Detection and Quantification of the Antioxidant Melatonin in Montmorency and Balaton Tart Cherries (*Prunus cerasus*)

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The antioxidant melatonin was recently identified in a variety of edible plants and seeds in high concentrations. In plants, as in animals, melatonin is believed to function as a free radical scavenger and possibly in photoperiodism. In this study, melatonin was detected and quantified in fresh-frozen Balaton and Montmorency tart cherries (*Prunus cerasus*) using high-performance liquid chromatography. Both cherry species contain high levels of melatonin compared to the melatonin concentrations in the blood of mammals. Montmorency cherries (13.46 ± 1.10 ng/g) contain ~6 times more melatonin than do Balaton cherries (2.06 ± 0.17 ng/g). Neither the orchard of origin nor the time of harvest influenced the amount of melatonin in fresh cherries. The implication of the current findings is that consuming cherries could be an important source of dietary melatonin inasmuch as melatonin is readily absorbed when taken orally. Also, previously published data and the results presented here show that melatonin is not only endogenously produced but also present in the diet.

Keywords: *Melatonin; fruit; Prunus cerasus; Balaton cherry; Montmorency cherry; antioxidant*

INTRODUCTION

Epidemiological studies indicate that constituents in fruits and vegetables are protective against a variety of diseases (*1*−*4*). The principal nutrients thought to provide the protection afforded by fruits and vegetables are antioxidants such as vitamin C, vitamin E, β-carotene, and flavonoids (including flavones, isoflavones, and anthocyanins).

Anecdotally, 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. However, there is little evidence indicating what compounds may be responsible for these alleged actions. Recently, reports have shown that the anthocyanins and cyanidin isolated from tart cherries exhibit in vitro antioxidative and anti-inflammatory activities (*5*, *6*). It was found that Montmorency tart cherries contain ~6 times more anthocyanins than do Balaton cherries (*7*). Although several polyphenolic compounds have been identified in tart cherries, the presence of other antioxidants in Balaton and Montmorency tart cherries has not been investigated (*8*−*10*).

Recently, melatonin (*N*-acetyl-5-methoxytryptamine), a secretory product of the vertebrate pineal gland (*11*), was reported to be a potent free radical scavenger and a broad-spectrum antioxidant (*12*, *13*). Free radicals, which are detoxified by antioxidants, are implicated in a number of pathophysiological processes including aging, inflammation, reoxygenation injury of ischemic tissues, atherosclerosis, and cancer. Melatonin detoxifies a variety of free radicals and reactive oxygen intermediates including the hydroxyl radical ($^{\bullet}OH$), the peroxynitrite anion ($ONOO^-$), singlet oxygen ($^1O_2$), and nitric oxide ($NO^{\bullet}$) (*12*, *13*). Melatonin possesses both hydrophilic and lipophilic characteristics (*14*, *15*), easily penetrates biological membranes, and enters all subcellular compartments. In many well-documented studies in vertebrates, melatonin reduced oxidative damage to macromolecules including lipids, proteins, and DNA (*16*). Besides production in the vertebrate pineal, melatonin has also been found in bacteria (*17*), protists (*18*), and plants (*19*). Relatively few plants have been examined for the quantity of melatonin they contain (*20*). Preliminary data indicate that the indolamine is present in plants; however, its concentration varies greatly among species and in different parts of a given plant. In some plants, especially in flowers and seeds (the reproductive organs that are most vulnerable to oxidative insults), melatonin concentrations are several orders of magnitude higher than those normally measured in vertebrate tissues (except for the pineal gland) (*21*). In plants, melatonin is believed to function, as in vertebrates, as a free radical scavenger and possibly in photoperiodism (*21*).

The consumption of plant materials that contain sufficiently high levels of melatonin could alter blood levels of the indole and provide protection against oxidative damage. The endogenous production of melatonin as well as its consumption could be important in limiting oxidative damage. Because the consumption of cherries is alleged to be associated with antioxidant and/or anti-inflammatory effects, the presence of melatonin in cherries was tested. The only other fruits examined to date, that is, banana and pineapple, were found to have low levels of melatonin (*21*, *22*). This paper describes the isolation and quantification of the antioxidant melatonin in tart cherries.

