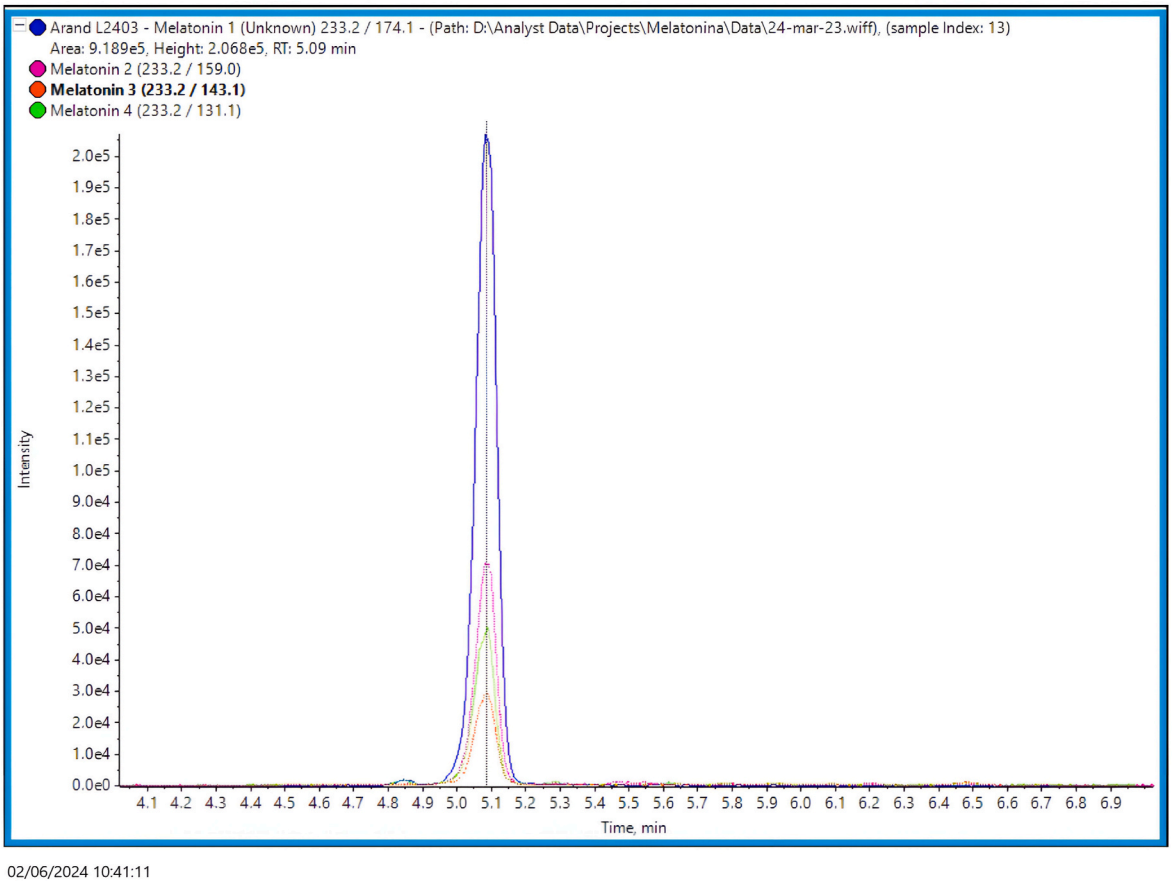

Fig. 4. LC-MS/MS chromatogram for melatonin extracted from blueberries.

each analysis appear in Table 5 and Table 6, respectively. The mean retention time of the MLT was 5.1 min (Fig. 4). The method was considered linear, with a mean R^2 value of 0.997.

