Extraction and quantification of melatonin in cornelian cherry (Cornus mas L.) by ultra-fast liquid chromatography coupled to fluorescence detector (UFLC-FD)

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ABSTRACT

Wild edible plants (WEPs) can be widely found in the world and defined as native species that grow naturally in their natural habitat. They have become part of the traditional food as human diet and used in folk medicine to treat diseases. They are very rich in terms of nutraceuticals. Melatonin is a natural hormone providing several benefits for human health. It has functions such as regulating growth and development and increasing tolerance to environmental stress factors in plants. It is stated that the serum melatonin level in humans increases after intake of foods containing melatonin. This study examined the presence of melatonin in wild grown cornelian cherry fruits by UFLC-FD and determined suitable extraction and chromatographic conditions. The optimum mobile phase, excitation/emission wavelength, and extraction solvent were determined as methanol: water: acetic acid, 275/345 nm, and methanol: water: HCl, respectively. Melatonin content in fruits ranged from 130.82 to 201.84 ng g\(^{-1}\) in fresh fruit.

KEYWORDS

Cornus mas L, extraction, melatonin, ultra-fast liquid chromatography-fluorescence detector

INTRODUCTION

Melatonin (N-acetyl-5-methoxytryptamine) is an endogenous indoleamine hormone structurally related to some substances such as tryptophan, serotonin, and indole-3-acetic acid. In mammals, melatonin is a biological regulator of various processes such as mood, hormonal regulation, circadian rhythms, sleep, immune system responses and sexual behaviour [1]. It also is a potent antioxidant scavenging free radical species and stimulating antioxidant enzymes [2]. This component discovered in plants in 1995 has a physiological role in processes such as flowering, photoperiodicity, and growth [3]. In studies investigating the biological effects of melatonin, its therapeutic effects and health benefits have been extensively studied. It has been emphasized that it has many bioactivities such as antioxidant activity, anti-inflammatory effect, boosting immunity, anticancer activity, cardiovascular protection, anti-diabetic effect, neuroprotective and anti-aging activity [4–7]. It has been stated that the consumption of foods containing melatonin increases the serum melatonin level and antioxidant capacity in humans [8].

Melatonin has been determined qualitatively and quantitatively in animal foods and edible plants in recent years. Researchers have detected melatonin in many fruits and vegetables such as wolfberry (Lycium barbarum L.), blackberry (Rubus fructicosus L.), black mulberry (Morus nigra), white mulberry (Morus alba), radish (Raphanus sativus L.), jujube (Ziziphus jujuba), clove (Syzygium aromaticum L.), tomato (Solanum lycopersicum), pepper (Capsicum annuum), tart cherry (Prunus cerasus), and sweet cherry (Prunus avium) [9–13].
The consumption of wild edible plants is an ancient phenomenon that predates agriculture [14]. These plants offer various benefits and opportunities to communities; for example, they enable communities to cope with food scarcity [15, 16]. In addition, wild edible plants increase the nutritional quality of rural diets for instance, micronutrients (vitamins and minerals) which are sometimes superior to those of domesticated varieties. Some of them also contain genes that can be sought to improve the productivity of cultivars [17, 18].

Consumption of natural products by people is increasing to help the treatment of diseases [19]. Cornelian cherry is a tree plant growing naturally. It belongs to the Cornaceae family, which includes approximately fifty-five species. Among Cornus species, Cornus mas L. are known as the European and Asiatic cornelian cherry and are grown for their edible fruits. Wild grown cornelian cherry trees are abundant in rural areas in mostly Balkan and east European countries and their fruits are generally consumed freshly or processed into jam, marmalade, syrup, jelly, compote, dried layers of fruit pulp, and juice. Besides, its fruit, leaf, root is used in folk medicine for centuries as an antipyretic, diarrhea preventive, and removing kidney stone. Cornelian cherry fruits have a rich content of anthocyanin, flavonoid, and phenolic compounds. Many studies have revealed the antiseptic, antioxidant, antidiabetic, and antimicrobial properties of the cornelian cherry [20–28]. The studies to reveal the therapeutic properties of plants are increasing, and cornelian cherry attracts attention from this aspect as well.

