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ORIGINAL PAPER

Detection and quantification of melatonin and serotonin in eight Sweet Cherry cultivars (*Prunus avium* L.)

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Abstract Melatonin functions as a free radical scavenger and controls the regulation of the sleep–wake cycle in mammals, while serotonin is the main intermediate in melatonin biosynthesis. In this paper, melatonin and serotonin have been detected and quantified for the first time in eight different sweet cherry cultivars using high performance liquid chromatography with mass spectrometry detection. The limits of detection of the proposed analytical method were 4.3 ng/mL for melatonin and 3.0 ng/mL for serotonin. An inverse relation between the contents of melatonin and serotonin was observed in the studied sweet cherry cultivars. The highest melatonin amounts were found in 'Burlat' sweet cherries (22.4 ± 1.3 ng/100 g of fresh fruit), while the highest serotonin contents were found in the cultivar 'Ambrunés' (37.6 ± 1.4 ng/100 g of fresh fruit). The results presented in this research allow us to know the amount of melatonin and serotonin bring to the diet.

Keywords Melatonin · Serotonin · *Prunus avium* L. · LC-MS · Antioxidant

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Introduction

The indolamine melatonin (MLT) (*N*-acetyl-5-methoxytryptamine) is an endogenous hormone found to be present in all vertebrates [1]. MLT (Fig. 1) is synthesized from tryptophan via 5-hydroxytryptophan, serotonin and *N*-acetylserotonin in vertebrate pineal gland. MLT has been shown to possess a great number of healthy benefits. The most well known function of MLT in mammals is the regulation of the sleep–wake cycle [2]. Its other functions in humans range from sexual maturation [3] to depression [4] and antioxidative defense [5]. Beside these properties, MLT was reported to be a potent free radical scavenger and a broad-spectrum antioxidant [6]. In addition, MLT detoxifies a variety of free radicals and reactive oxygen intermediates, including the hydroxyl radical, the peroxynitrite anion, singlet oxygen and nitric oxide [6]. Hattori et al. [7] demonstrated that MLT consumed in plant products is absorbed, enters the circulation, and could have physiological effects via receptor- or non-receptor-mediated processes.

The presence of MLT in plants and fruits has been previously described due to the current interest to know the natural sources of MLT in human diets. High MLT levels were found in medicinal plants such as feverfew, among other Chinese medicinal herbs, in concentrations of 10 ng per gram dry mass [8]. The presence of MLT in different plants such as tomatoes, cucumbers, sunflowers seeds, banana, pineapple among others [9–11] was previously studied. Burkhardt et al. [12] have detected and quantified the amount of melatonin in 'Montmorency' and 'Balaton' tart cherries. The reported concentrations were in the ng/g FW levels.

Serotonin (SERO) (5-hydroxytryptamine) is a heterocyclic amine that was first isolated by Ersparmer and Vialli in

1937. SERO (Fig. 1) is a principal intermediate in MLT biosynthesis. SERO plays an important role as neurotransmitter, and also controls mood behaviors and body temperature. Quantitative amounts of SERO were found in edible fruits, vegetables and nuts [13]. The amount of SERO in these natural products has not been perfectly established due to the great variability of this amine according to the fruit variety and its degree of ripeness during the harvest period [14].

The consumption of cherries and its products was reported to be health promoting, particularly to alleviate arthritic pain and gout, and to reduce the incidence of cancer [15].

Sweet cherries for fresh consumption are an important crop in Spain that has expanded rapidly over the past decades. The Valle del Jerte area (7,500 ha.) is a deep and narrow valley and cherries are cultivated mainly on terraces at different altitudes between 700 and 1,200 m. The combination of different cultivars planted at different elevations results in a large harvest window. A significant percentage of sweet cherries grown in this area belong to a group of late maturing cultivars collectively called "Picotas" like 'Ambrunés', 'Pico Colorado', 'Pico Limón Negro' or 'Pico Negro' harvested until mid July. In fact, those cultivars represent one of the latest ripening sweet cherry cultivated worldwide [16]. Other important characteristic of Valle del Jerte sweet cherries is the taste that combines high sugar content with a slight acidity, a firm pulp and the release of the peduncle at harvest. Some of the autochthonous cherry cultivars grown in this area are considered as Protected Designation of Origin (PDO) sweet cherries.

