MELATONIN IN TART CHERRIES:
METHODS OF EXTRACTION AND DETECTION

A Thesis

Presented to the Faculty of the Graduate School

of Cornell University

In Partial Fulfillment of the Requirements for the Degree of

Master of Science

by

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August 2008

ABSTRACT

Melatonin, a powerful antioxidant, offers potential human benefits in the fields of medicine, nutrition, and food science. While best understood in a mammalian system, melatonin has been identified in plants and dietary melatonin has been shown to increase circulating levels in the blood. Thus, there exists a great interest in extracting and detecting melatonin present in edible plant matrices. Extraction techniques such as liquid/liquid, solid phase, and solid/liquid extraction were investigated and compared to determine the best approach for isolating melatonin from fruit. Enzyme-linked immunoassay (ELISA), fluorescence, and mass spectrometry were investigated for their use as detection methods for melatonin originating in fruit systems. Additionally, the stability of melatonin in a pH 3.5 buffered model system was studied to gain preliminary information regarding melatonin heat and light stability. It was determined that melatonin is both heat stable and light stable for up to one hour (85ºC and 17 par, investigated separately). Solid/liquid extraction using ethyl acetate as a solvent was determined to be the best extraction procedure while high performance liquid chromatography-mass spectrometry with the use of a deuterated internal standard was the preferred detection method. A significant amount of work remains in the area of quantification of melatonin from fruit systems.

BIOGRAPHICAL SKETCH

Megan was raised in Rochester, NY, the daughter of a mechanical engineer and a Family and Consumer Sciences teacher. Her interest in food science stemmed from the logical, scientific influence of her father and the creative, culinary influence of her mother. During the time that her interest in food science began to surface, she attended Cottey College in Nevada, MO and earned an Associates of Science in chemistry and an Associates of Arts. She transferred to Cornell University to pursue a bachelor's degree in Food Science and continued directly on to her master's research. During her time at Cornell, she participated in several product development competitions, researched as a Summer Scholar, interned with Kraft Foods, Inc., and served as the president of the Graduate Student Association of the Geneva Experiment Station.

ACKNOWLEDGMENTS

This work would not have been possible without the support and inquisitive mind of my advisor, Dr. Olga Padilla-Zakour. The balance in which she held both myself as a student and this project allowed for maximum growth of my scientific skills. I am also appreciative of my minor advisory, Mary Tabacchi, for her support, interest, and enthusiasm for the project. I am deeply grateful to the IR-4 lab, including Michele Humiston, Roxanne Fish, Chris Lam, and Wlodzimierz Borejsza-Wysocki, for their expertise in analytical measurements, use of their LC-MS instrument, and all around support for this project. I am thankful to Randy Worobo for the use of his lab and for his support, and also to John Churey and Herb Cooley for their immense knowledge of the Food Research Lab Building and innovative approach to research challenges. I'd like to thank Ramon Mira de Orduña for the use of his HPLC-FLD and Sandra Christen for her expertise in operating the instrument. Every member of the Food Science and Technology department at Cornell University has helped me with this project at one point or another and I am grateful for the support, ideas, and assistance. Thank you to Olga's girls, both past and current for their support and certainly not least, thank you to David Manns. His understanding of chemistry, academia, graduate studies and his friendship have been invaluable to my experience at the Geneva Experiment Station.

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CHAPTER 1

INTRODUCTION

Justification

A diet rich in plant foods promotes human health by providing necessary nutrients in addition to beneficial phytochemicals (Potter and Hotchkiss 1995). Plants have been used throughout history as both a source of medicine and of poison, yet it is only within the last century that a particular group of plant chemicals, phytochemicals, have been identified and studied (Macıas et al. 2007). A phytochemical is a nonnutritive bioactive plant substance, and those of particular interest to food scientists often have a beneficial effect on human health. Most plant foods undergo processing prior to human consumption during which phytochemicals may be destroyed. Thus, it is in the interest of human health to identify beneficial phytochemicals in foods as well as the stability of these compounds during typical processing measures.

Melatonin as a phytochemical of interest

Melatonin, a mammalian hormone and antioxidant, was first identified in plants in 1995 by Dubbels, et al. Since then, it has been identified in numerous plants, many of which are human foods, such as olive oil, walnuts, pineapples, tomatoes, ginger, and others (Badria 2002; Reiter and Tan 2002; Reiter et al. 2005a; De La Puerta et al. 2007). Furthermore, dietary melatonin has been shown to increase melatonin levels and total antioxidant status of human serum (Hattori et al. 1995; Benot 1999). Melatonin's free radical scavenging abilities allow it to be classified as a phytochemical and make it a compound of particular interest (Tan et al. 2002).

Melatonin and human health

Melatonin has traditionally been considered a neurohormone. It is produced in mammals in the pineal gland and regulates circadian rhythm, sleep cycles, and seasonal reproduction (Morera and Abreu 2006). The role of light in relation to circadian rhythms is well documented. Exposure to light decreases circulating melatonin; melatonin is decreased by 50% after 39 minutes of exposure to incandescent light at night (Nunes and Pereira 2008). Beyond diurnal regulation, its role also includes that of potent antioxidant and free radical scavenger, potential anti-inflammatory agent, immunomodulatory agent, and inhibitor of carcinogenesis (Cardinali et al. 1997; Blask et al. 2004; Mayo et al. 2005; Tan et al. 2007b). A decrease in melatonin leads to metabolic disruption, increased oxidative stress, reduced immune function, increased risk of cancer, and changes in reproduction (Nunes and Pereira 2008). The beneficial effects of melatonin have led to promising research in the areas of human health.

In humans, melatonin production decreases with age. As age increases, oxidative disease risk also increases. While the system is too complex to draw correlations between the two trends, the potential use of melatonin as a therapy for oxidative disease treatment or prevention is being investigated. Researchers have studied the effects of light therapy and melatonin supplementation as a means of treating Alzheimer's disease (Wu and Swaab 2005). They have postulated that the decline in melatonin production with age, which disturbs the sleep/wake pattern and circadian rhythms, may contribute to mental decline. Thus, reestablishing the circadian rhythm through the use of melatonin supplements may be beneficial. Additionally, the

free radical scavenging capabilities of melatonin are a promising tool in the prevention and treatment of the disease (Wu and Swaab 2005). In the prevention and treatment of cardiovascular diseases, melatonin has been investigated due to its free radical scavenging capability, its indirect antioxidant activity, and its regulatory effects on enzymes – upregulating antioxidant enzymes and downregulating pro-oxidative enzymes (Tengattini et al. 2008). Studies have shown that melatonin exhibits anticancer activity both *in vivo* and *in vitro* and may improve the efficacy of typical cancer treatments when used in conjunction (Blask et al. 2004). Blask and others demonstrated that melatonin ingestion delayed tumor onset in a study of rat hepatoma and that melatonin was sequestered in the tumors. Alzheimer's, cardiovascular disease, and cancer are all oxidative diseases and the primary evidence of the benefits of melatonin in these systems adds to the interest surrounding melatonin.

Melatonin has also been shown to have antibiotic and antiviral activity. It was found to inhibit methicillin-resistant *Staphylococcus aureus* (MRSA), *Acinetobacter baumannii* and *Pseudomonas aeruginosa* at levels as low as 250 *μ*g/ml and 125 *μ*g/ml *in vitro* (Tekbas et al. 2008). When melatonin was paired with SB-73, a mixture of magnesium, phosphate, and fatty acids extracted from *Aspergillus* sp., the combination resulted in fewer adverse side effects and shorter lesion healing time than traditional medications for the treatment of the Herpes simplex virus (Nunes and Pereira 2008).

It is speculated that melatonin may play a role in determining the onset of puberty and that lifestyle changes may be influencing melatonin levels thus causing earlier onset of puberty in girls. Circumstantial evidence indicates that there are several receptor sites for melatonin on the human hypothalamus; melatonin levels are high throughout childhood and fall during puberty (Yun et al. 2004). Melatonin is thought to inhibit the hypothalamus-pituitary-gonadal (HPG) axis, which controls the onset of puberty (Murcia 2002). Therefore, if melatonin levels are reduced, it could potentially lead to the early onset of puberty. A greater understanding of the pathways involved in puberty is needed before conclusions can be drawn. However, melatonin appears to have a regulatory role within the human reproductive system.

Studies on medicinal herbs have found many to be very high in melatonin concentration, some in excess of 1000 ng/g of dry mass (Badria 2002; Chen et al. 2003). Typical physiological levels range from 10-60 pg/ml of plasma (Chen et al. 2003). Herbs may be an excellent natural source of pharmacological levels of melatonin. While melatonin has antioxidant capacity at both physiological and pharmacological levels, pharmacological levels may be more efficacious for the treatment of disease (Reiter et al. 2005b).

Melatonin chemistry and mechanisms of action

Melatonin is produced in both mammals and plants through the tryptophan metabolic pathway with serotonin being the immediate precursor (Murch et al. 2000). Melatonin is an indolamine containing an indole ring with an alkyl amide side chain and a benzene ring with a methoxy sidechain.

Melatonin is small, 232 g/mol, and although it has been reported to pass through membranes and enter all parts of the cell, it is difficult to dissolve from powdered form in aqueous solution (Shida et al. 1994; Reiter and Kim 1999). However, Shida and others found that melatonin is readily soluble in aqueous media when first dissolved in solvent and then taken to dryness. The basis for this observation is unknown but it is speculated that the physical arrangement of melatonin in a thin film may increase the interactions with water. They also note that melatonin is highly stable in solution when kept at 4ºC (Shida et al. 1994). Although the molecule contains both lipophilic and hydrophilic moieties, it has been reported that, preferentially, melatonin is located in the polar headgroup region of reversed micelles and in the aqueous phase (60%) of micelles (Ceraulo et al. 1999; Mekhloufi et al. 2007).

Melatonin is a light sensitive molecule. It absorbs light at 254 nm and thus is susceptible to UV degradation (Bromme et al. 2008). Researchers report that 0.1 mg/ml of melatonin exposed to a 400 W UV lamp reduced melatonin to 18% of its original concentration within 20 minutes and was complete degradation of melatonin after 2 hours (Maharaj et al. 2002). The end product of photo-degradation of melatonin has been identified as N^1-acetyl-N^2-formyl-5-methoxykynuramine (AMFK), which has been shown to maintain antioxidant activity (Tan et al. 2001; Maharaj et al. 2002).

Membrane receptor sites for melatonin, such as ML1, have been identified. It has been demonstrated that melatonin also interacts with nuclear receptor sites and intracellular proteins (Cardinali et al. 1997). Ouyang and Vogel (1998) found that the melatonin receptors are coupled to other signaling pathways such as the Ca^{2+} signaling pathway modulated by calmodulin which has been shown to bind melatonin.

Melatonin is particularly effective at inhibiting oxidation. Through the AMFK pathway, melatonin has been shown to scavenge up to 10 reactive species, extending its ability to scavenge free radicals through its quaternary metabolites (Tan et al. 2007a). The indole ring is the primary source of oxidant interaction due to both the high resonance stability of the ring and its low activation energy barrier. The amide side chain is responsible for scavenging a second reactive species and then forms a new five-membered ring while the methoxy group prevents pro-oxidative activity (Tan et al. 2002). The free radical scavenging capabilities of melatonin metabolites are also of interest. For example, AMFK, the end product of the photo-degradation of melatonin, can donate two electrons to scavenge free radicals and thus reduce lipid peroxidation and reduce neuronal death by hydrogen peroxide, glutamate, or amyloid (Tan et al. 2001). Research with melatonin and the ABTS cation radical have led to the discovery of derivatives of AMFK, although the degradation pathway and properties of those molecules have not yet been determined (Rosen et al. 2006).