Our results are similar to those reported by Verde et al., (2016), in their study in which they obtained an amount of MLT in blueberries of 0.250 ng/g (Verde Rodríguez, 2016). However, the technique used by Verde et al., (2016) differs greatly from the one described in this study, as they use chloroform as solvent and do not perform the SPE process. Despite the similarity of results between those obtained by Verde et al., (2016) and those obtained in this study, if we compare them with those reported for other blueberry species, such as cranberry (Vaccinium macrocarpon), the latter has much higher concentrations than blueberry, used in this study, containing 96 μg/g (Brown et al., 2012). In the study by Brown et al. (2012) also does not use the same extraction solvent as the one used here, since they use 200 μl of methanol: water: formic acid (80: 20: 1 v/v). Studies that analyze the concentration of MLT contained in blueberries are almost non-existent, making it difficult to make comparisons of results and establish, precisely, the amount of this indoleamine in this species of red fruit. If we compare the results obtained in our analyzes with those reported for other fruits, it could be said that blueberries do not contain a high amount of MLT, since, for example, cherries present approximate concentrations of 15.050 ng/g (Mannino et al., 2021). Likewise, goji berries contain about 530 ng/g of MLT (Mannino et al., 2021). However, certain species of strawberries, such as *Fragaria magna*, have a somewhat lower concentration of MLT (0.136 ng/g) (Badria, 2002) than the blueberries in our study. However, this is variable depending on the species, as another strawberry species, *Fragaria ananassa*, can contain MLT in the range of 1.4–11.26 ng/g (Stürtz et al., 2011).

Regarding the percentage of recovery, we cannot compare it with the studies in which the concentration of MLT in blueberries has been evaluated, since none of them report this percentage. Our results are similar to those of Verde et al., (2016), so perhaps the recovery percentage we obtain is similar to that obtained by those authors. However, if we compare our recovery with that obtained by other authors, who have analyzed the MLT content in cherries, we observe that the percentage obtained in our study is lower, since in cherries the recoveries obtained are in a range of 60–75% (Burkhardt et al., 2001; González-Gómez et al., 2009; Kocadağli et al., 2014; Rosado et al., 2017). Yet, in other matrices, such as rice and different nuts, recovery percentages of almost 100% are achieved (Setyaningsih et al., 2015; Verde et al., 2022). This may be due to the fact that red fruits are very complex matrices and may affect the recovery of indolamine. Likewise, the extraction method used can also cause variability in the results, so a standardized method should be developed for the analysis of this type of matrices.

The technique used in this study has been designed by our research team, with the aim of establishing a valid procedure for the determination of the MLT content in blueberries. This is the main novelty of the present study, being, finally, an extraction technique very similar to that carried out by Rosado et al. (2017). However, both techniques differ in some aspects such as the volume of solvent or the SPE cartridge used. Furthermore, in the case of Rosado et al. (2017) the matrix used is cherry, whose composition is slightly different from that of blueberries, which have almost 90% water, compared to the 83% that cherries contain. Likewise, blueberries contain 16% fat, while cherries only contain 7% (AESAN/BEDCA, 2018). This is something to take into account, since MLT, due to its amphipathic nature, is soluble in lipids (Verde et al., 2022), which would justify the lower recovery percentage obtained in our study (49.91%) compared to those obtained by Rosado

et al. (2017) (61–75%). However, more studies are necessary to improve the recovery percentage obtained, since it is below that reported for other species of red fruits, although this may be due to the nutritional composition of blueberries.

4. Conclusions

Taking into account all the results obtained, we determine that:

1. A better efficiency of the process is obtained with the use of MeOH than with the use of AcN.
2. Of the three cartridges used for solid phase extraction (SPE OASIS WCX, SPE BOND ELUT PLEXA and SPE EBH), the highest percentages of recovery and efficiency were obtained with the PLEXA cartridge.
3. With all this and after performing the chromatographic analysis of the samples, we obtained that the blueberries used in this study contained an average of 0.173 ± 0.028 ng/g of MLT, with a recovery percentage of 49.91%. The analytical results obtained in this study demonstrate that the developed procedure is reliable for the determination of MLT in blueberries, since they are similar to the results obtained by other studies in which this matrix is analyzed.

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CRediT authorship contribution statement

Ana Muñoz-Jurado: Writing – original draft, Investigation. **Daniel López:** Investigation, Data curation. **Begoña M. Escribano:** Writing – review & editing, Validation, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

The authors do not have permission to share data.

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