Many chromatographic and non-chromatographic techniques have been developed and improved for MLT and SERO determination and measurement. At present, gas chromatography with mass spectrometry and reversed-phase high-performance liquid chromatography with fluorescence or electrochemical detection are widely used for MLT and SERO determination [12, 17, 18]. In addition, it has been reported radioimmunoassay as a detector for MLT in edible plant samples [10].

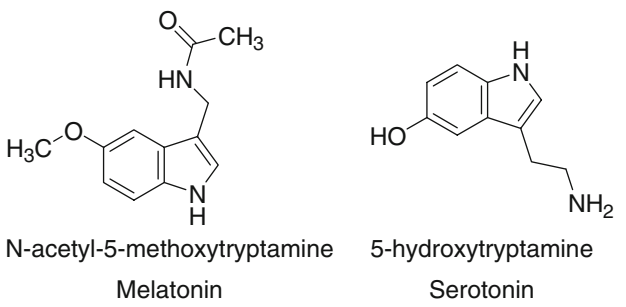

Fig. 1 Chemical structure for *N*-acetyl-5-methoxytryptamine (melatonin) and 5-hydroxytryptamine (serotonin)

The amounts of MLT and SERO were estimated for the first time in eight different sweet cherry cultivars. The distribution of MLT and SERO in these sweet cherry cultivars according to their ripeness stage was also evaluated. Five of the studied cultivars belong to the Valle del Jerte PDO. For this purpose, a fast and accurate analyte extraction procedure has been optimized increasing the literature recovery rates [12].

Materials and methods

Apparatus

Ultra pure water was provided by MillQ-Millipore system. For sample pretreatment, an Omni-Mixer homogenizer (Omni International, GA, USA) and a Beckman Coulter centrifuge (Beckman-Coulter Inc, CA, USA) were used. Fresh cherries were lyophilized using a Virtis-Genessis freeze dryer (SP Industries Inc, PA, USA). The chromatographic separations were carried out on an Agilent 1100 series instrument equipped with a MS-ESI-quadrupole detector. The mass spectrometer parameters were a nitrogen flow of 12 mL/min as drying gas at 350 °C. The capillary voltage optimization was performed by direct infusion of a standard solution of MLT and SERO. The optimized voltage allowed us to monitor the precursor ion of MLT and precursor ion as well as its major fragment ion for SERO, and it was set to 70 V. Chromatographic data processing was done using Agilent ChemSation software.

Chemicals

All the chemicals but standards were purchased from Panreac-Quimicas SA, Spain. The chromatographic grade solvents were previously filtered using a 0.45 μm nylon filter. Stock solutions of MLT (100 μg/mL) and SERO (100 μg/mL) were prepared by dissolving the standards (Sigma-Aldrich Spain) in ultra pure water (milli-Q quality). Working solutions were prepared daily by diluting the necessary amount of the stock solution in the running buffer. Stock and working solutions were stored at 4 °C avoiding direct light exposure, to prevent degradation.

Sample selection and storing

For this research, eight sweet cherry cultivars were studied: 'Burlat', 'Navalinda', 'Van', 'Pico Limón Negro', 'Sweetheart', 'Pico Negro', 'Ambrunés' and 'Pico Colorado'. The selection of the sweet cherry cultivars was made according to their harvesting time. 'Burlat' is one of the earliest harvested cultivar (around middle May). The other cultivars were harvested from May to July following the sequence

'Navalinda' (6 days after 'Burlat'), 'Van' (18 days after 'Burlat'), 'Pico Limón Negro' (31 days after 'Burlat'), 'Sweetheart' (33 days after 'Burlat'), 'Pico Negro' and 'Ambrunés' (37 days after 'Burlat') and 'Pico Colorado' (44 days after 'Burlat'). 'Burlat' and the last four cultivars represent the highest incomes for the local farmers since these cultivars are the first and the last fruits to be available for groceries markets. The cultivars 'Pico Colorado', 'Pico Negro', 'Pico Limón Negro', 'Navalinda' and 'Ambrunés' belong to Valle del Jerte (Extremadura, Spain) PDO sweet cherries.