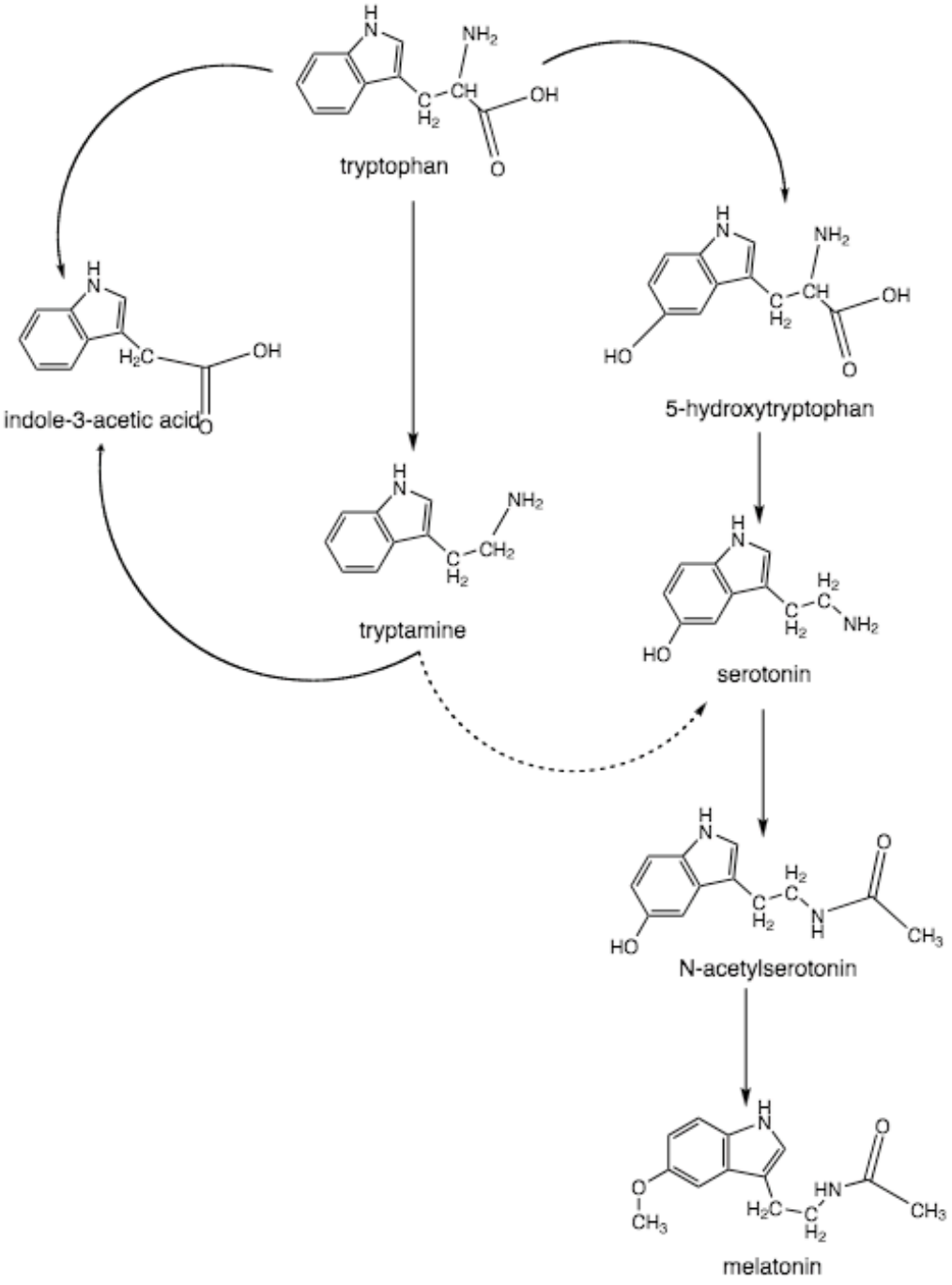
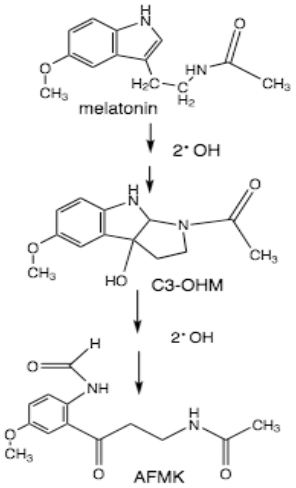


Figure 1.1. Metabolic pathways of tryptophan in mammals and plants as proposed by Murch et al., 2000.

(a)



(b)

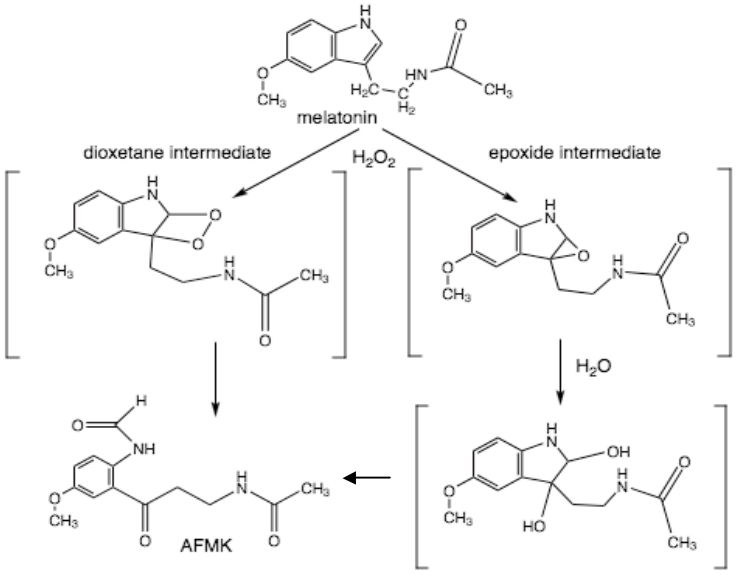


Figure 1.2. (a) Proposed mechanism for melatonin oxidation by •OH through C30OHM intermediate to AFMK. (b) Proposed mechanism for melatonin oxidation by H_2O_2 resulting in the formation of AMFK (Martinez et al. 2005).

Many oxidative diseases also involve dysfunction of the apoptic process involving the mitochondria. It has been suggested that melatonin scavenges oxygen and nitrogen reactive species in the mitochondria which leads to improved mitochondrial respiration and increased ATP synthesis during stress, which in turn may prevent the apoptic cascade (Leon et al. 2005). Melatonin can then be viewed as an indirect regulator of programmed cell death. On the genomic level, melatonin has been shown to regulate gene expression by influencing antioxidant enzyme activity and cellular mRNA levels of enzymes under both basal and elevated stress conditions (Rodriguez et al. 2004; Tomas-Zapico and Coto-Montes 2005).

Cyclooxygenase-2 (COX-2) is a key enzyme in the inflammatory response system and over-expression is associated with many diseases and cancer. Melatonin and its metabolites may regulate COX-2 and have been shown to prevent COX-2 activation. In addition, they prevent the oxidation of inducible nitric oxide synthase, thus playing an important role in the regulation of the inflammatory response (Mayo et al. 2005).

Melatonin in plants

The role of melatonin in plants is not yet well understood. The compound has been identified in numerous plants, including edible varieties. Studies on the distribution of melatonin within a plant, within a day, and within a growing season have been conducted to gain more information about the potential role of melatonin in plants.

Table 1.1 Summary of published values for melatonin in edible plants.

Plant	Tissue	Method[a]	Concentration, pg/g[b]	Reference
Apple	Fruit	RIA	48	Hattori et al., 1995
Asparagus	Shoot	RIA	10	Hattori et al., 1995
Balaton Cherries	Fruit	HPLC-ECD	1000-2000	Burkhardt et al., 2001
Banana	Fruit	RIA and GC-MS	500	Dubbels et al., 1995
Barley	Seed	RIA	378	Hattori et al., 1995
Cabbage	Leaf	RIA	107	Hattori et al., 1995
Cabernet Franc Grape	Skin	HPLC-FD and ELISA	5	Iriti et al., 2006
Cabernet Sauvingon Grape	Skin	HPLC-FD and ELISA	422	Iriti et al., 2006
Carrot	Fruit	RIA	55	Hattori et al., 1995
Chinese Herbs	Leaf	HPLC-FD and HPLC-MS	12,000-3,771,000	Chen et al., 2003
Cucumber	Fruit	RIA and GC-MS	90	Dubbels et al., 1995
Cucumber	Fruit	RIA	25	Hattori et al., 1995
Ginger	Root	RIA	584	Hattori et al., 1995
Indian Spinach	Leaf	RIA	39	Hattori et al., 1995
Japanese Radish	Root	RIA	657	Hattori et al., 1995
Kiwi	Fruit	RIA	24	Hattori et al., 1995
Montmorency Cherries	Fruit	HPLC-ECD	5000-20,000	Burkhardt et al., 2001
Oat	Seed	RIA	1796	Hattori et al., 1995
Olive	Oil	ELISA	53-120	C de La Puerta et al., 2006
Onion	Root	RIA	32	Hattori et al., 1995
Pineapple	Fruit	RIA	36	Hattori et al., 1995
Rice	Seed	RIA	1006	Hattori et al., 1995
Sangiovese Grape	Skin	HPLC-FD and ELISA	332	Iriti et al., 2006
Strawberry	Fruit	RIA	12	Hattori et al., 1995
Sweet Corn	Seed	RIA	1366	Hattori et al., 1995
Tomato	Fruit	RIA and GC-MS	100-500	Dubbels et al., 1995
Tomato	Fruit	RIA	32	Hattori et al., 1995
Tomato	Fruit	RIA and GC-MS	0-15	Van Tassel et al., 2001
Walnuts	Seed	RIA	3500	Reiter et al., 2005

[a] Methods were: radioimmunoassay (RIA), high performance liquid chromatography with electrochemical detection (HPLC-ECD) or with fluorescence detection (HPLC-FD), enzyme linked immunoassay (ELISA), gas chromatography with mass spectrometry detection (GC-MS)

[b] Values reported are estimates and have been converted to pg/g from the published values

In vertebrates, melatonin is known to act as a night signal, regulating circadian rhythm through response to light. A study by Van Tassel and O'Neill sought to investigate this property of melatonin in plants and found inconsistent evidence that melatonin increases at night in tomatoes and morning glory. However, they found that melatonin in fruit increased during the ripening period and continued to increase as the fruit became overripe (Van Tassel and O'neill 2001; Van Tassel et al. 2001). In addition to fruit, melatonin is found in roots, stems, leaves, and seeds. It is generally unequally distributed within a given plant and varies widely with plant type (Reiter and Tan 2002). Melatonin is derived from the tryptophan pathway as are other catecholamines such as auxin and indoleacetic acid which have been shown to promote tissue growth and flowering (Kuklin and Conger 1995). Root development, mitosis, and mitotic spindle formation are all affected when the concentration of melatonin in plant tissues is artificially changed, suggesting that melatonin may be a potential regulator of plant growth and development (Murch and Saxena 2002).

Methods of quantifying melatonin

Many of the instrumental methods which exist for quantifying melatonin were originally designed for use with mammalian samples such as blood serum and plasma, saliva and urine. There are several challenges to quantifying melatonin in plants: the analysis is nearly always destructive and that plants contain compounds, such as phenolics, that can interfere with melatonin analysis. Therefore, sample preparation is particularly crucial in analyzing plant material.

Liquid-liquid extraction is a common sample preparation step following primary extraction from plant material. Solvents include water, pH controlled buffers, methanol, chloroform, dichloroethane, and diethyl ether (Dubbels et al. 1995; Kolar et al. 1997; Kim et al. 1999; Burkhardt et al. 2001; Cao et al. 2006). Solid phase extraction has also been used, in which melatonin is eluted with a solvent such as methanol or acetone (Rolcik et al. 2002; Chen et al. 2003; Papy-Garcia et al. 2003; Iriti et al. 2006; Pape and Luening 2006).

Immunoassays such as enzyme-linked immunoassay (ELISA), and radioimmunoassay (RIA), are popular due to their specificity and the availability of kits (Kennaway et al. 1977; Fraser et al. 1983; Poeggeler et al. 1991; Itoh et al. 1994; Poeggeler and Hardeland 1994; Dubbels et al. 1995; Iriti et al. 2006; Pape and Luening 2006). However, interferences with melatonin-specific antibodies have been cited, which necessitates that immunoassays be validated by another analytical method (Van Tassel and O'neill 2001). High performance liquid chromatography (HPLC) is commonly used in melatonin detection due to its ability to further separate compounds beyond sample preparation. It has been paired with electrochemical detection, fluorescence detection and mass spectroscopy detection, in the order of specificity (Itoh et al. 1994; Poeggeler and Hardeland 1994; Dubbels et al. 1995; Kolar et al. 1997; Iinuma et al. 1999; Burkhardt et al. 2001; Papy-Garcia et al. 2003; Tomita et al. 2003; Hamase et al. 2004; Hirano et al. 2004; Martinez et al. 2005; Cao et al. 2006; Iriti et al. 2006; Pape and Luening 2006). Deuterated internal standards for melatonin are readily available and have been used with mass spectroscopy. Detection methods including immunoaffinity chromatography, solid phase enzyme immunoassay, capillary

electrophoresis, and general chromatography are less popular methods for melatonin detection (Yie et al. 1993; Kim et al. 1999; Rolcik et al. 2002; Ayano et al. 2007).

Native melatonin fluoresces at an excitation of 280 nm and emission of 345 nm (Itoh et al. 1994; Poeggeler and Hardeland 1994; Iinuma et al. 1999; Tomita et al. 2003; Hamase et al. 2004; Hirano et al. 2004; Iriti et al. 2006). A higher fluorescent response is given by the controlled oxidation of melatonin by hydrogen peroxide to the degradation product *N*-[(6-methoxy-4-oxo-1,4-dihydroquinolin-3-yl)methyl]acetamide (6-MOQMA) under basic conditions (Hamase et al. 2004). The new compound fluoresces at an excitation of 245 nm and emission of 380 nm and the response is estimated to be 6.8 times higher than that of native melatonin (Tomita et al. 2003). Challenges with this method include adding additional sources of error through derivatizing melatonin, in which not enough is known about the degradation products of melatonin to ensure that all melatonin is converted to 6-MOQMA or that other compounds present in the sample are not degrading to interfering compounds.

Mass spectrometry, either paired with gas chromatography or liquid chromatography is a sensitive and reliable detection method which allows for the use of a stable isotope internal standard for validation. Liquid chromatography offers a benefit over gas chromatography because a derivatization step is not necessary. In liquid chromatography, the parent ion is monitored at 233 *m/z* (molecular weight of melatonin is 232 g/mol plus the addition of one hydrogen becomes 233) and the daughter ion (fragment ion) is measured at 174 *m/z* when using electrospray ionization (ESI) (Cao et al.

2006). A deuterated internal standard would have a mass to charge ratio (*m/z*) dependent on the number of substituted deuterium atoms. The use of liquid chromatography-tandem mass spectrometry, LC-MS/MS, gives even more specificity in that it monitors the transition between the parent and daughter ion whereas LC-MS is only capable of monitoring either the parent or daughter ion separately (Dass 1997).

New York State fruit production

New York State is the fourth largest producer of tart cherries after Michigan, Washington, and Utah, and is expected to produce 9.20 million pounds in 2008 (2008). Additionally, of the total U.S. tart cherry production (262 million pounds per year, average 2005-2007), 97% (255 million pounds per year, average 2005-2007) is processed into products such as juices and jams. Fruits such as apples, grapes, strawberries, peaches, and berries are also important to New York State both for their fresh market value and for processing (2008).