Studies investigating melatonin content in Cornus species are limited with the study of Chen et al. [10] in Cornus officinalis. No finding of melatonin content in the C. mas species was found in the literature. Since plants contain trace levels of melatonin, there are difficulties in their accurate measurement. Factors such as extraction technique, solvent selection, and analytical detection method affect the determination of melatonin in foods. The present study aimed to establish suitable solvent and time parameters for melatonin extraction from wild grown cornelian cherry (C. mas L.) fruit. In the study performed using UFLC-FD, optimum excitation and emission wavelengths were determined before extraction, and two different mobile phases were tested.

EXPERIMENTAL

Reagents and standards

The solvents ethanol, methanol, acetic acid, and acetonitrile were obtained from Merck (Darmstadt, Germany) and were of HPLC analytical grade. Melatonin (pure reagent) from Merck (Darmstadt, Germany) were purchased. Sodium acetate (≥99% purity) was obtained from the same supplier. Ultrapure water was prepared by a Millipore Direct-Q 3 UV-R water purification system (Molsheim, France).

Sampling

In the study, fruits of four different wild cornelian cherry (C. mas L.) genotypes grown in Malatya ecology in Turkey were used as material. The fruit samples were stored at −20 °C until analysis.

Extraction

Melatonin extraction from fruit samples removed kernel and homogenized was carried out in ultrasonic bath at room temperature. In the study, six different solvent/solvent mixtures [ethanol, methanol, methanol: water: HCl (70:29.9:0.1 v/v/v), ethanol: water: HCl (70:29.9:0.1 v/v/v), methanol: water (50:50 v/v), and ethanol: water (50:50 v/v)] and three different times (10, 20, and 30 min) were tested for extraction, and the most suitable extraction solvent/solvent mixture and time were determined. For extraction, 1 g of fruit sample was weighed into a capped test tube, and 10 mL of solvent/solvent mixture was added to it. After the extraction procedure, the mixture was centrifuged at 4,500 rpm for 4 min, and the supernatant was filtered through a 0.45 μm filter. An aliquot (20 μL) of the filtrate was used for injection into the UFLC-FD system.

UFLC-FD conditions

The analyses were performed using an ultra-fast liquid chromatography (Shimadzu Technologies, Kyoto, Japan), equipped with a DGU-20A5 model vacuum degasser, 20 ADXR solvent pump, and RF-20A fluorescence detector (FD). Separations were performed using a Welch Welchrom C18 5 μm reversed-phase column (250 × 4.6 mm). Isocratic elution was performed with 1 mL min−1 flow rate at 25 °C, and the injection volume was 20 μL. Two different mobile phases [methanol: water: acetic acid (55:44.9:0.1 v/v/v) and 75 mM sodium acetate-acetonitrile (72:28 v/v)] and six different excitation/emission wavelengths (275/345, 280/350, 280/384, 280/310, 245/380, and 270/340) were tested, and the most suitable mobile phase and excitation/emission wavelengths were determined.

The method was validated with parameters the limit of detection (LOD), the limit of quantitation (LOQ), linearity, precision, and accuracy. LOD and LOQ were calculated according to following equations:

\[
\text{LOD} = X + 3SD \\
\text{LOQ} = X + 10SD
\]

where X is the mean concentration of the blank and SD is standart deviation of the blank. Linearity was performed by preparing melatonin standard solutions at various concentrations (0.5, 1, 10, 100, 200, 300, 400, and 500 ng mL−1) and injecting in triplicate into the UFLC-FD system. The calibration curve was plotted with the peak area against melatonin concentration and correlation coefficient (R²) calculated. The precision of method was verified with parameters of repeatability and reproducibility. For repeatability, it was analysed the sample spiked with 100 ng mL−1.
melatonin standard, and the relative standard deviation (RSD) was calculated. Reproducibility was determined analysing the same sample for three days, and RSD was calculated. For the accuracy of the method, recovery studies were performed by analysing the spiked samples at the 100 and 200 ng mL$^{-1}$ concentration levels.