All the sweet cherry cultivars used in this research were obtained from Valle del Jerte local farmers (Extremadura, Spain). For each cherry cultivar, around 10 kg were harvested in the same day from a group of trees at commercial maturity. All the analyses were done in triplicate. According to their color, cherries were classified in three ripening stages. The commercial mature stage was established for those cherries showing the most intense color. The samples were frozen and vacuum packed using plastic bags and stored at −20 °C for further analysis. For MLT and SERO studies, samples were defrosted, keeping the sample at room temperature for 30 min. Sweet cherries were pitted using a commercial kitchen tool and a homogenate was prepared with an Omni-Mixer. The homogenates were lyophilized and the samples were stored in vacuum dark containers.

Melatonin and serotonin fruit extraction

In order to extract melatonin and serotonin from fruit, 10 mL of phosphate buffer (pH 8.0) was added to 2.0 g of lyophilized fruit (2.0 g of lyophilized fruit correspond to 10.0 g of pitted fresh fruit). Following the sample homogenization, it was centrifuged for 10 min at 4 °C at 1,000 rpm. The sample was filtered and rinsed with 5 mL of phosphate buffer (0.05 M, pH 8.0). 3 mL of chloroform and 300 μL of KOH (0.1 M) were added to the aqueous phase and the mixture was shaken for 5 min. After that, the organic phase was separated and evaporated using gas nitrogen steam. 200 μL of formic acid (0.45%) solution was used for sample reconstitution and it was injected in the HPLC system. During the extraction procedure, light must be avoided since MLT and SERO are highly light sensitive and extracted samples have to be analyzed as soon as the extraction is completed.

Chromatographic conditions for melatonin and serotonin separation

The separation of MLT and SERO was accomplished with an Xterra C_{18} HPLC column (100 mm × 2.1 mm, 3.5 μm; Waters) heated at 30 °C. The mobile phase used for the separation was formic acid (0.45%) and acetonitrile in gradient mode. From minute 0 to minute 5 an isocratic elution was applied (95% formic acid, 0.45% and 5% of acetonitrile). Following the SERO elution, a linear gradient was used for MLT elution, from minute 5 to minute 8 the acetonitrile portion of mobile phase went from 5 to 100%, and it was kept in this proportion until minute 16. The separation was followed by 15 min re-equilibration period. The injected volume was 50 μL.

Once the peaks were characterized, the determination of melatonin and serotonin was performed by SIM of its generated ions (m/z 233 for MLT and m/z 177 for SERO). Quantitative calculations were conducted with the peak areas of MLT and SER. ACOC software was used for calibration and statistics calculations [19]. Agilent ChemStation software was used for data acquisition and peak areas integration.

Statistical analysis

Data are expressed as mean values. The error associated to each measure was listed as relative standard deviation (RSD). Data were analyzed using a one-way analysis of variance ($P < 0.05$). When ANOVA detected significant differences between mean values, mean values were compared using LSD Tukey test ($P < 0.05$). For statistical studies, SPSS 13.0 software was used.

Results and discussion

To evaluate the extraction recovery rate, a set of five standard solutions of MLT and SERO, in a concentration range from 5 to 40 ng/mL, were prepared by dissolving the necessary amount of the stock solution in ultrapure water. The MLT and SERO of each solution were extracted by following the procedure described in Melatonin and Serotonin fruit extraction section. To determine the recovery, the extracted MLT and SERO from standards were compared with the MLT and SERO measured without extraction. The recoveries were 70.7% (±3.2%) for MLT and 72.0% (±2.9%) for SERO ($n = 5$). This extractive procedure allows us to increase the recovery rate of 60% reported by Burckhardt et al. [12]. The critical point to increase the recovery rate was the fixation of the pH value over 9 by adding the appropriate amount of KOH right before chloroform addition.