Juice processing

Much of the fruit grown in the United States is used for juice processing, however, processing can affect the nutritional quality of the product (Yeom et al. 2000). Some compounds present in the fruit, such as vitamin C, may quickly degrade under processing conditions, while other compounds may become more bio-available, such as lycopene in tomatoes (Unlu et al. 2007).

There are five basic steps to processing fruit into juice: sorting and washing, pressing, filtering, pasteurizing, and packing (Nelson 1980). The fruit is prepared by removing stems, leaves and pits and may be washed in cold water. Any spoiled or damaged fruit is removed during sorting. The fruit is then pressed, either hot (65ºC) to extract more color, or cold to preserve the fresh fruit flavor. Cold pressed juice is quickly heated to between 88-93ºC to inactivate enzymes that can decrease quality. Pectinase enzymes may be used to increase juice yields. Following pressing, the juice is filtered, typically through a rack and frame filter press, to create a bright, clear juice. During pasteurization, juice is typically brought to 85ºC for 1 minute, then hot packed into bottles or cans and cooled.

Juice producers must balance the destruction of pathogens and spoilage organisms, to ensure safety and stability, with maintaining a high quality juice and retaining heat sensitive compounds and nutrients. Therefore, it is of interest to juice producers to understand both how much melatonin is present in fruits as well as the stability of the compound under processing conditions.

Objectives

The analysis of melatonin from mammalian tissues and fluids is well established in the literature. In recent years, researchers have applied and modified these methods for use with plant materials. Fruits, in particular, pose a challenge for melatonin analysis due to their high concentration of sugars, acids, and phenolic and colored compounds, which can cause difficulties in extraction and interferences with detection. Yet, due to the potential health

benefits of melatonin in the human diet, established methods for measuring melatonin in fruit systems are needed. This study aims to:

1. Develop a method for extracting melatonin from fruit using Montmorency tart cherry as a model system
2. Detect melatonin in the range of 1-10 parts-per-billion (ng/mL) in the Montmorency matrix
3. Investigate the stability of melatonin under processing conditions in a pH buffer controlled model system

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CHAPTER 2

MELATONIN ANALYSIS

Abstract

Interest in melatonin comes from nutrition and food processing as well as medical fields due to the chemical's free-radical scavenging ability and the demonstrated increase in circulating melatonin in mammalian systems by an increase in dietary melatonin. While methods for detection and quantification of melatonin in mammalian tissues and biological fluids are well-established, work remains to be done with regard to plants. Liquid/liquid, solid phase, and solid/liquid extraction were compared as means of isolating melatonin from the complex matrix of fruits. Enzyme-linked immunoassay (ELISA), fluorescence, and mass spectrometry were compared for specificity and sensitivity as detection methods for melatonin. Initially, a variety of fruits were tested and Montmorency tart cherry was selected as a model fruit system when constraints prevented the testing of more than one fruit variety. The results of this study show that solid/liquid extraction with ethyl acetate is an effective method for extracting melatonin from Montmorency cherries and that mass spectrometry detection gave the most reliable quantification of melatonin concentrations.

Introduction

While melatonin detection and quantification are well established for mammalian tissues and fluids, much less is known about melatonin in plants. Recently, dietary melatonin has been shown to increase circulating levels of melatonin in humans, which in addition to offering a beneficial source of

antioxidants, increases the importance of understanding how much melatonin is present in edible plant tissues (Reiter et al. 2001).

Edible plant tissues contain many complex bioactive compounds such as anthocyanins and other polyphenols which can interfere with extraction and detection of melatonin and require innovative methods for isolating melatonin from the matrix (Ferrari 2004). Reported values for melatonin in the literature contain discrepancies, which may be due to both the difficulty of accurately measuring melatonin as well as the extreme variation in fruit components both within a given plant and between plants (Dubbels et al. 1995; Hattori et al. 1995; Van Tassel et al. 2001). Reported values for melatonin in plants are consistently low, in the parts-per-billion or parts-per-trillion. Therefore very sensitive detection methods are needed to quantify such low amounts, complicated by the high number of interfering compounds present in fruits (Chegini et al. 1995; Dubbels et al. 1995; Reiter and Tan 2002).

This study sought to highlight some of the challenges in extracting and quantifying melatonin from fruit matrices and offer solutions to some of the challenges presented. Additionally, the stability of melatonin during exposure to heat or light was investigated to eliminate sources of potential degradation during experimental procedures. This information, although preliminary, also benefits fruit processors by eliminating sources of melatonin degradation during processing.

Materials

Chemicals

Reagent grade chemicals: monopotassium and dipotassium phosphates, concentrated hydrochloric acid, formic acid, and HPLC grade solvents: methanol, water, ethyl acetate, chloroform, hexane and acetonitrile were obtained from Fisher Scientific (Fair Lawn, NJ). Melatonin was obtained from MP Biomedicals (Solon, OH) and deuterated melatonin (d4-Melatonin) was obtained from CDN Isotopes (Pointe-Claire, Quebec, Canada).

Fruits

All fruits were obtained from the New York State Agricultural Experiment Station orchards (Geneva, NY). Tart cherry varieties: Balaton, North Star, Schattenmorelle, Oblacinska, and Sumadinka belong to the USDA Federal Tart Cherry Collection. All other fruits were grown on research plots. Table 2.1 displays the type of fruit and varieties obtained.

Table 2.1 Fruit type, variety, and source used to screen for melatonin.

Species	Variety	Obtained From
Tart Cherries *Prunus cerasus*	Montmorency	Research North Farm, NYSAES, Geneva, NY
	Balaton North Star Oblacinska Schattenmorelle Sumadinka	Federal Tart Cherry Collection, NYSAES, Geneva, NY
	Topas Karneol	Lucy Farm, NYSAES, Geneva, NY
Sweet Cherries *Prunus avium*	Lapin Hedelfingen Regina Sweetheart	Herb Cooley, NYSAES, Geneva, NY
Plums *Prunus*	Cacak's Best Pozegaca French Damson Blues Jam Stanley NY 1456 Mirabellior	Research South Farm, NYSAES, Geneva, NY
Strawberries *Fragaria*	Lamour NY 99-15 Jewel	Courtney Weber,NYSAES, Geneva, NY
Black Raspberries *Rubus occidentalis*	Jewel Mac Black	Courtney Weber, NYSAES, Geneva, NY
Black Currant *Ribes nigrum*	Titania	RH Rhodes & Son, Inc., Penn Yan, NY

Melatonin Stability

Heat Treatment

A 1000 ng/mL solution of melatonin in potassium phosphate buffer (0.05M, pH 3.5 with concentrated HLC) was made by adding 0.5 ml of a 100 μg/mL stock solution of melatonin in methanol and bringing the total volume to 50 mL with buffer. This solution was loaded into five capillary tubes (Kimax-51, Fisher Scientific, Fair Lawn, NJ) at 40 μL each for 200 μL total per heat treatment level for a total of 30 tubes. The capillaries were sealed by flame and stored under refrigeration and protected from light until the treatment was applied. Five test tubes (17 x 100 mm) filled with water were allowed to equilibrate in an 85ºC water bath (Haake L circulator/D2 temperature controller water bath, Paramus, NJ). An additional five tubes filled with water were allowed to equilibrate in an ice bucket.

To apply the heat treatment, sets of capillary tubes were dropped into the heated test tubes in the water bath. The test tubes, each containing five capillary tubes, were removed after the given treatment time (2, 5, 15, 30, and 60 minutes, respectively) and immediately deposited into the cooled test tubes in the ice bucket. The control sample was deposited into the capillary tubes and kept on ice.

The samples were prepared for analysis by crushing the capillary tubes in bottles containing 1.8 mL of water and 50 ng/mL of d4-melatonin. The diluted samples, at a calculated concentration of 100 ppb melatonin and 50 ppb d4-melatonin, were passed through PTFE syringe filters (13 mm, 0.45 μm pore,

Whatman, Springfield Mill, UK) to remove glass particulates and loaded into amber HPLC vials (Fisher Scientific, Fair Lawn, NJ).

Light Treatment

A 100 ng/mL solution of melatonin in potassium phosphate buffer (0.05M, pH 3.5 with concentrated HLC) was made by adding 5 ml of a 1.0 μg/mL stock solution of melatonin in methanol, 2.5 mL of a 1.0 μg/mL solution of d4–melatonin in methanol and bringing the total volume to 50 mL with buffer.

Treatment was applied by depositing a 1.0 mL drop of melatonin solution per treatment level on a strip of parafilm under bright ambient lighting. The light intensity was measured as 16.78 par (μmol of photons/m^2/s) using a Li-Cor 250 light meter with a LiCor Quantum sensor (Lincoln, NE). Following the treatment (2, 5, 15, 30, and 60 minutes, respectively), drops were recovered using a pipette, passed through a PTFE syringe filter, and deposited into amber HPLC vials.

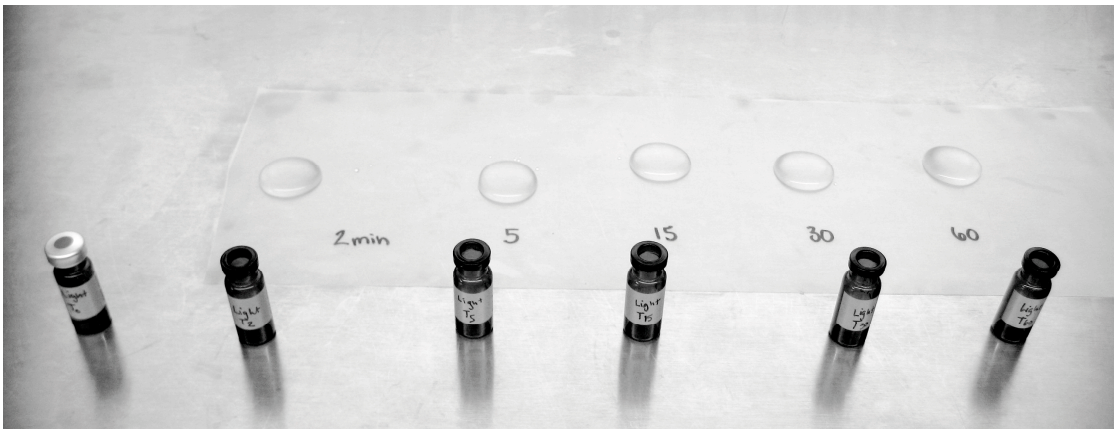

Illustration 2.1 Light treatment application.

Melatonin Extraction

Sample Preparation

Fruit samples were harvested between June 13th and September 21st, 2007 at ripeness indicated by appropriate ºBrix level. Strawberries were hulled and halved, and immediately frozen at –40ºC until lyophilized. Cherries were destemmed and pitted using a retail-type cherry pitter (Walmart, Rogers, AR), flash frozen using liquid nitrogen, and stored at –40ºC until lyophilized. Black raspberries were frozen whole. Plums were halved and pitted and then frozen at –40ºC. Frozen fruits were all stored at –40ºC and protected from light until freeze-drying when they were loaded into a Virtis SR50-C freeze dryer (Virtis Co., Gardiner, NY). Following lyophilization, fruits were stored at 2ºC and protected from light. Prior to analysis, dried fruits were ground in a Waring blender (Waring Laboratory & Science, Torrington, CT) and returned to storage at 2ºC, protected from light.

Liquid-Liquid Extraction

Approximately two grams of freeze-dried fruit sample was reconstituted to its original moisture content. A vortex (Vortex Genie 2, Scientific Industries, Bohemia, NY) was applied to the fruit for 30 seconds to ensure proper mixing and allowed to sit at room temperature for 5 minutes to ensure good rehydration. The sample was then centrifuged (Sorvall RC-5B with SS-34 rotor, DuPont Instruments, Wilmington, DE) for 10 minutes at 5000 rpm and 4ºC. An aliquot of 500 μL of the supernatant (juice) was decanted into 1.7 mL eppendorf tubes (Polypropylene, Fisher Scientific, Fair Lawn, NJ) in replicates of three per sample. Either chloroform or ethyl acetate was added to the eppendorf tubes at 700 μL to extract the melatonin. The tubes were capped

and shaken vigorously by hand for 10 minutes at room temperature. The solvent layer was decanted into clean eppendorf tubes and the extraction was repeated. The first and second solvent extracts were combined and dried in a vacuum centrifuge (Speedvac Plus SC110A, Savant, Fullerton, CA) for 20-35 minutes or until dryness. Each tube was reconstituted with 100 μL of distilled, deionized water.

Derivatization of Melatonin

A solution of melatonin, at less than or equal to 25 ppb and 100 μL, was combined with 10 μL of 300 mM sodium carbonate and 10 μL of 10 mM hydrogen peroxide. The resulting solution was heated in a 90^{o}C water bath for 30 minutes to allow for the conversion of melatonin to *N*-[(6-methoxy-4-oxo-1,4-dihydroquinolin-3-yl)methyl]acetamide (6-MOQMA) (Hamase et al. 2004). A potential internal standard, 5-methoxyindole-3-acetic acid (MIAA) was derivatized under the same conditions.

Solid Phase Extraction (SPE)

Fruit samples were prepared similarly to those used for the liquid-liquid extraction method. The freeze-dried, ground samples were reconstituted, mixed vigorously and allowed to rehydrate for five minutes at room temperature before being centrifuged for 10 minutes at 5000 rpm at 4^{o}C. The supernatant (juice) was decanted and filtered through a fine mesh screen and divided into three 2 mL aliquots in glass test tubes. For each fruit sample, the three treatments were designated replicate 1, replicate 2, and recovery spike. A 1 μg/mL solution at 100 μL of d4-melatonin in methanol (50 ppb) was added to each treatment as an internal standard. The recovery spike treatment

received an additional 100 μL of 2 μg/mL melatonin in methanol (100 ppb). All samples were protected from light and refrigerated if not extracted immediately. Refrigerated samples were brought back to room temperature approximately 10 minutes before extraction.