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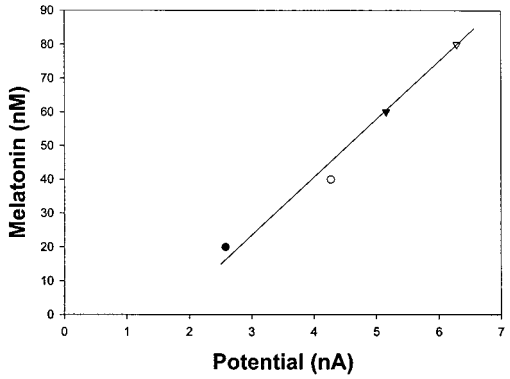

Figure 1. Melatonin dose−response curve. The melatonin concentrations used to obtain this curve ranged from 20 to 80 nM.

MATERIALS AND METHODS

Chemicals. Melatonin was a gift from Helsinn Chemical Co. (Biasca, Switzerland). All other chemicals were purchased from Sigma (St. Louis, MO).

Cherry Fruits. Montmorency and Balaton tart cherries (*Prunus cerasus* L. Rosaceae) were obtained from commercial growers (Agriculture Experimental Station, Traverse City, MI). The fruits were harvested at different times and degrees of ripeness (July and August) and from two different orchards (orchards 1 and 2), both located in Traverse City, MI (latitude 47° N). The fruits were immediately frozen and stored at −80 °C.

Sample Preparation. The cherry samples (2 g) including rind and meat but excluding the seed were homogenized (in groups of ∼20 cherries) in 1 mL of 0.05 M potassium phosphate buffer (pH 8.0). The homogenates were centrifuged at 4 °C and 3000 rpm for 5 min. Five hundred microliters of the supernatants was mixed with 25 μL of a 1 M KOH solution to increase the pH of the samples; 700 μL of chloroform was added, and the samples were horizontally shaken for 10 min. The water phase was discarded, and the chloroform phase was dried under vacuum. The residues were dissolved in 120 μL of the HPLC mobile phase, and 30 μL was injected into the HPLC-EC system.

Melatonin Dose—Response Curve. With the current HPLC-EC method for melatonin measurement, the ratio of signal to noise was greater than 3, and the least detectable level of melatonin was 20 pg/injection. On the basis of a dose−response curve (Figure 1) the melatonin concentrations were calculated as follows:

$$C = Y^0 - 24.752 + 16.339 \times \text{Amp}/2.4 \qquad (1)$$

(2.4 is a final concentration factor).

Melatonin Recovery Rate. Melatonin was dissolved in a concentration range from 20 to 80 nM in 0.05 M potassium phosphate buffer (pH 8.0). Five hundred microliters of the samples was mixed with 25 μL of a 1 M KOH solution to increase the pH; 700 μL of chloroform was added, and the samples were horizontally shaken for 10 min. The water phase was discarded, and the chloroform phase was evaporated under vacuum. The residues were dissolved in 120 μL of the HPLC mobile phase, and 30 μL was injected into the HPLC-EC system. As a melatonin standard, 30 μL of melatonin solution in the concentration range from 20 to 80 nM was directly (without chloroform extraction) injected into the HPLC-EC system. To determine the recovery of the chloroform extraction method used in this study, melatonin concentrations with extraction were compared with melatonin concentrations measured without extraction (Figure 2).

HPLC Analysis of Melatonin in Cherries. An ESA HPLC system equipped with an eight-channel CoulArray 5600 detector was used; the column used was a YMC-BD (4.6 mm × 250 mm, Partisil 5 μm OD53; Milford, MA), and the eluent (pH 4.5) was 0.1 M potassium phosphate buffer with acetonitrile (20%) at a flow rate of 1 mL/min. Applied potentials were initiated at 200 mV for channel 1 and were increased by 100 mV for each of the other channels, resulting in 900 mV at channel 8. By using the potential differentiation method, any substance that may possess the same retention time as melatonin could still be differentiated from melatonin because of its different oxidative potential. Melatonin peaks were obtained only from channel 8 at a potential of 800 mV.

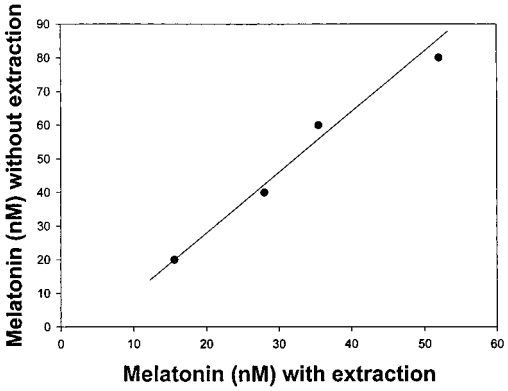

Figure 2. Melatonin recovery curve shows a comparison of known melatonin concentrations in samples before homogenization and after extraction with chloroform; $R = 0.99$. The extraction efficiency was determined to be 60%.