The chromatographic separation mode proposed allowed us to separate both analytes and matrix components in less than 15 min using a gradient elution. For gradient optimization, spiked cherry solutions were used to take into account the matrix complexity. Elution time for SERO was 1.8 min in the established conditions. Once the SERO is eluted, the

mobile phase polarity was changed by increasing the non-polar solvent proportion (from 5 to 100% of acetonitrile). In order to separate analyte compounds from cherry matrix components, a soft gradient was used. In these conditions, MLT elution was achieved at minute 11.5. Peak identifications were accomplished by comparing retention time of MLT and SERO in a standard solution with the chromatograms obtained from fruit extract. This information was corroborated with co-chromatographic experiments by using spiked cherry homogenates and the MS scan-spectra of the chromatographic peaks. Figure 2 shows the chromatograms of melatonin and serotonin standard solution, fruit extracts and spiked fruit extracts. Once the peaks were characterized, the SIM mode was used as it was explained in the chromatographic conditions section. Mass spectra for MLT and SERO chromatographic peaks for standard solution and 'Pico Colorado' sweet cherry cultivar are represented in Fig. 3.

Calibration curves were established at five concentration levels in triplicate and analytical figures of merit were calculated for MLT and SERO. Results are summarized in Table 1. To take into account the complexity of the studied sample, the LOD according to Long and Winefordner criteria [20] was calculated being 4.3 ng/mL for MLT and 3.0 ng/mL for SERO. According to literature data [12], the method is sensitive enough for MLT and SERO detection and quantification in sweet cherries. Good linearity was observed between MLT and SERO concentration and peak area in the studied interval (from 5 to 40 ng/mL of MLT and SERO). The precision of the method was determined by calculating the intraday repeatability and interday precision (Table 1) expressed as standard deviation of peak areas and retention time. Intraday repeatability was conducted by the injection of three replicates containing 10 ng/mL of MLT and SERO. These experiments were carried out during 3 days to evaluate the method interday precision. No statistical differences were established when the two experiments were compared. The method accuracy was evaluated by spiking sweet cherry homogenate with three different amounts of MLT and SERO and it was expressed as the agreement of nominal and found concentrations. In addition, no statistical differences were found after slope comparison of external standard calibration with standard addition calibration curve, therefore, no matrix effects were observed.

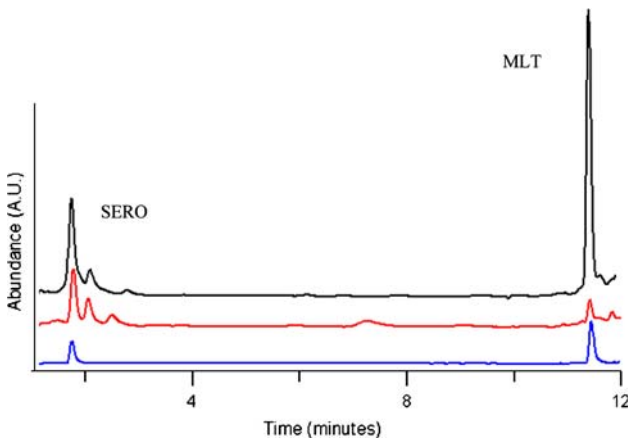

Fig. 2 Chromatograms of SERO (Rt = 1.80 min) and MLT (Rt 11.5 min) (20 ng/mL) standard solution (*blue line*), 'Pico Colorado' sweet cherry cultivar (*red line*) and 'Pico Colorado' sweet cherry cultivar spiked with MLT and SERO (*black line*) (100 ng/mL). All traces are represented in the same scale. Mass spectrometer was used as chromatographic detector