The solid phase extraction cartridges, C18, 500 mg, 3 mL (Thermo Scientific, Fisher Scientific, Fair Lawn, NJ) were conditioned by subsequently passing 3 mL methanol, 3 mL HPLC grade water, 3 mL ethyl acetate, and 3 mL HPLC grade water through the cartridges utilizing a vacuum manifold holding four cartridges and without allowing the material to go dry. The samples were slowly loaded onto the cartridges, washed with 3 mL of HPLC grade water, and dried under a stream of nitrogen for 20-30 minutes or until dry. Melatonin was eluted from the cartridges with 2 mL of ethyl acetate and collected in 2.2 mL eppendorf tubes. The bed was allowed to fully saturate with ethyl acetate before the solvent was forced through the cartridge using a plunger and eluting in a drop-wise fashion.

Solid-Liquid Extraction

Freeze-dried, ground fruit samples, 1 g, were spiked with 100 μL of 1 μg/mL d4-melatonin in methanol. Recovery spike treatments were also spiked with 100 μL of 2 μg/mL melatonin in methanol and both samples and recovery spikes were allowed to dry for 15 minutes at room temperature. Ethyl acetate at 2 mL was added to each dry sample which was then placed in a sonicator bath (Branson 2200, Fisher Scientific, Fair Lawn, NJ). The samples were removed individually and homogenized (Ultra Turbax, T25 Basic, Ika-Werke, Wilmington, NC) for 1 min at 22000/min and then returned to the sonicator

bath. The samples underwent sonication for approximately 10 minutes and reached approximately 35ºC. Each sample was briefly shaken using a vortex before being centrifuged at 5000 rpm for 5 minutes at 4ºC. The ethyl acetate layer was decanted into 2.2 mL eppendorf tubes and dried in an evaporation centrifuge for approximately 30 minutes. The solid material was extracted a second time with another 2 mL aliquot of ethyl acetate and the solvent extract was added to the same eppendorf tube as the first solvent layer and dried to dryness. The residue was reconstituted with 2 mL of HPLC mobile phase, 50:50 acetonitrile and water, and then injected onto the HPLC-MS system for analysis.

Melatonin Detection

ELISA

The use of enzyme-linked immunoassay was investigated for the detection of melatonin. A kit from IBL International was used (Hamburg, Germany) and the centrifuge procedure was followed.

HPLC-FLD

The detection of melatonin, both derivatized and non-derivatized, was investigated using high performance liquid chromatography with fluorescence detection. In either case, a Symmetry C18 column, 250 x 4.6 mm, 5 μm pore size (Waters, Millford, MA) was used along with a Hewlett Packard 1100 series HPLC system with a G1321A fluorescence detector (Agilent, Santa Clara, CA). An isocratic chromatographic system was used for both native and derivatized melatonin and the column temperature was maintained at 25ºC. The mobile phase for native melatonin was 10 mM sodium acetate,

0.01 mM ETDA, and 20% acetonitrile at pH 4.5 and was run at 1 ml per minute with a 100 μL injection volume (Iriti et al. 2006). Detection was measured at an excitation of 280 nm and emission of 345 nm. Additional work was performed on native melatonin using a Shimadzu 20A HPLC system with a RF10A-XL fluorescence detector (Shimadzu Scientific Instruments, Columbia, MD) using a 30 min gradient of acetonitrile and water (ACN:H_2O, 0 min – 20:80, 18 min – 90:10, 20 min – 90:10, 22 min – 20:80) with a flow rate of 0.4 ml per min. The mobile phase for derivatized melatonin was 100 mM sodium phosphate buffer and 12% acetonitrile at pH 7 with a flow rate of 1 ml per minute and a 100 μL injection volume (Tomita et al. 2003). Detection of derivatized melatonin was measured at an excitation of 247 nm and emission of 384 (Iinuma et al. 1999).

The use of an internal standard was investigated with HPLC-FLD. The compound, 5-methoxyindole-3-acetic acid (MIAA) follows fluorescence spectral shifts during oxidation similar to melatonin. The derivatized product is 5-methoxy-4-quinolone (6-MOQ) (Hirano et al. 2004). MIAA was derivatized in vitro with melatonin and its value as an internal standard was evaluated.

HPLC-MS

High performance liquid chromatography with mass spectroscopy was performed on an Agilent 1100 series LC/MSD using a Zorbax SB-C18 column, 5 μm pore size, 150 x 4.6 mm (Agilent, Santa Clara, CA). A 14.2 minute gradient with a 3 minute post run was performed according to Table 2.2

Table 2.2 HPLC Gradient system for melatonin analysis using MS detection, Zorbax SB-C18 column, 150 x 2.6 mm, 5 μm pore size, and including a 3 minute post run.

Time (min)	% Acetonitrile	% 0.5% Formic Acid	Flow (mL/min)
Pre run	20	80	0.4
0	20	80	0.4
2	20	80	0.4
12	90	10	0.4
14	90	10	0.4
14.1	20	80	0.4

The column was held at 40ºC with nitrogen as the drying gas at 350ºC and 10 L/min, 20 psi nebulizer pressure, and 4000 V capillary voltage. Detection was performed in atmospheric pressure ionization – electrospray (API-ES) ionization mode, monitoring for ions 233 (melatonin) and 237 (d4-Melatonin internal standard) with 100 V fragmentor voltage in selected ion monitoring (SIM) mode with positive polarity. Separately, ions 174 (melatonin) and 178 (d4-melatonin) were monitored using a 200 V fragmentor voltage.

Figure 2.1 Spectral scan of Montmorency cherries with 100 ng/mL melatonin added under (a) 100 v fragmentor voltage and (b) 200 V fragmentor voltage.

(a)

```
Sample Name : 100 ppb melatonin in montmorency juice
================================================================================
Acq. Operator   : mch                                  Seq. Line :   2
Acq. Instrument : Instrument 1                        Location : Vial 12
Injection Date  : 6/23/2008 1:45:37 PM                    Inj :   1
                                                     Inj Volume : 25  1
Acq. Method     : C:\CHEM32\1\DATA\MELATONIN\70 1\MELATONINSCAN.M
Last changed    : 6/23/2008 1:41:10 PM by mch
Analysis Method : C:\CHEM32\1\METHODS\MELATONIN174.M
Last changed    : 6/27/2008 8:03:17 AM by mch
                  (modified after loading)
```