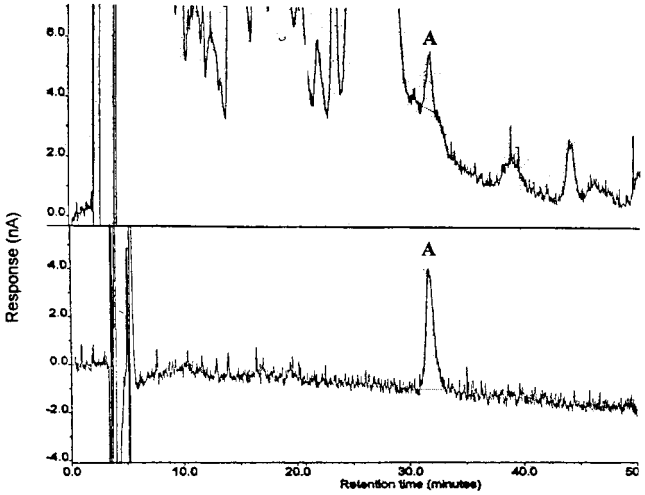

Figure 3. HPLC chromatogram of melatonin from tart cherries and the melatonin standard. The retention time of melatonin in this system was roughly 32.2 min. "A" in the upper panel represents the peak of melatonin in tart cherries, whereas "A" in the lower panel represents the peak of melatonin standard (40 ng).

Statistical Analysis. Data are expressed as means ± SE and were analyzed using a one-way analysis of variance (ANOVA) followed by a Student−Newman−Keuls test. A $p < 0.05$ was considered to be statistically significant.

RESULTS

The melatonin recovery rate, using the presently described protocol for extraction, was in the range of 60%; this represents a comparison of the known melatonin concentration in samples before homogenization and extraction with chloroform (Figures 2 and 3). The resulting curve had an R value equal to 0.99.

On the basis of a dose−response curve (Figure 1) the melatonin concentrations were calculated according to eq 1.

High melatonin concentrations were present in all Balaton and Montmorency tart cherries (Figure 3).

Table 1. Concentrations (Mean ± SEM) of Melatonin in Montmorency and Balaton Tart Cherries (*P. cerasus*) Harvested near Traverse City, MI[a]

	Montmorency	Balaton
melatonin concn (ng/g of tissue)	13.46 ± 1.10	2.06 ± 0.17
time of harvest		
early	13.51 ± 1.11	1.07 ± 0.35
mid	15.43 ± 1.75	2.18 ± 0.26
late	13.96 ± 1.31	2.03 ± 0.29
influence of orchard		
1	12.77 ± 2.09	
2	14.14 ± 0.75	
influence of tree		
1	5.57 ± 0.38	
2	19.59 ± 2.76	
3	12.85 ± 4.04	

[a]Besides comparing the melatonin levels (measured by HPLC with electrochemical detection) in the two tart cherry varieties, the effect of the time of harvest (degree of ripeness) and the effect of the orchard and the tree from which the cherries were derived were examined.

There was, however, a strain difference; Montmorency cherries contain ~6 times more melatonin (13.46 ± 1.10 ng/g) than do Balaton cherries (2.06 ± 0.17 ng/g) (Table 1).

The amount of melatonin in the cherries, either Balaton or Montmorency, did not vary according to when they were harvested (July and August). Melatonin levels measured for early, mid, and late harvests of either Montmorency or Balaton tart cherries, harvested on July 17 and 26 and August 7, had melatonin concentrations that were equivalent (Table 1). Likewise, there were no significance differences in the melatonin concentrations found in Montmorency tart cherries when harvested from two different orchards in Traverse City, MI (Table 1).

When Montmorency cherries were harvested from three individual trees (from orchard 1) on July 11, melatonin levels did vary (Table 1). The fruits from the three different trees had mean melatonin levels that differed significantly (Figure 8), but the mean value for the three trees (12.61 ng/g) was equivalent to the mean value of Montmorency cherries harvested in orchard 1 (14.28 ng/g).

DISCUSSION

Diets that contain an abundance of fruits and vegetable are protective against a variety of diseases, particularly cancer (*23*−*26*) and cardiovascular (*27*−*30*) and cerebrovascular diseases (*31*, *32*). In attempts to unravel the underlying mechanisms of this protection, various approaches have been taken. The antioxidant vitamins E and C and β-carotene (provitamin A) have received considerable attention for their potential role in the prevention of such diseases (*33*, *34*). Although vitamins are generally considered to function as antioxidants, they also exhibit toxicity and prooxidative actions under some conditions (*35*, *36*). Besides the vitamins, anthocyanins, which belong to the flavinoid family, are widely distributed in flowers, fruits, and vegetables and have been shown to have some positive therapeutic effects. Some of the conditions in which they have been found to be beneficial include diabetic retinopathy (*37*), fibrocystic disease of the breast in humans (*38*), and vision (*39*, *40*). Wang and colleagues (*5*, *6*) reported that anthocyanins and cyanidin, isolated from tart cherries, possess in vitro antioxidant and anti-inflammatory activities.