Fresh fruit at commercial maturity, previously defrosted, were treated according to the methodology optimized for this research and three different extractions and analyses were done. The relation between MLT and SERO contents in all the cultivars can be observed in Fig. 4. The correlation between MLT and SERO contents in the sweet cherry cultivars was evaluated by the Pearson correlation coefficient and was significant ($P < 0.05$, $r = -0.619$). This inverse correlation could be due to the MLT biosynthetic route. MLT can be formed via *O*-methylation of SERO and subsequent *N*-acetylation of 5-methoxytryptamine [6]. Dubbels et al. [10] also concluded that the quantity of SERO is related to the amount of MLT, as we report in this research. In addition, Underfriend et al. [21] studied the amounts of SERO and MLT in fruits and plants finding a strong parallelism between the amounts of SERO and MLT.

MLT and SERO concentration values found in the studied sweet cherry cultivars are listed in Table 2 according to harvesting time. The standard deviation is expressed in terms of RSD. According to the ANOVA results, the amounts of MLT and SERO were statistically significantly different ($P < 0.001$). The Tukey's test ($P < 0.05$) groups the cherry cultivars in several categories according to the MLT and SERO contents (represented with different letters in Table 2). 'Burlat' cherries showed more than double amount of MLT than SERO. 'Ambrunés' was the cultivar with the major SERO content and MLT was not detected. No correlations between MLT and SERO contents with harvesting time were found for any sweet cherry cultivar. Thus, the different amounts of MLT and SERO should be a cultivar characteristic. These results are in agreement with those of Burkhardt et al. [12]. They reported that the amount of melatonin in 'Montmorency' and 'Balaton' tart cherries (*Prunus cerasus*) did not vary according to harvesting time. They also reported that 'Montmorency' contents had almost six times MLT than 'Balaton'.

The influence of ripening stage was studied in four sweet cherry cultivars, 'Sweetheart', 'Pico Colorado', 'Pico Negro' and 'Ambrunés'. The higher amounts of MLT were