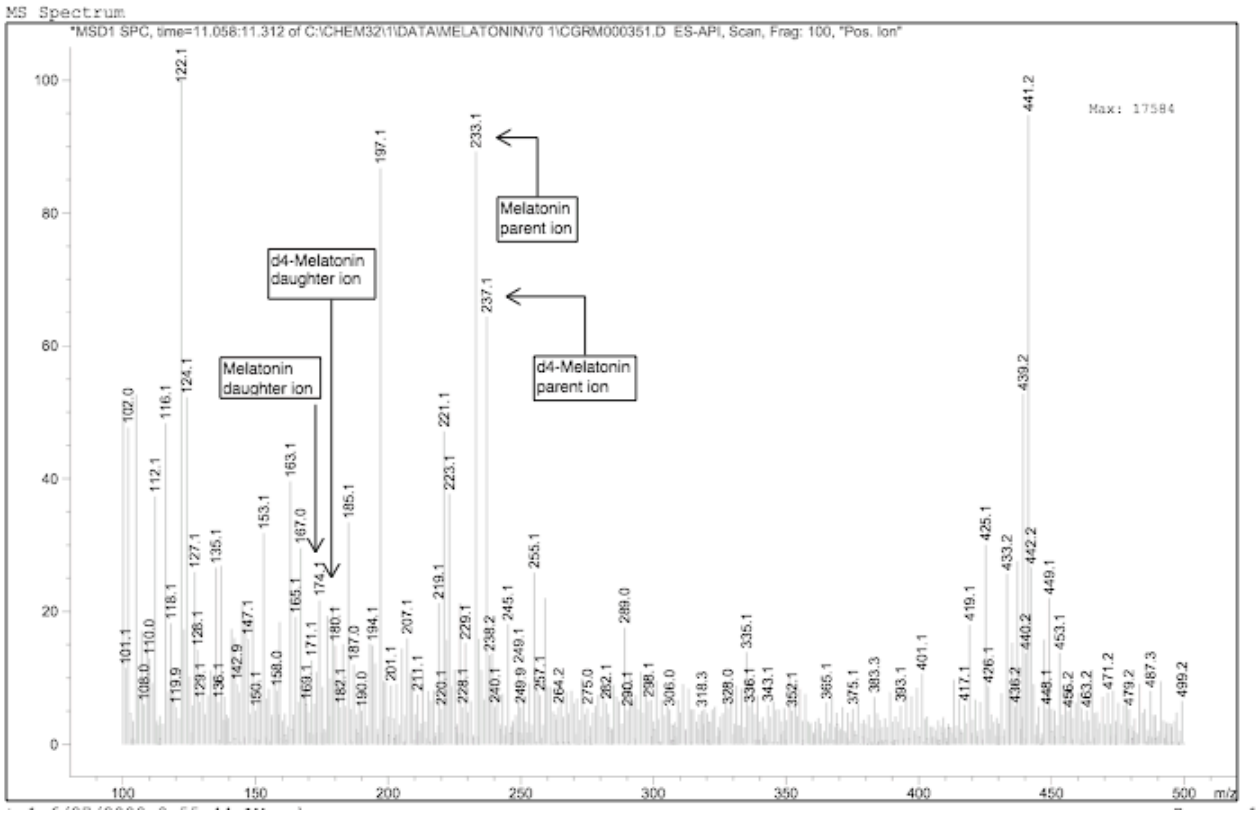

Figure 2.1 (Continued) (b)

```
Sample Name : 100 ppb melatonin in montmorency juice
================================================================================
Acq. Operator   : mch                                 Seq. Line :   1
Acq. Instrument : Instrument 1                       Location : Vial 12
Injection Date  : 6/23/2008 2:16:14 PM                  Inj :   1
                                                    Inj Volume : 25  l
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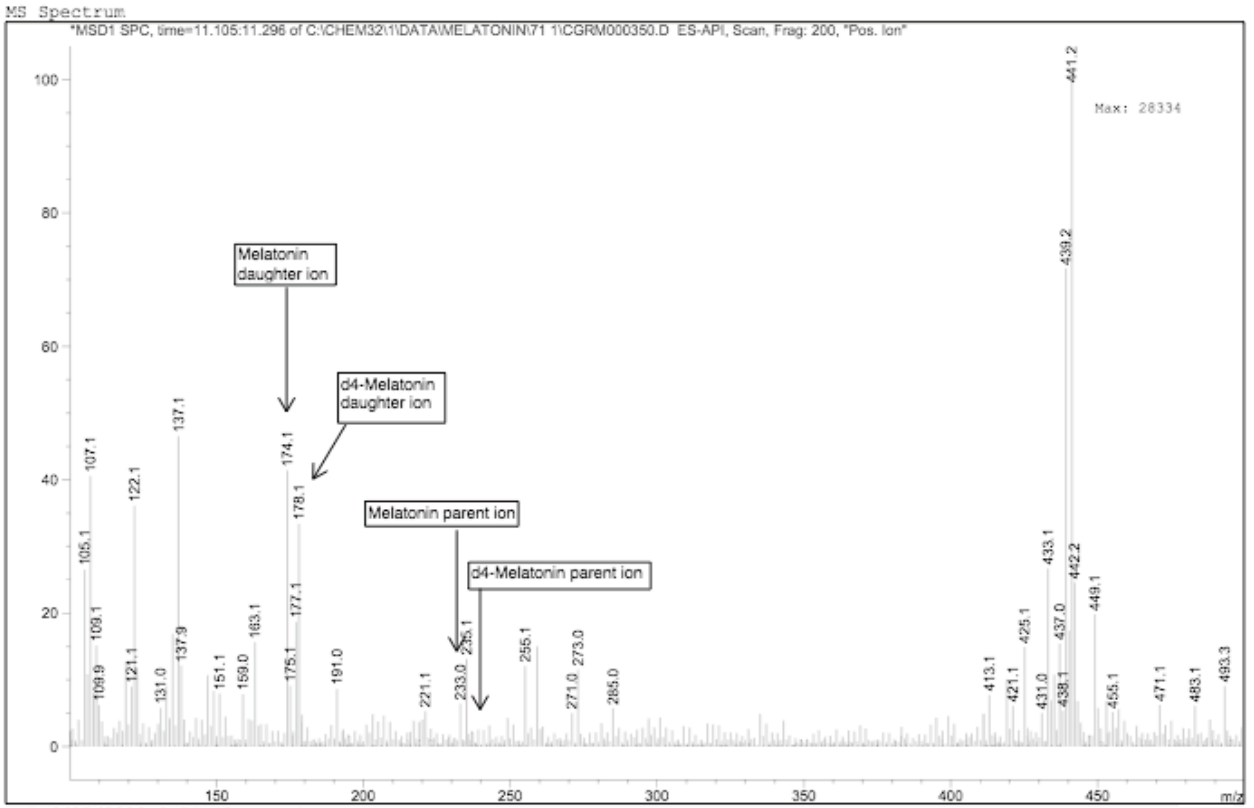


Results and Discussion

Melatonin Stability

The method used to investigate the heat stability of melatonin was modified from that used to determine microbial thermal death rates. Treatment times were chosen to create a model of melatonin kinetics within a one hour window of interest to determine whether melatonin was affected by environmental conditions during analysis. The pH 3.5 buffered system was selected as a model system to mimic the behavior of melatonin in juice and eliminate the interference of other compounds typically found in juice, such as anthocyanins and other phenolic compounds. A final concentration of 100 ng/mL of melatonin was selected since it fell at the high end of the standard curve used when measuring native melatonin in the HPLC-MS system.

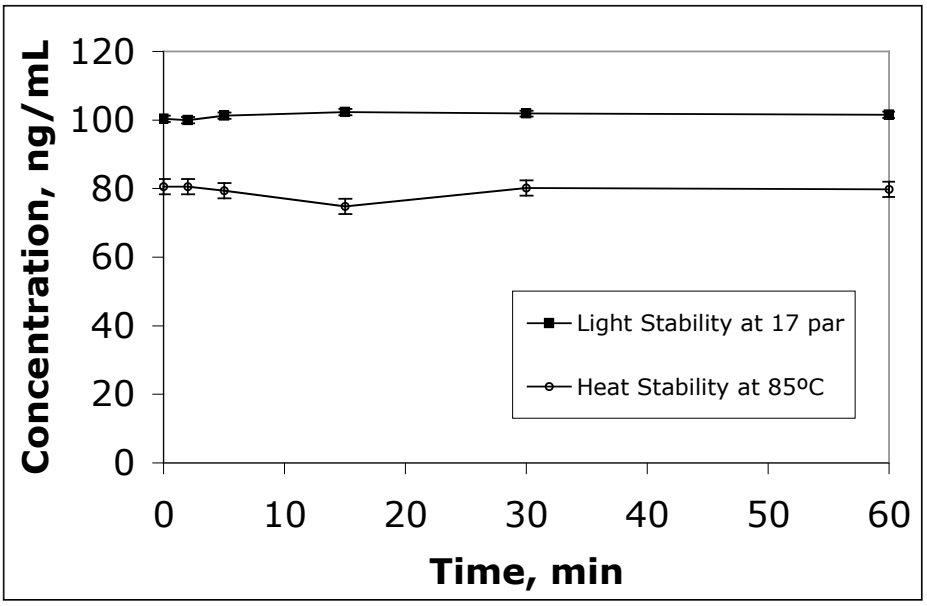


Figure 2.2 Melatonin stability in 0.05 M potassium phosphate buffer, pH 3.5, at 85ºC and at 17 par.

During the heating study, loading of the capillary tubes, followed by the crushing of the tubes and dilution step led to a melatonin loss of 20%. This effect was most likely due to a portion of the melatonin solution remaining in the pieces of the crushed capillary tubes. All heat-treated samples showed the same percent loss, with a standard deviation of 2.22 ng/mL, while light treated samples showed no loss with a standard deviation of 0.9 ng/mL (Figure 2.2). Based on this prelimary investigation, melatonin appears to be heat stable at 85ºC and light stable at 17 par for up to one hour when no other bioactive compounds are present. The light stabililty results presented contradict those reported by Bromme et al., (2008) and Maharaj et al., (2002). However, their work utilizes aeration of the samples during the light treatment. Melatonin is degraded through an oxidative pathway. It is plausible that melatonin was degraded in the studies above due to aeration rather than exposure to light, however, the effects of oxygenation or aeration were not explored in this study.

Melatonin Extraction

Chloroform was initially chosen as a solvent for the extraction of melatonin based on published material (Burkhardt et al. 2001). However, due to low extraction efficiency, below 60%, ethyl acetate was explored as an alternative. Figure 2.3 shows the relative extraction efficiency by ethyl acetate versus chloroform on the same melatonin solution in water. The ethyl acetate was approximately 3 times more effective at extracting melatonin from a water solution (liquid/liquid) than chloroform.

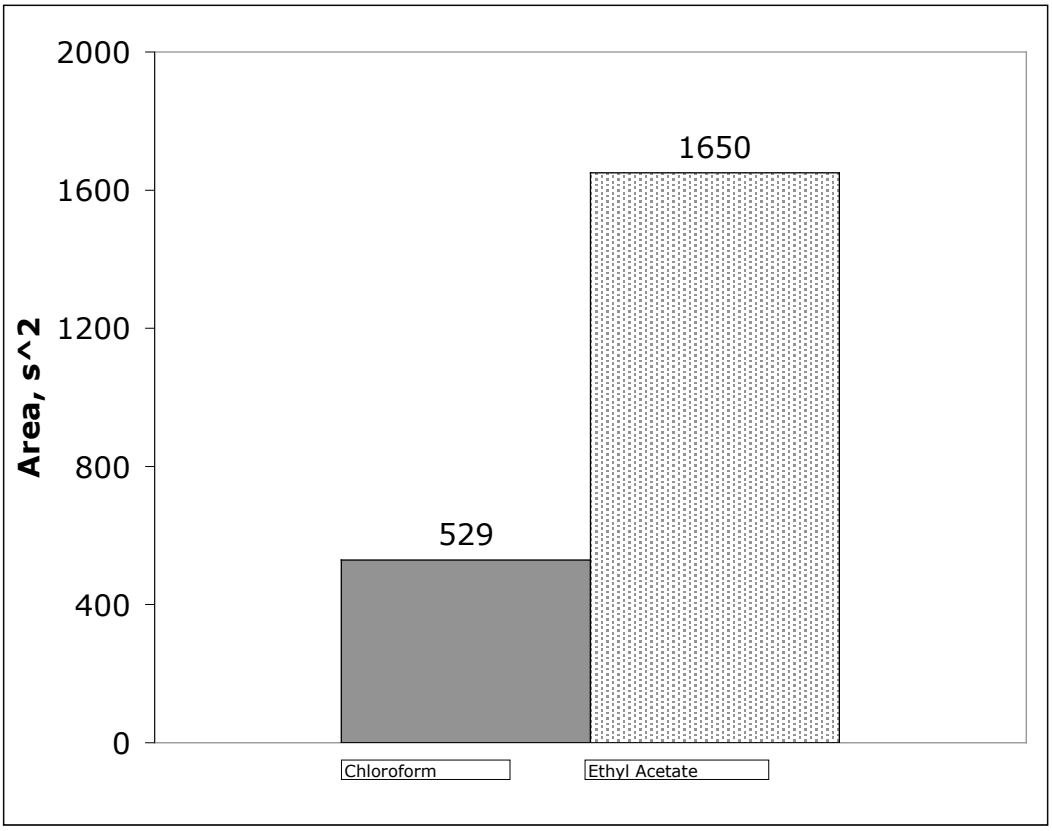


Figure 2.3 Extraction efficiency of 261 ng/mL of melatonin in water extracted with chloroform or ethyl acetate, derivatized and measured using HLPC-FLD.

During the evaluation of MIAA as an internal standard for melatonin analysis by HPLC-FLD with derivatization, an interfering peak in the MIAA spectra appeared. Work with MIAA was then discontinued. However, during work with MS detection, it was discovered that nylon filters were giving an extra peak in the deuterated internal standard spectra. Nylon filters were also used in the preparation of MIAA samples and it is reasonable to conclude that the interfering peak was due to the use of nylon filters in the case of fluorescence detection as well.

The high sugar content of the supernatant allowed for the formation of emulsions which presented a challenge for liquid/liquid extraction of the fruit samples. When the emulsion was broken by centrifugation, a plug formed at the interface between the water phase and the ethyl acetate phase and very low melatonin recovery rates were observed (less than 30%). It has been shown that melatonin is preferentially located at the interface between polar and non-polar phases and is particularly associated with membranes (Ceraulo et al. 1999). Therefore, solid phase extraction (SPE) was investigated as a means of extracting melatonin from fruit samples.

The extraction efficiency of melatonin from fruit using SPE was approximately 50% when the sample was spiked directly before loading onto the Sep-pak. This was determined by comparing the peak area for a melatonin standard to the peak area for melatonin spiked into fruit at the same concentration measured by both the molecular and fragment ion using HPLC-MS. The melatonin peak measured by the fragment ion was relatively broad and, at times, a shoulder was observed. Investigation of the method by measuring the fragment ions at m/z 174 and 178 confirmed that the substance identified as melatonin based on retention time and m/z ratio in molecular ion mode was an artifact and the amount of melatonin identified in the fragment chromatogram was below the detection limit of 0.13 ng/mL when extracted from juice using solid phase extraction.