Melatonin has been shown, in a variety of studies, to possess anti-inflammatory, antioxidant, and anticancer properties (*16*, *41*, *42*). Melatonin is known to be a direct scavenger of the hydroxyl radical ($^{\bullet}OH$) (*42*, *43*) and hydrogen peroxide (H_2O_2) (*44*). It also can scavenge the peroxynitrite anion ($ONOO^-$), nitric oxide (*45*), and possibly the peroxyl radical ($LOO^{\bullet}$) and singlet oxygen ($^1O_2$) (*16*, *42*, *43*).

Measurement of the antioxidant melatonin in plants is a novel area of research. Melatonin has been identified in a variety of edible plants and fruits including cabbage, tomato, rice, orange, apple, and banana (*19*). The presence of melatonin in a plant is believed to be for its own protection from free radicals generated due to environmental or metabolic events, such as the process of photosynthesis (*21*). In support of this, leaves of different varieties of *Nicotiana tabacum* are differentially sensitive to ozone (a free radical generator) damage, with the sensitivity being reduced in leaves with the highest melatonin concentrations (*19*). This is consistent with the idea that melatonin in plants, as in animals, functions as an antioxidant. High amounts of melatonin have also been found in seeds of edible plants (*21*). It is hypothesized that melatonin in seeds may be essential for protecting germ and reproductive tissues from oxidative damage due to ultraviolet light, drought, extremes in temperature, and environmental toxins.

In this work, Montmorency and Balaton tart cherries were collected at different times during the harvest season. The earliest harvested cherries in July were less ripe than those harvested in August. Other beneficial nutrients have levels that are affected by conditions of growing, time of year, and maturity of the plant when harvested (*46*). According to R. Dubbels (personal communication), the concentration of melatonin in tomatoes is inversely related to the degree of ripeness of the fruit. By comparison, increasing maturity of blueberries at harvest yielded higher levels of the antioxidant anthocyanin, as well as the total phenolic content of the berries (*44*). In the present study, no correlation between the melatonin concentration and the ripeness of the fruit was found. Both tart cherry species had equivalent levels of melatonin regardless of their degree of ripeness when harvested during July and August.

Montmorency cherries had significantly different melatonin concentrations when harvested from different trees, although the mean concentration for the three trees was equivalent to the mean concentration of the orchards. The reason for this variation may be related to genetic differences or to different parts of the trees from which the cherries were picked. Also, due to their location in the orchard, the fruits may have been subjected to different environmental stressors. There is evidence in one plant species (St. John's wort) that melatonin synthesis in plants can be induced by light (*45*). In contrast to the tree variations, no significant differences were found when mean melatonin levels from pooled tart cherries from two orchards were compared.

When diets of plant products containing measurable amounts of melatonin were fed to chicks, levels of melatonin significantly increased in the circulation of the birds and the ingested melatonin bound to melatonin receptors (*20*). Clearly, the implication of these findings is that melatonin consumed in plant products is absorbed, enters the circulation, and could have physiological effects via receptor- or non-receptor-medi-

ated processes. Because the consumption of cherries is reported to produce anti-inflammatory and antioxidative effects, these effects may be related to the level of melatonin and/or other antioxidants these fruits contain. Therefore, the alleged beneficial effects of cherries may depend on the combination of antioxidants they possess.

In the present study, high concentrations of melatonin were found in tart cherries. The highest levels were detected in Montmorency tart cherries (13.46 ng/g). Although the melatonin concentrations in Balaton tart cherries (2.06 ng/g) are 6 times lower than in Montmorency cherries, the concentrations are still higher than those measured in other fruits. Because previous research (*20*) found that the consumption of plant products with low melatonin concentrations increased circulating melatonin levels (*20*) and because levels of melatonin in the blood correlate with the total antioxidant status (*48*), it seems reasonable that eating cherries with high melatonin concentrations would increase the antioxidant capacity of the organism. On the basis of the current findings, cherries, which contain substantial amounts of melatonin, as part of a healthy diet may be beneficial in counteracting some conditions in which free radical damage is a component of the condition.

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