**Statistical analysis**

Analyses were performed with three replicates. All data were subjected to variance analyses. Significant differences among applications were determined according to the least significant difference (LSD) multiple comparison test at $P < 0.05$.

**RESULTS AND DISCUSSION**

Liquid chromatography technique has been used widely for melatonin analysis. AnFD is sensitive and versatile to quantify melatonin in samples that contain low melatonin levels, and it has low limits of detection and quantification [4, 29–31]. In the literature review, it is seen that different excitation/emission wavelengths such as 285/345 [1], 280/350 [2], 280/348 [10], 280/310 [30], 290/330 [21], and 275/345 [32] are used in many studies. In the present study, the six different excitation/emission wavelengths included these wavelengths were tested, and the results are shown in Fig. 1. In the measurements performed with the 75 ng mL$^{-1}$ melatonin standard solution, the peaks with the highest peak area were obtained statistically at 275/345 (A) and 270/340 (B) nm (Fig. 1). 275/345 (A) nm was chosen as the optimal excitation/emission wavelengths. These excitation/emission wavelengths were used in the later studies.

In this study, two kinds of mobile phases [methanol: water: acetic acid (55:44.9:0.1 v/v/v) and 75 mM sodium acetate-acetonitrile (72:28 v/v/v)] were tested to the optimal elution. The successful separation of melatonin peak was acquired using methanol: water: acetic acid (55:44.9:0.1 v/v/v) mixture as mobile phase (Fig. 2A). In many studies, solvent/solvent mixtures containing buffer or acid besides solvents such as methanol, water, and acetonitrile were used in the elution of melatonin in liquid chromatography [1, 2, 4, 5, 13, 31, 32]. Making the mobile phase choice depends on the characteristics of the analyte and the column (stationary phase) [33]. As seen in Fig. 2A, a sharper and higher peak was acquired in the elution with a mixture of methanol: water: acetic acid (55:44.9:0.1 v/v/v) in the C18 reversed-phase column used in the study.

Many conditions such as extraction solvent and extraction time can significantly affect the extraction of melatonin from cornelian cherry fruits. The extraction of target compounds from plant samples is dependent on the polar properties and diffusivity of the solvent employed. The solvent polarity affects the extraction depending on the polar properties of the compound that will be extracted [31]. Extraction procedure was performed with 6 extraction solvents/solvent mixtures: ethanol, methanol, methanol: water: HCl (70:29.9:0.1 v/v/v), ethanol: water: HCl (70:29.9:0.1 v/v/v), methanol: water (50:50 v/v), and ethanol: water (50:50 v/v) to assess their effectiveness in melatonin extraction from cornelian cherry fruits. The effect on melatonin extraction of these solvents/solvent mixtures is presented in Fig. 3. The results showed that the methanol: water: HCl solvent mixture as the extraction solvent was more efficient than other solvents/solvent mixtures (Fig. 3). Escriva et al. [6] employed methanol, ethanol, methanol: water solvent/solvent mixtures for melatonin extraction in spiked aphid samples and obtained the highest recovery in the methanol: water solvent mixture. Lazar et al. [34] and Murc et al. [35] employed a solvent mixture of methanol: water: formic acid (80:20:1 v/v/v) for melatonin extraction in Chara cells and grapes.

In a study examining the effects of pH, temperature, and light on the stability of melatonin, the melatonin standard was stored at different pHs (1, 4, 7, 10, and 13), and the degradation of melatonin was investigated. The pH values of the solutions were adjusted by 0.1 M HCl and 0.1 M NaOH. As a result, while the degradation of melatonin was 34% at