Based on the SPE extraction results, it was unclear if all the melatonin was extracted from the fruit. Thus, solid-liquid extraction was used to determine if melatonin was being held in the solid fruit matter. Initially, the fruit was first

reconstituted with water, the water phase was removed and the remaining fruit solids were extracted with ethyl acetate. However, this led to residual water contaminating the ethyl acetate extract and the method was revised. Adding ethyl acetate directly to the freeze dried material eliminated the contamination of the solvent layer with water and water soluble compounds. Ethyl acetate preferentially excludes anthocyanins thus giving a relatively colorless extract.

The extraction efficiency of solid-liquid extraction was approximately 45% and the use of an internal standard allowed for the detection of low amounts of melatonin. The concentrations measured in the whole fruit using the fragment ion were, on average, six times higher than the concentrations observed in the juice extracted with solid phase extraction, accounting for melatonin recovery rates. This method demonstrates that melatonin is not preferentially located in the water phase following centrifugation of ground cherries and thus an all-encompassing and stronger extraction method is needed.

Melatonin Detection

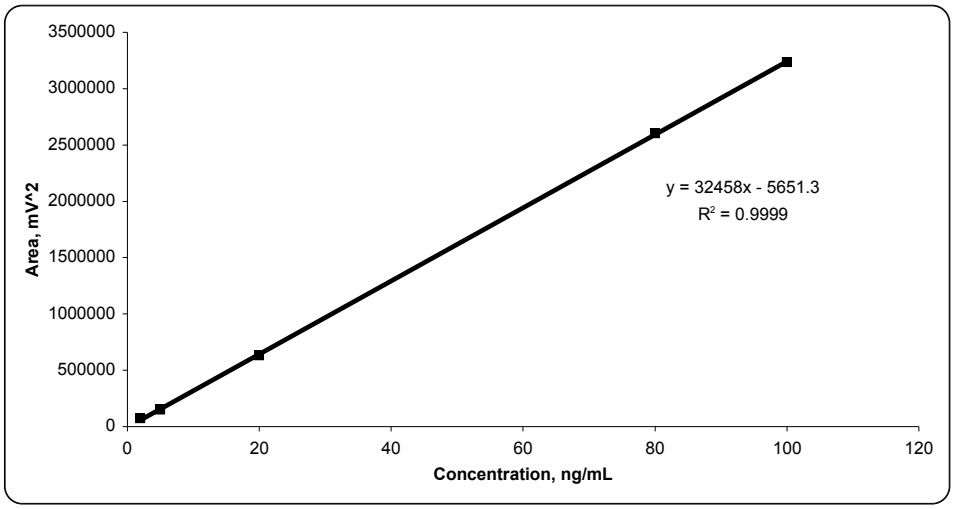
The ELISA kit from IBL International was designed for use with mammalian blood plasma and serum samples and was not able to be modified for use with fruit serums within the available time. Therefore, chromatographic methods of detection were investigated.

Fluorescence detection has high specificity since it detects molecules using both excitation and emission. The sensitivity of fluorescence is governed by Stokes shift (hv_{EX}-hv_{EM}) and allows for emission photons to be detected as isolated from excitation photons, giving low background signal. The sensitivity

can be deeply compromised by the presence of signal background. Signal background can result from reagent fluorescence or non-specific sample fluorescence. Additionally, a higher density sample will lead to light scattering, decreasing the penetration of excitation light and distorting the overall signal (2007).

The melatonin standards (2-100 ng/mL) were detected with great sensitivity and followed a linear pattern (Figure 2.4 (a)). However, the detection of melatonin in fruit samples showed a high rate of signal suppression. The calculated recovery rate of melatonin was below 7% with fluorescence, in contrast to greater than 45% using mass spectrometry. A chromatogram of Montmorency tart cherries can be found in Figure 2.4 (b). The apparent signal suppression suggests that a more specific sample clean-up regimen and extraction procedure is needed to make use of fluorescence detection for melatonin. Although, the excitation and emission wavelengths for melatonin were obtained from published literature, tuning the instrument to the melatonin standards, which may require deviating from published values, may increase the sensitivity of the detection method.

(a)



(b)

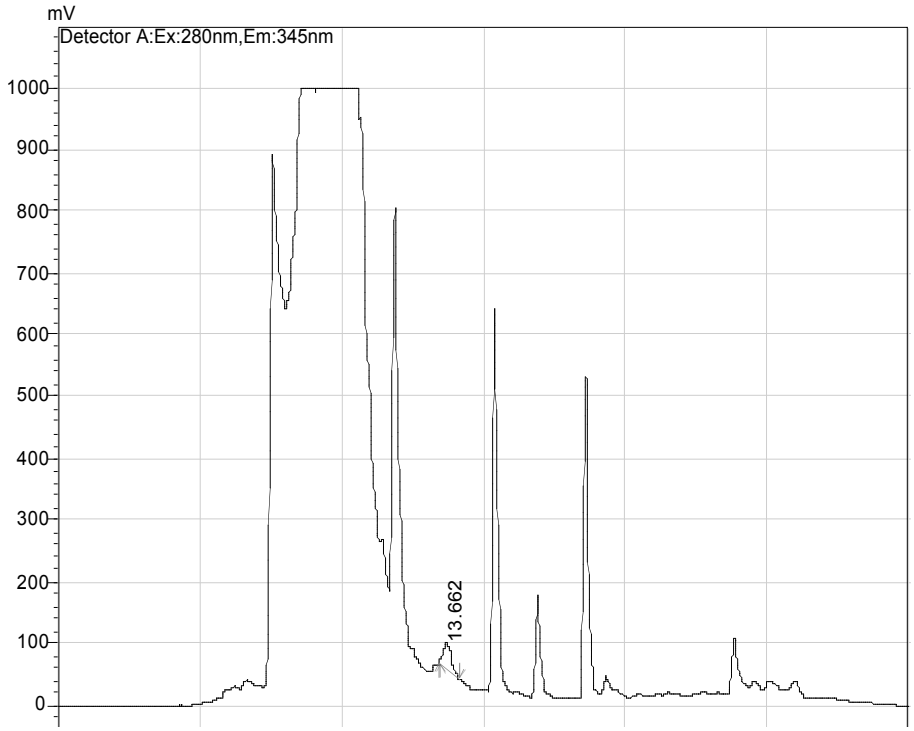


Figure 2.4 (a) Dose-response curve for melatonin standards with HPLC-FLD. (b) Chromatogram of Montmorency tart cherries showing signal suppression and/or quenching of melatonin with HPLC-FLD.

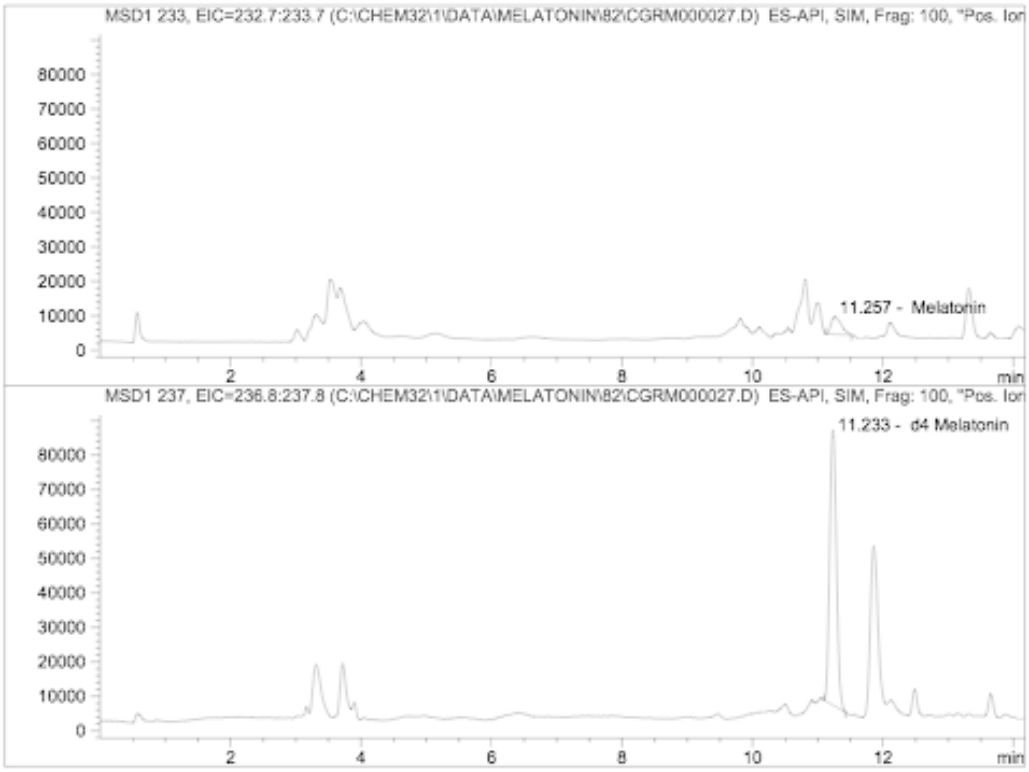
The use of mass spectrometry was the best method studied for detecting melatonin in fruit matrices. It allowed for the use of an internal standard, d4-melatonin, which both corrected for losses in analyte and provided a retention time marker. Although the instrument was a single quadrupole, both the molecular ion and the fragment ion for melatonin were able to be investigated, increasing the confidence level for determining the concentration of melatonin.

Initially, samples were filtered through nylon filters (0.45 μm, 13 mm, Fisher Scientific, Fair Lawn, NJ). However, the use of nylon filters led to the appearance of an additional peak in the d4-melatonin chromatogram. This problem was corrected by switching to PTFE filters. The chromatograms showing the extra peak from the nylon filters and the lack of peak using PTFE filters are presented in Figure 2.5.

Fruit samples prepared by solid phase extraction and analyzed for molecular ions gave melatonin concentrations presented in Figure 2.6. The levels detected in Montmorency cherries are within the range reported previously (Burkhardt et al. 2001). The melatonin peak identified in the mass chromatogram for m/z 233 appear to have an interference across all samples. Therefore, a verification of detection was performed by monitoring the fragment ions of melatonin and d4-melatonin, m/z 174 and 178, respectively.