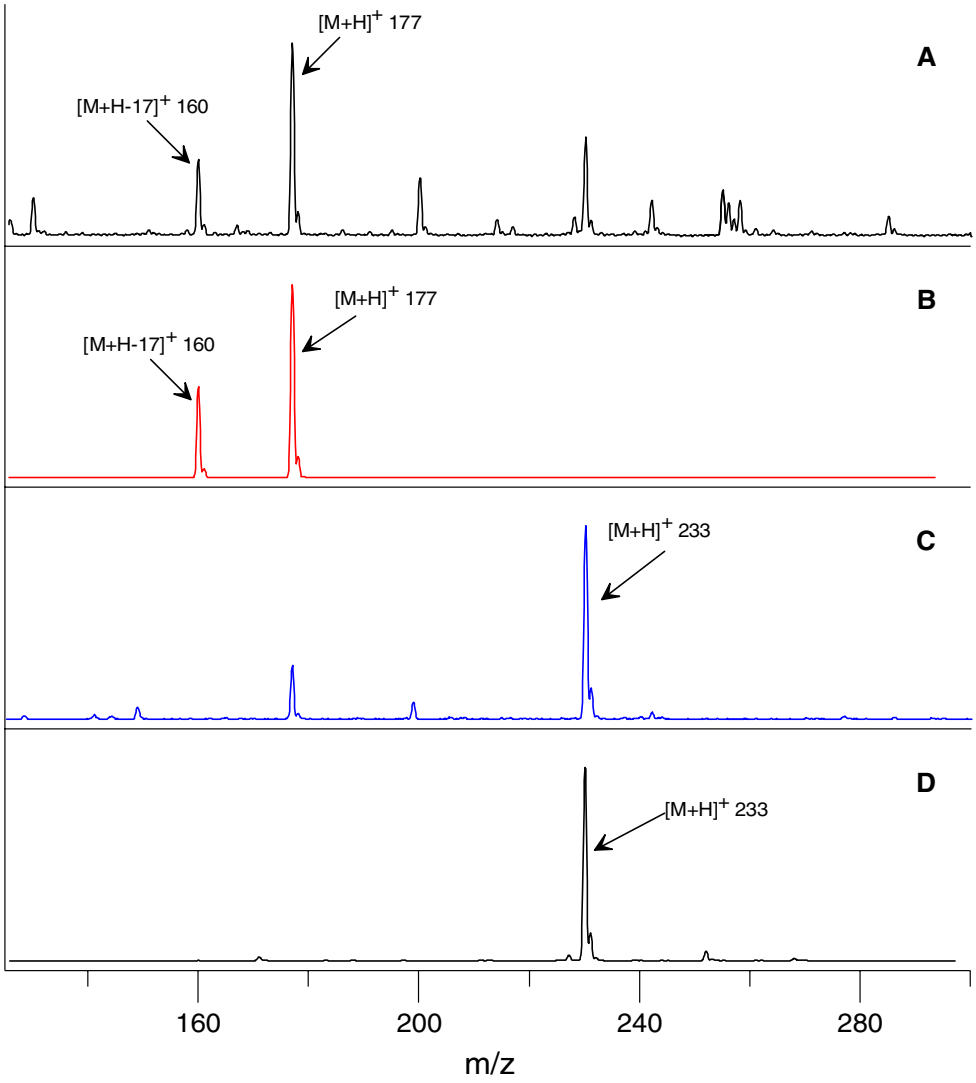

Fig. 3 Mass spectra for serotonin chromatographic peak (Rt 1.80 min) for 'Pico Colorado' sweet cherry and standard solution sample (**a**, **b**), and for melatonin (Rt 11.5 min) for 'Pico Colorado' sweet cherry and standard solution sample (**c**, **d**)

Table 1 Analytical figures of merit for MLT and SERO

	MLT	SERO
Slope ± σ (arbitrary units mL/ng)	$9.73 \times 10^3 \pm 0.59 \times 10^3$	$4.74 \times 10^3 \pm 0.09 \times 10^3$
Intercept ± σ	$13.0 \times 10^3 \pm 3.8 \times 10^3$	$6.36 \times 10^3 \pm 4.75 \times 10^3$
Regression coefficient (R)	0.989	0.998
Linearity (%)	94.0	98.0
Sensitivity (ng/mL)	2.32	2.23
LOD (ng/mL)	4.30	3.00
Intraday assay	Rt 11.522 ± 0.002	Rt 1.829 ± 0.001
	$W_{1/2}$ 0.108 ± 0.001	$W_{1/2}$ 0.119 ± 0.001
Interday assay	Rt 10.531 ± 0.018	Rt 1.830 ± 0.023
	$W_{1/2}$ 0.170 ± 0.011	$W_{1/2}$ 0.120 ± 0.003
Accuracy[a]	0.640 ± 0.010	0.676 ± 0.012

[a] Expressed as the ratio between the found and the average nominal concentration for three different replicates

found in the ripest fruit while no quantifiable or very low amounts of MLT were found for the other ripening stages. For 'Ambrunés', no MLT was found at any stage. No statistical differences were found in the SERO contents in 'Ambrunés' cultivar for the three ripening stages studied. For 'Pico Colorado', 'Pico Negro' and 'Sweetheart'

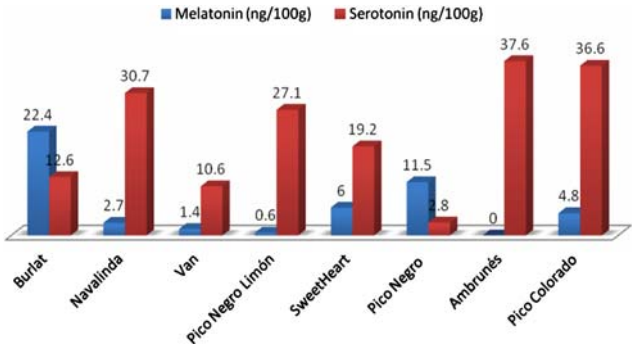

Fig. 4 Distribution of melatonin (MLT) and serotonin (SERO) in Valle del Jerte sweet cherry cultivars. Amounts are expressed by 100 g of fresh weight