![Fig. 1. Results of excitation/emission wavelengths for 75 ng mL$^{-1}$ melatonin standard solution tested in UFLC-FD Conditions: mobile phase methanol: water: acetic acid (55:44.9:0.1 v/v/v); flow rate 1 mL min$^{-1}$; injection volume 20 μL; column temperature 25 °C. Values are mean ± SD ($n = 3$). Means followed by a different letter are significantly different at an alpha level of 0.05 according to an LSD test. ($P < 0.05$)](image_url)
Fig. 2. UFLC chromatograms of mobile phases (A and B) for 75 ng mL\(^{-1}\) melatonin standard solution, nonspiked sample (C), sample spiked with 100 ng mL\(^{-1}\) melatonin (D), and 200 ng mL\(^{-1}\) melatonin standard solutions (E). Conditions for chromatogram A: mobile phase methanol: water: acetic acid (55:44.9:0.1 v/v/v); flow rate 1 mL min\(^{-1}\); injection volume 20 μL; column temperature 25 °C. Conditions for chromatogram B: mobile phase 75 mM sodium acetate-acetonitrile (72:28 v/v); flow rate 1 mL min\(^{-1}\); injection volume 20 μL; column temperature 25 °C. Conditions for chromatograms C, D, and E: mobile phase methanol: water: acetic acid (55:44.9:0.1 v/v/v); flow rate 1 mL min\(^{-1}\); injection volume 20 μL; column temperature 25 °C.
pH 1, degradation of over 95% occurred at other pH values [36]. To minimize melatonin degradation during the extraction, HCl was added to the solvent mixtures in the present study. As melatonin has both hydrophilic and lipophilic properties, it needs a solvent mixture with a wide range of polarities rather than a single solvent for extraction. Therefore, methanol: water: HCl solvent mixture was selected as an appropriate solvent for extracting melatonin from cornelian cherry fruits.

One factor that influenced melatonin extraction from cornelian cherry fruits is also extraction time. 10, 20, and 30 min were tested to determine optimal extraction time. It can be seen from Fig. 4 that as the extraction time increased, the amount of melatonin extracted increased and reached a maximum at 20 min but decreased after 20 min. Exposure to the extraction solvent during a certain time results in a higher extraction rate [37]. However, increasing extraction times might also promote the degradation of extracted melatonin. In previous studies, times such as 10 [12], 15 [35], 20 [1, 38], 30 [10], and 40 [5] minutes were applied for melatonin extraction. Oladi et al. [39] evaluated the effect of time on melatonin extraction from pistacia seeds in the range of 5–50 min. They stated that the optimum extraction time was 20 min, which is consistent with the result of the present study.

Finally, the optimum excitation/emission wavelength, mobile phase, extraction solvent, and extraction time were determined as 275/345 nm, methanol: water: acetic acid (55:44.9:0.1, v/v/v), methanol: water: HCl (70:29.9:0.1 v/v/v), and 20 min, respectively. Under these conditions, fruits of four different cornelian cherry (C. mas L.) genotypes were extracted, and the melatonin content was measured by UFLC-FD.

Analytical characteristics (retention time, LOD, LOQ, linear range, and $R^2$) of the extraction and chromatographic procedures are presented in Table 1. The precision and
accuracy results are reported in Table 2. In the precision studies performed on the same day (repeatability) and the different days (reproducibility), RSD was 1.09% for repeatability and 1.83% for reproducibility. For accuracy, recovery was calculated by comparing the measured values to the theoretical concentrations. In the recovery studies performed at two different concentrations, recovery and RSD were 90.24% and 1.98% for 100 ng g\(^{-1}\) \(C_0\), 95.16% and 2.13% for 200 ng g\(^{-1}\) \(C_0\), respectively. To quantify fruit melatonin content, the melatonin standard curve was established using the UFLC-FD within the range of 0.5\(–\)500 ng mL\(^{-1}\) (Fig. 5).