Figure 2.5 Montmorency cherries filtered through (a) nylon filters (0.45 μm, 13 mm Fisher Scientific) and (b) PTFE filters (0.45 μm, 13 mm, Whatman)

(a)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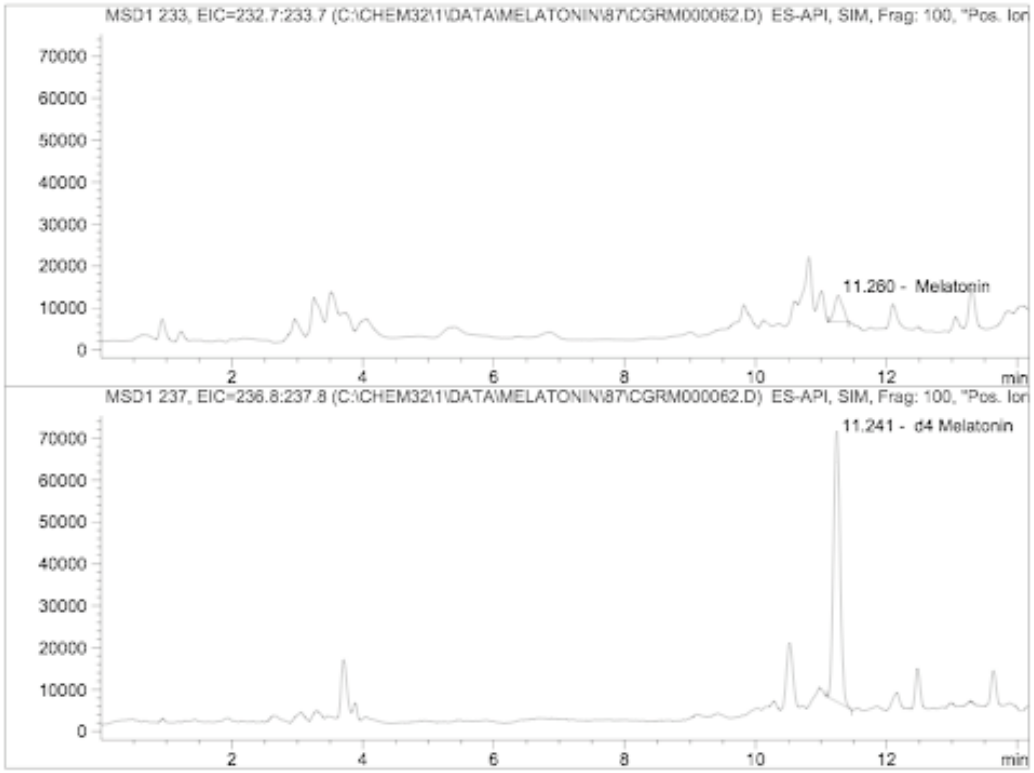
80000
70000
60000
50000
40000
30000
20000
10000
0
11.257 - Melatonin
2
4
6
8
10
12
min
MSD1 237, EIC=236.8:237.8 (C:\CHEM32\1\DATA\MELATONIN\82\CGRM000027.D) ES-API, SIM, Frag: 100, "Pos. Ion
11.233 - d4 Melatonin
80000
70000
60000
50000
40000
30000
20000
10000
0
2
4
6
8
10
12
min

Figure 2.5 (Continued)

(b)



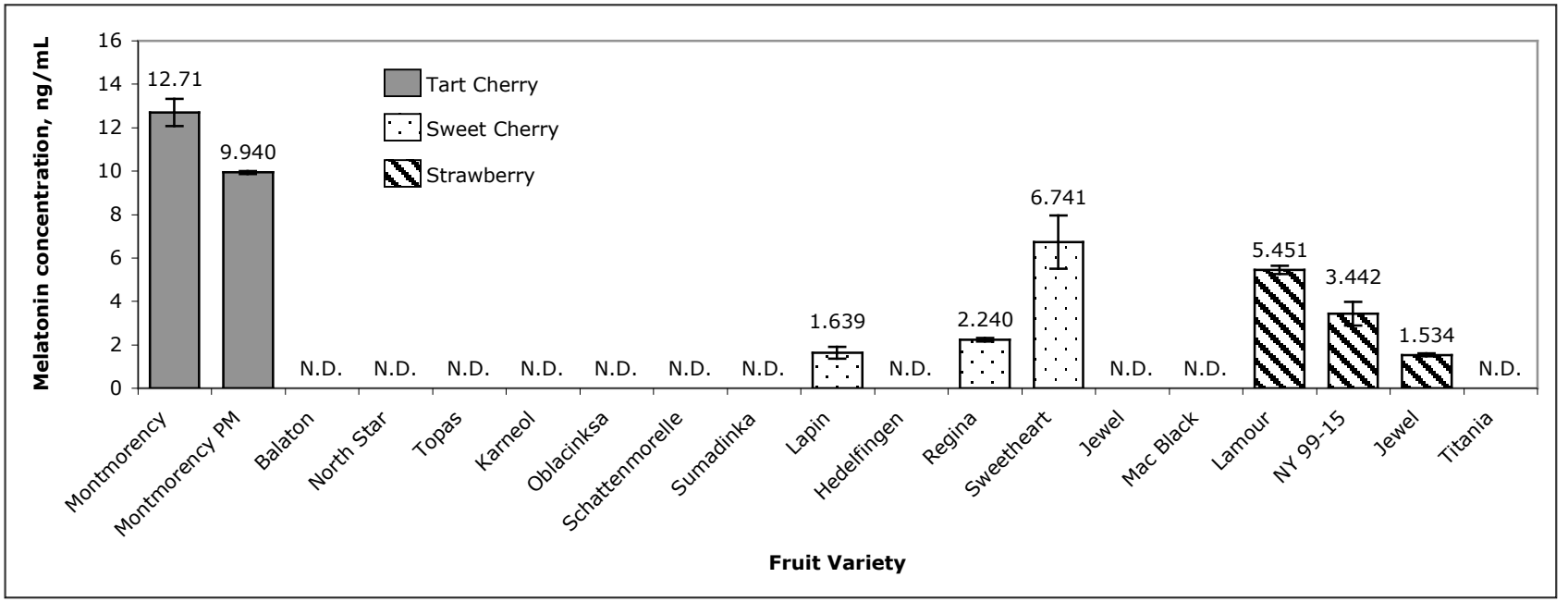


Figure 2.6 Apparent melatonin concentration detected in fruit varieties after solid phase extraction and by HPLC-MS under the molecular ion of melatonin, m/z 233 and d-4 Melatonin, m/z 237.

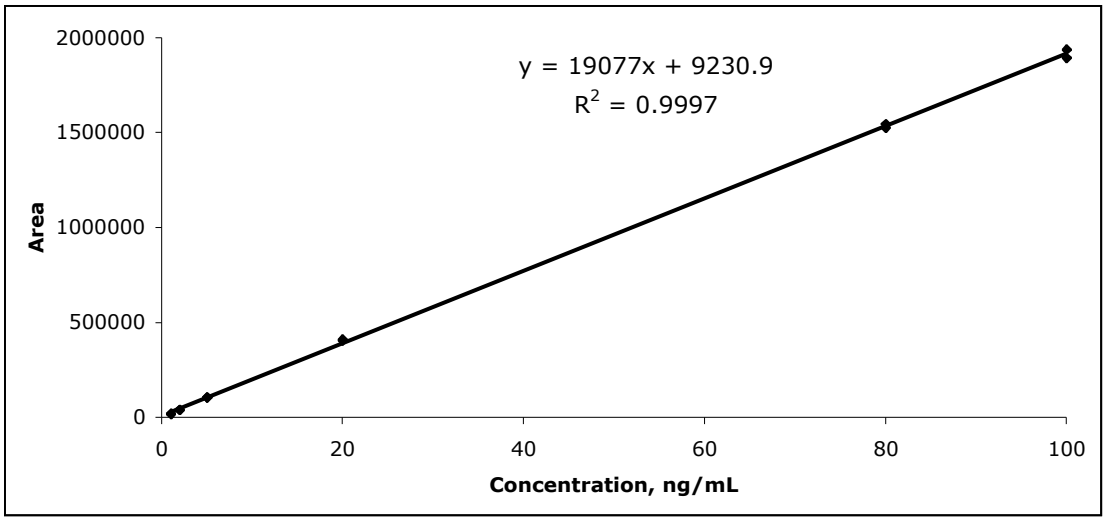


Figure 2.7 Dose-response curve for melatonin standards 1-100 ng/mL measured by HPLC-MS under fragment ion, m/z 174.

An investigation of the fragment ion of melatonin revealed good sensitivity. The limit of detection for the method was 0.13 ng/mL melatonin, calculated by the Pallesen method using a signal-to-noise ratio of 10:1 (Berthouex and Brown 2002). Measuring the response of the molecular ion and the fragment ion in the same extract of Montmorency cherries confirmed that an artifact was present in the molecular ion. The chromatograms demonstrating the discrepancy between measurement of the same sample of Montmorency cherries by molecular and fragment ion are presented in Figure 2.8. Time constraints prevented the analysis of all fruit extracts under the fragment ion and with solid-liquid extraction. However, melatonin, extracted by solid-liquid extraction, was detected in Montmorency cherries under the fragment ion in the range of 0.4-0.8 ng/mL with an average of 0.6 ± 0.3 ng/mL. The average concentration of melatonin detected by both the molecular ion and the fragment ion is presented in Figure 2.9.

Figure 2.8 Demonstration of melatonin artifact present in molecular ion chromatogram: Montmorency cherries, extracted by solid phase extraction and detected by (a) molecular ion (m/z 233) and (b) fragment ion (m/z 174)

(a)

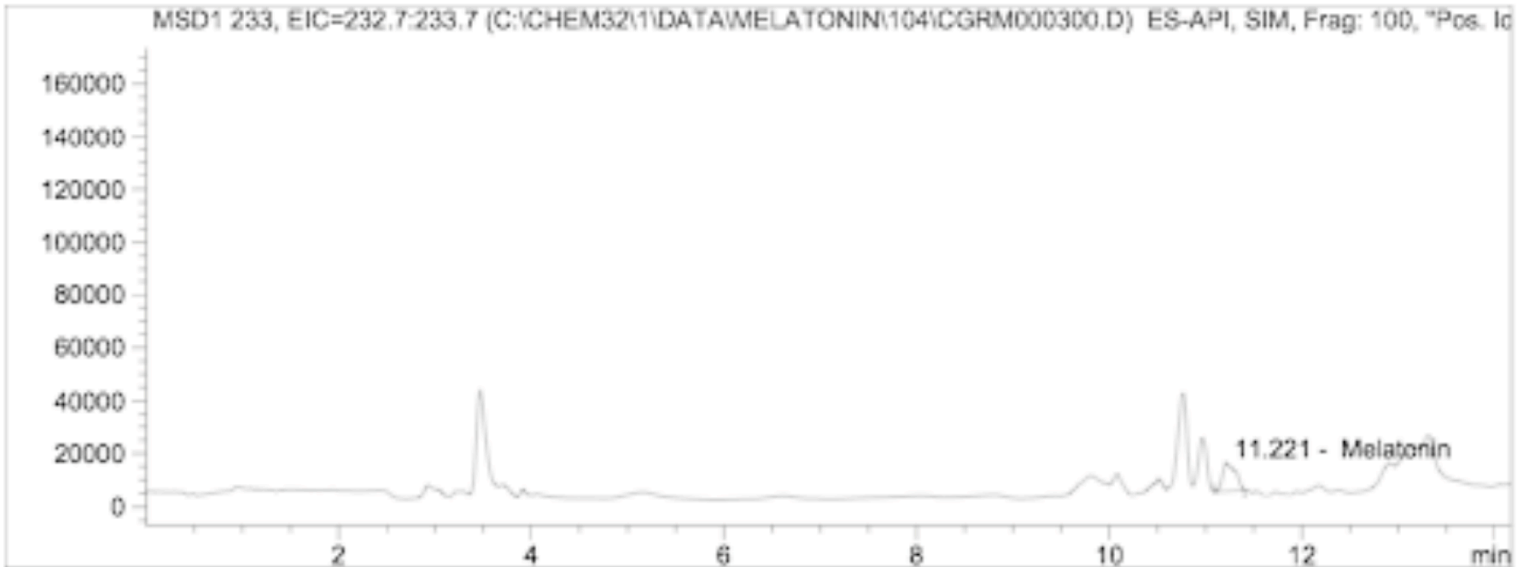
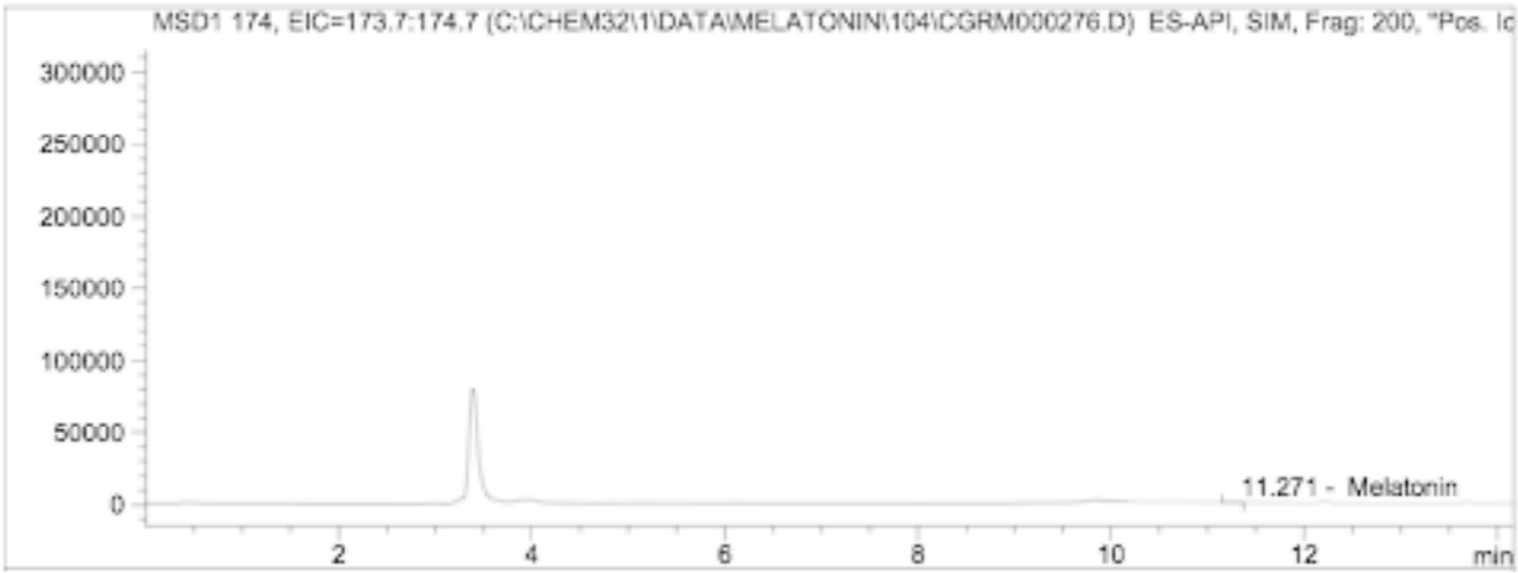

MSD1 233, EIC=232.7:233.7 (C:\CHEM32\1\DATA\MELATONIN\104\CGRM000300.D) ES-API, SIM, Frag: 100, *Pos. Io
160000
140000
120000
100000
80000
60000
40000
20000
0
2
4
6
8
10
12
min
11.221 - Melatonin

Figure 2.8 (Continued)

(b)



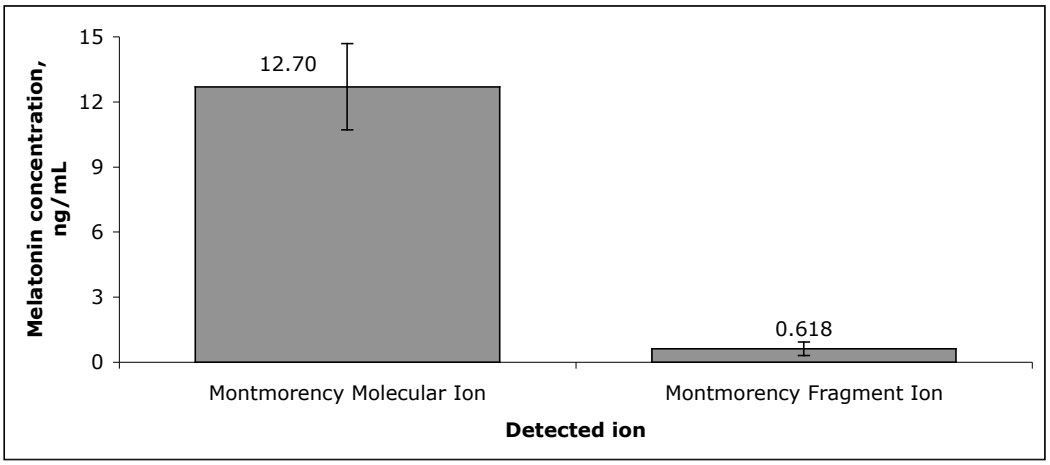


Figure 2.9 Detected concentration of melatonin in Montmorency cherries by measuring molecular ion (m/z 233) versus fragment ion (174).

Montmorency cherries, extracted by solid phase extraction, were measured under the molecular ion (m/z 233) and fragment ion (m/z 174) with the deuterated internal standard. In order to investigate method validity, melatonin was spiked into Montmorency cherries at the levels of 2 ppb and 5 ppb (n=3 per level). On average, 94.3% of the 2 ppb spike and 88.3% of the 5 ppb spike were recovered and measured by the fragment ion. The average concentration of melatonin for each spike level by measurement of the fragment ion are presented in Figure 2.10. No discernable trend was found under the molecular ion. Chromatograms of the fragment ion for each level are presented in Figure 2.11.

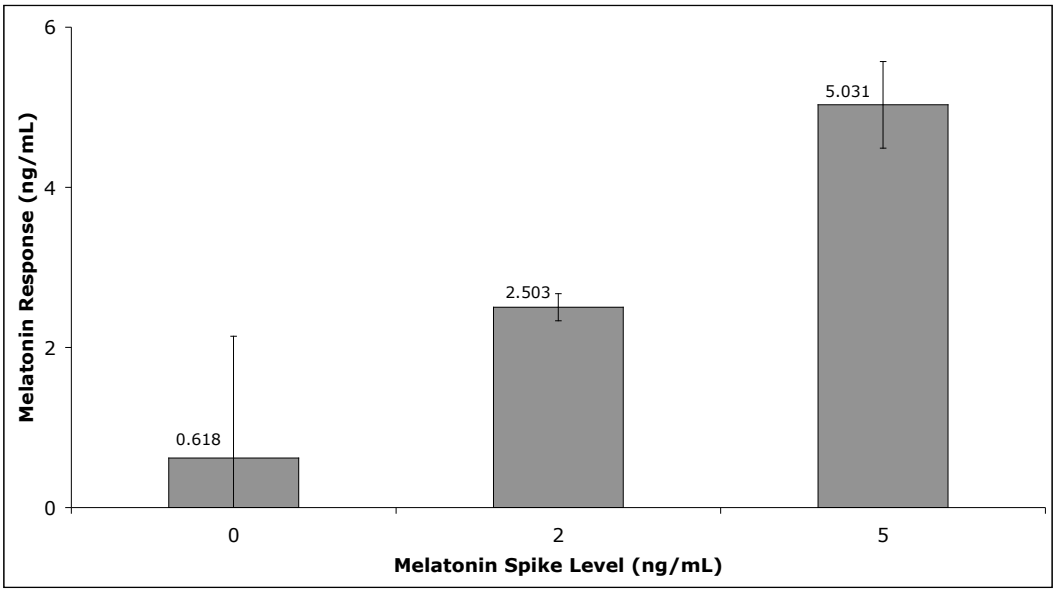
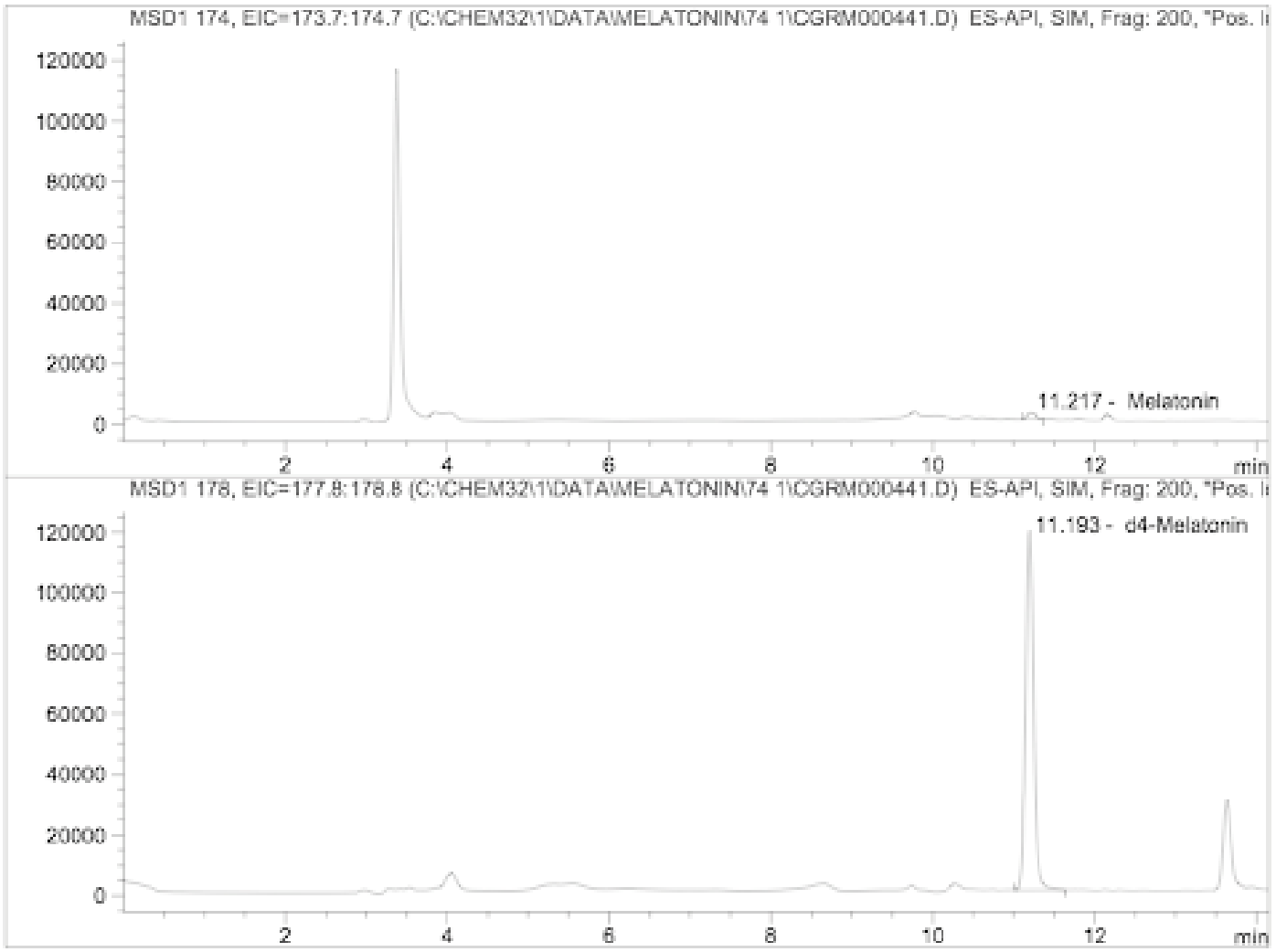


Figure 2.10 Mean values of melatonin recovered from Montmorency cherries (solid/liquid extraction) for spike levels 0, 2, and 5 ng/mL.

Figure 2.11 Chromatograms for 0, 2, and 5 ng/mL melatonin spiked into Montmorency cherries, extracted with solid/liquid extraction, measured by fragment ion (m/z 174, d-4 melatonin m/z 178).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120000
100000
80000
60000
40000
20000
0
11.217 - Melatonin
2
4
6
8
10
12
min
MSD1 178, EIC=177.8:178.8 (C:\CHEM32\1\DATA\MELATONIN\74 1\CGRM000441.D) ES-API, SIM, Frag: 200, "Pos. I
11.193 - d4-Melatonin
120000
100000
80000
60000
40000
20000
0
2
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10
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min

Figure 2.11 (Continued)

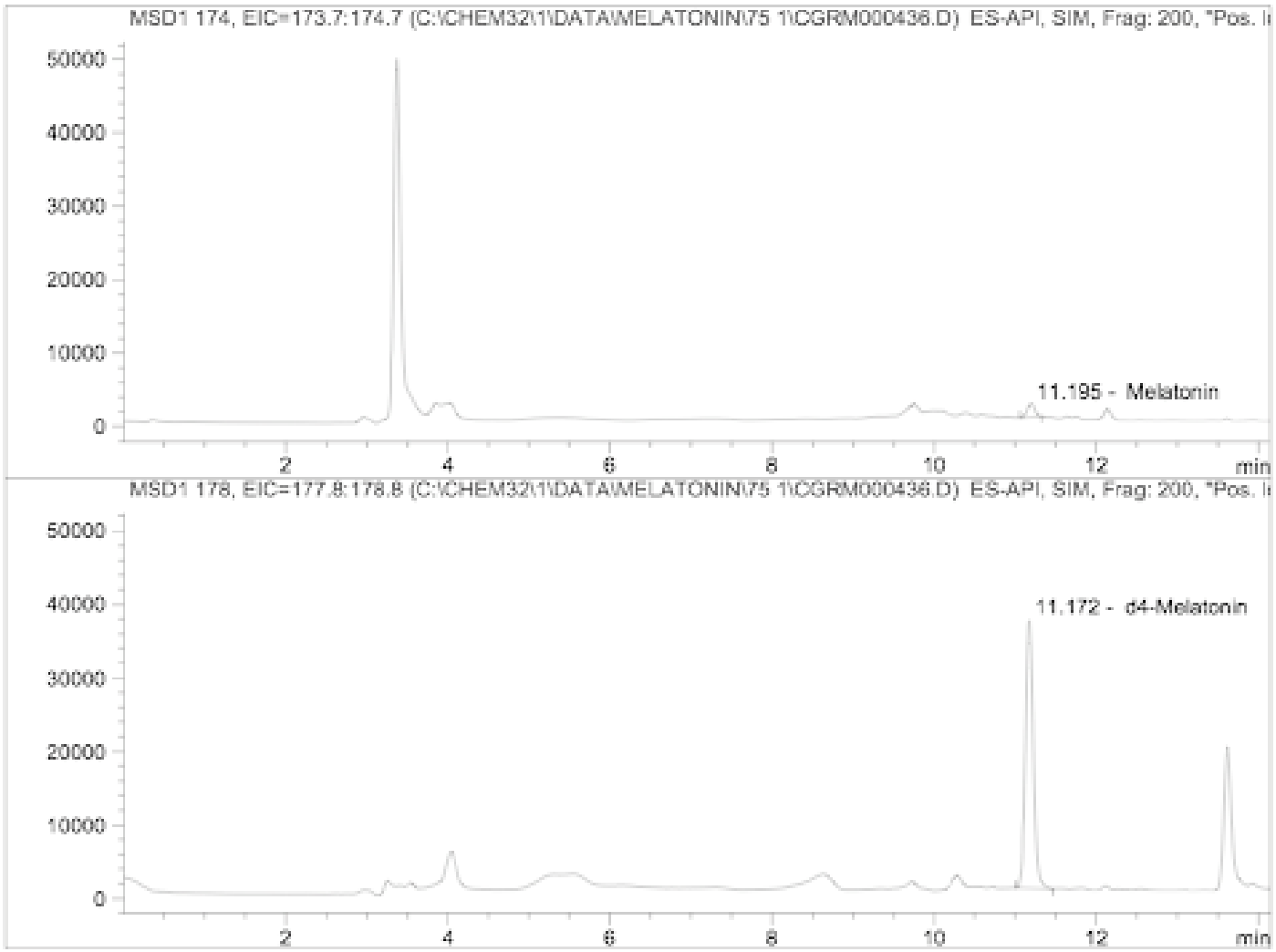
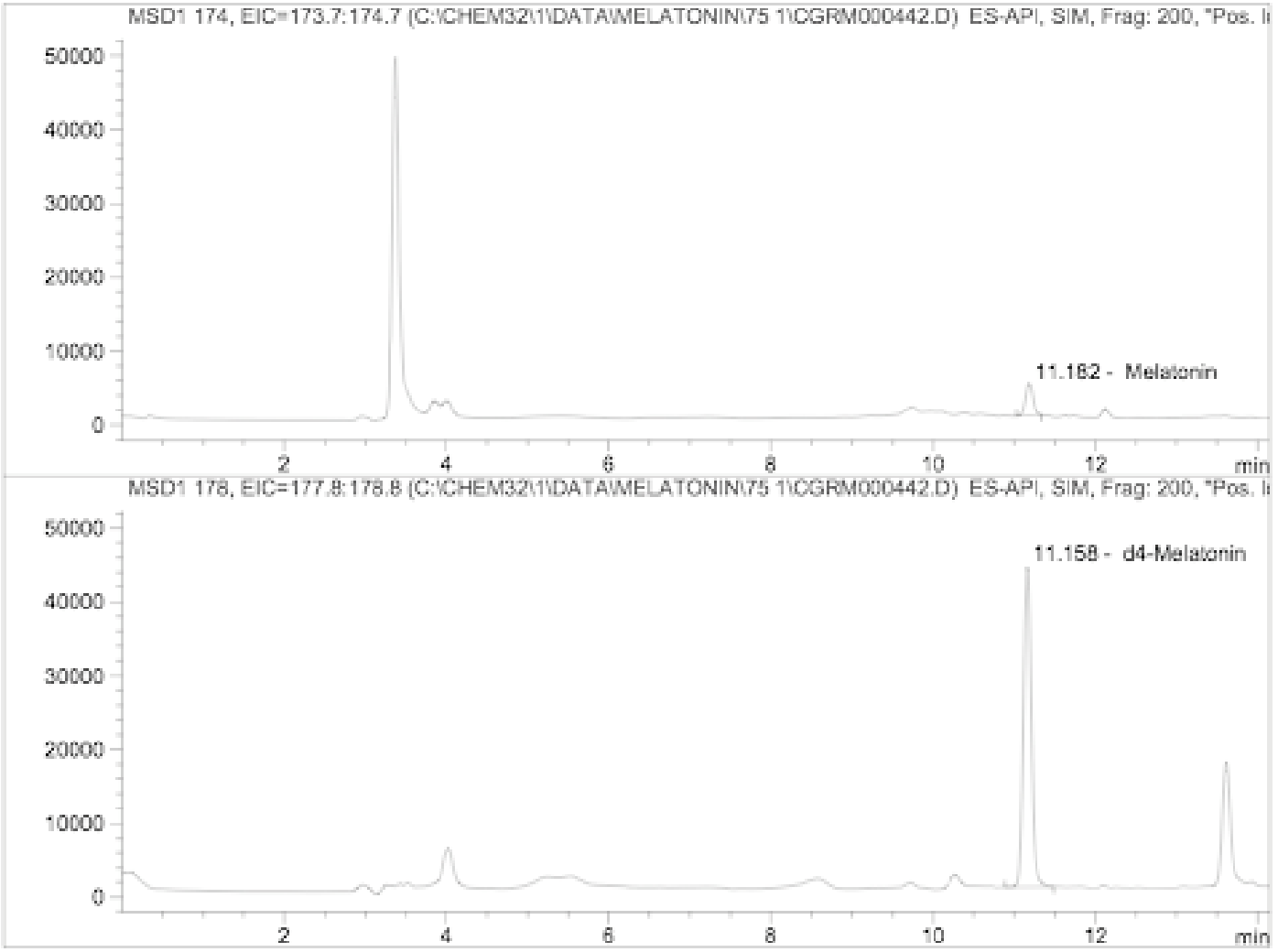


Figure 2.11 (Continued)



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Iinuma, F., K. Hamase, S. Matsubayashi, M. Takahashi, M. Watanabe and K. Zaitsu (1999). "Sensitive Determination of Melatonin by Precolumn Derivatization and Reversed-Phase High-Performance Liquid Chromatography." Journal of Chromatography A **835**(1-2): 67-72.

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CHAPTER 3