Table 2 Concentrations (Mean ± RSD) of MLT and SERO in Valle del Jerte sweet cherries cultivar at commercial maturity

Cultivar	Melatonin (ng/100 g fwt)	RSD (%)	Serotonin (ng/100 g fwt)	RSD (%)
Burlat	22.4d	1.2	12.6bc	1.3
Navalinda	2.7b	2.4	30.7de	0.9
Van	1.4ab	0.9	10.6b	1.1
Pico Limón Negro	0.6a	0.7	27.1d	1.1
Sweetheart	6.0c	2.0	19.2c	1.4
Pico Negro	11.5d	3.3	2.8a	3.7
Ambrunés	0.0a	0.0	37.6e	1.6
Pico Colorado	4.8c	2.2	36.6e	1.8
Significance	***		***	

By columns, numbers followed by different letters are significantly different (Tukey Test, $P < 0.05$)

RSD Relative standard deviation

*** $P < 0.001$

cultivars statistical differences were observed in the SERO content for the three ripening stages, although there was not a clear tendency but a random SERO distribution. This information is summarized in Table 3.

These results will provide value information in order to know the amount of MLT and SERO sweet cherries bring to diet, and to select the appropriate sweet cherry cultivar according to the consumer's needs. The administration of melatonin to humans can change mood performance, sleepiness and sleep onset and it alleviates the symptoms of jet lag [22]. A melatonin dose of 1.0–1.5 ng/g is sufficient to raise daytime blood melatonin levels to high values normally seen at night in humans [23]. In addition, for industrial purposes, it will be important to know the distribution of MLT and SERO for the preparation of natural dietary complements.

Table 3 MLT and SERO distribution in 'Pico Negro', 'Pico Colorado', 'Ambrunés' and 'Sweetheart' cultivars according to fruit ripening stage

Cultivar	RS	Melatonin (ng/100 g)	RSD (%)	Serotonin (ng/100 g)	RSD (%)
Pico Negro	1	–	–	12.4b	1.1
	2	–	–	18.4a	1.3
	3	16.0	2.9	8.5c	2.5
Pico Colorado	1	1.0	0.7	24.2a	4.3
	2	–	–	16.1b	3.5
	3	4.8	2.2	17.6b	5.0
Ambrunés	1	–	–	18.7a	3.7
	2	–	–	22.2a	5.6
	3	–	–	19.1a	7.6
Sweetheart	1	–	–	20.5a	1.5
	2	–	–	10.1b	0.5
	3	6.0	2.0	22.7a	6.4

For the same cultivar, by columns, numbers followed by different letters are significantly different (Tukey Test, $P < 0.05$) (Significance $P < 0.001$)

RSD Relative standard deviation, *RS* Ripening stage, 1 unripe, 2 intermediate ripe, 3 ripe

Conclusions

For the first time, MLT and SERO have been simultaneously determined and quantified in different sweet cherries cultivars (*Prunus avium* L.). The optimized extractive procedure allowed us to quantitatively recover MLT and SERO from cherries. A sensitive and reproducible chromatographic method has been established for MLT and SERO determination and quantification in a reasonable analysis time. This method has been applied to study the MLT and SERO distribution in eight different sweet cherry cultivars from Valle del Jerte Protected Origin Designation area, showing statistical differences in the MLT and SERO concentrations. The MLT and SERO contents of different sweet cherry cultivars have been evaluated. The highest MLT and SERO concentrations were found for 'Burlat' (22.4 ng MLT/100 g FW) and 'Ambrunés' (37.6 ng SERO/100 g FW), respectively. The other studied cultivars showed intermediate values. Based on the current research, sweet cherries, which contain substantial amounts of MLT and SERO, may have a great number of health benefits and would be important to incorporate in a healthy diet.

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