Table 1. Some analytical characteristics of melatonin analysis by UFLC-FD detection

<table>
<thead>
<tr>
<th>Compound</th>
<th>Retention time (min)</th>
<th>LOD (ng mL(^{-1}))</th>
<th>LOQ (ng mL(^{-1}))</th>
<th>Linear range (ng mL(^{-1}))</th>
<th>(R^2)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Melatonin</td>
<td>5.383</td>
<td>1.64</td>
<td>2.32</td>
<td>0.5–500</td>
<td>0.9999</td>
</tr>
</tbody>
</table>

Table 2. Precision and accuracy studies of melatonin

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Means</th>
<th>RSD (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Repeatability ((n = 6))</td>
<td>92.85</td>
<td>1.09</td>
</tr>
<tr>
<td>Reproducibility ((n = 6))</td>
<td>91.10</td>
<td>1.83</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Amount added (ng g(^{-1}))</th>
<th>Recovery (%)</th>
<th>RSD (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>100</td>
<td>90.24</td>
<td>1.98</td>
</tr>
<tr>
<td>200</td>
<td>95.16</td>
<td>2.13</td>
</tr>
</tbody>
</table>

Melatonin concentration was calculated based on the UFLC peak areas. The melatonin content in cornelian cherry fruits was presented in Table 3.

The presence of melatonin in *Cornus officinalis* species was reported by Chen et al. [10]. In the present work, the presence of melatonin in *C. mas* species was reported for the first time. Chen et al. found as 821 ng g\(^{-1}\) dry herb the melatonin content of *C. officinalis*. In previous studies, it was reported that melatonin content in sweet cherry (*P. cerasus*) and sour cherry (*P. avium*) species ranged from 2.06 to 13.46 ng g\(^{-1}\) [40, 41] and 0.01–20 ng g\(^{-1}\) [13, 40–42] in fresh fruit, respectively. In other studies, it was stated that melatonin content of fruit kinds such as pineapple (*Ananas comosus*), kiwi (*Actinidia chinensis*), blackberry (*Rubus fruticosus*), strawberry (*Fragaria ananassa*), apple (*Malus domestica*), and mulberry (*M. alba*) was 0.28 [43], 0.02 [44], 233.86 [9], 11.26 [45], 5.00 [46], and 183.29 ng g\(^{-1}\) [9] in fresh fruit, respectively. There are differences in melatonin concentrations of fruit kinds, even *Cornus* species. The ecological factors such as climate and growth conditions may be the reasons for the variety in melatonin concentration in plants. Apart from these factors, the extraction and analytical methods employed also play an important role in the difference in melatonin levels.

Extraction techniques such as solid-liquid extraction [1, 6, 11, 13], solid-phase extraction [2, 5, 10], pressurized liquid extraction [8], and extraction using a commercial special kit [47] are employed for melatonin extraction from various samples. These techniques mainly included extract evaporation under a nitrogen stream or vacuum, and the residue was resuspended final steps. In the present study, melatonin

![Calibration curve of melatonin](https://example.com/calibration_curve.png)

Table 3. Melatonin content of cornelian cherry fruits (ng g\(^{-1}\) fresh fruit)

<table>
<thead>
<tr>
<th>Genotypes</th>
<th>Compound</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Melatonin(^\ast)</td>
<td>198.57±11.48(^a)</td>
<td>130.82±4.06(^b)</td>
<td>173.35±2.62(^b)</td>
<td>201.84±4.97(^a)</td>
</tr>
</tbody>
</table>

\(^\ast\)Values are mean ± SD \((n = 3)\). Means followed by a different letter are significantly different at an alpha level of 0.05 according to an LSD test. \((P < 0.05)\).
extraction was performed successfully in a solvent mixture without passing a solid-phase extraction cartridge and the need for extract evaporation.

CONCLUSIONS

For the first time, melatonin was extracted in C. mas species and determined by the UFLC-FD system. It was determined suitable solvent (methanol: water: HCl) and time (20 min) parameters for melatonin extraction from cornelian cherry (C. mas L.) fruit. It was designated the optimal excitation/emission wavelengths (275/345 nm) and a mobile phase (methanol: water: acetic acid) for melatonin quantification in the UFLC-FD system. Results demonstrated that cornelian cherry fruits contain high amounts of melatonin by comparison to many fruits such as sour cherry, sweet cherry, pineapple, kiwi, strawberry, apple, and mulberry. Studies have shown that melatonin has many bioactivities. Therefore, the consumption of foods containing melatonin may provide health benefits. More trials are needed to reveal the effects of food containing melatonin on human health.

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