CONCLUSIONS AND FUTURE WORK

Melatonin Stability

Melatonin was stable for up to one hour in 0.05 M potassium phosphate buffer, pH 3.5 at both 85ºC and 17 par. Further work on melatonin stability remains. The effect of pH should be investigated to determine if pH plays a role in the heat or light stability of melatonin. Furthermore, the light treatment performed used ambient lighting. Modifiying the study to include multiple wavelengths of light (particularly at 254 nm, the wavelength at which melatonin has been reported to absorb) would give useful information for protecting melatonin during analysis and processing (Hirano et al. 2004; Navara and Nelson 2007; Bromme et al. 2008).

To address the interest in dietary melatonin, the compound should be investigated in its natural environment (within the fruit) and the effects of individual fruit components are worth considering. For example, cherries are known to oxidize quickly which leads to the degradation of some polyphenols and anthocyanins. It would be interesting to investigate the stability of melatonin in the presence of these molecules. What types of reactions in fruit prompt melatonin to engage in antioxidant activity? How does melatonin act in the presence of other antioxidative molecules?

Additionally, the stability of melatonin should be investigated over longer periods of time. For example, if melatonin is determined to be stable in juice throughout processing, it would be important to determine stability over the product shelf life.

The effect of aeration and oxygenation on melatonin should also be investigated. As demonstrated by Bromme and Maharaj, melatonin concentrations decrease relatively quickly when exposed to light during aeration (Maharaj et al. 2002; Bromme et al. 2008). The effect of aeration on melatonin must therefore be investigated separately from exposure to ultra-violet light in order to understand the cause of the melatonin degradation.

Extracting Melatonin from Fruit Matrices

For the purposes of this study, solid/liquid extraction with ethyl acetate from freeze-dried samples gave the most complete quantification of melatonin. However, the resulting extract was not clean enough for use with fluorescence detection. Further clean-up may allow for the use of fluorescence as a sensitive and specific detection method. Possible techniques include the use of different solvents, combinations of solvents, and combinations of extraction methods, such as solid/liquid extraction followed by solid phase extraction. Another option for purification that should be considered is using chromatography to separate and collect the melatonin fraction to eliminate signal suppression or quenching from other compounds during fluorescence detection.

Further investigation into chloroform as a solvent for extraction is warranted as a means to eliminate the artifact detected with HPLC-MS. Also, characterization of the interference may provide useful information for determining the best extraction methods for melatonin from fruit matrices.

Melatonin is the acetylated form of serotonin, which is part of the melatonin pathway (Murch et al. 2000). Therefore, it is feasible that in extracting with ethyl acetate, melatonin is artificially increased through the acetylation of melatonin precursors or other compounds present in the matrix. Further investigation into the melatonin pathway and the effect of exposure to ethyl acetate or other solvents on matrix components is necessary.

A greater understanding of melatonin's location within fruit, its forms, and the types of associations it has with other compounds would be a great asset in designing an extraction regimen. For example, if melatonin is located within membranes, protein and lipid extraction techniques may be useful, whereas if it is associated with the nucleus, DNA extraction techniques may be beneficial.

Detecting Melatonin

During the course of this study, detection of melatonin was found to be most reliable with mass spectrometry,. However, fluorescence detection is a viable option that, with further investigation, offers increased sensitivity over mass spectrometry. Tuning the fluorescence detector to a melatonin standard, as opposed to relying on published values, may decrease signal suppression and increase the sensitivity of the system. Furthermore, in conjunction with more developed extraction procedures, fluorescence may provide an advantage over mass spectrometry.

The chromatographic system used with mass spectrometry is worth investigating to determine whether the artifact interfering with melatonin detection under the parent ion can be eliminated or separated out.

Additionally, the use of a tandem mass spectrometer would give detailed information on the transition of melatonin from the parent ion to the daughter ion and, in doing so, may give additional information regarding interference.

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