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Simultaneous analysis of serotonin, melatonin, piceid and resveratrol in fruits using liquid chromatography tandem mass spectrometry

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ABSTRACT

An analytical method was developed for the simultaneous quantification of serotonin, melatonin, *trans*- and *cis*-piceid, and *trans*- and *cis*-resveratrol using reversed-phase high performance liquid chromatography coupled to mass spectrometry (HPLC–MS) with electrospray ionization (ESI) in both positive and negative ionization modes. HPLC optimal analytical separation was achieved using a mixture of acetonitrile and water with 0.1% formic acid as the mobile phase in linear gradient elution. The mass spectrometry parameters were optimized for reliable quantification and the enhanced selectivity and sensitivity selected reaction monitoring mode (SRM) was applied. For extraction, the direct analysis of initial methanol extracts was compared with further ethyl acetate extraction. In order to demonstrate the applicability of this analytical method, serotonin, melatonin, *trans*- and *cis*-piceid, and *trans*- and *cis*-resveratrol from 24 kinds of commonly consumed fruits were quantified. The highest serotonin content was found in plantain, while orange bell peppers had the highest melatonin content. Grape samples possessed higher *trans*- and *cis*-piceid, and *trans*- and *cis*-resveratrol contents than the other fruits. The results indicate that the combination of HPLC–MS detection and simple sample preparation allows the rapid and accurate quantification of serotonin, melatonin, *trans*- and *cis*-piceid, and *trans*- and *cis*-resveratrol in fruits.

1. Introduction

Melatonin (N-acetyl-5-methoxytryptamine) and serotonin (5-hydroxytryptamine) are indolamines (Fig. 1). Melatonin is a neurohormone produced by the pineal gland of animals, synthesized from the amino acid L-tryptophan via serotonin [1]. It has many physiological effects on humans [2,3] including ones that influence circadian rhythm, sleeping disorders, jet lag [4], free radical disorders [5], and cancer [6,7]. Serotonin mediates a wide range of activities in various animal cells as a neurotransmitter, and as a hormone and mitogenic factor [8]. It also affects mood behaviours [9] and body temperature [10]. These two indoleamines have recently been reported to have widespread occurrence in many edible plants at varying but significant concentrations [11].

Resveratrol (3,4′,5-trihydroxystilbene) and its 3-O-β-D-glucoside, piceid, are stilbene phytoalexins (Fig. 1). They are two of many secondary metabolites produced by plants that may contribute to the potential health benefits attributed to some food plants. As a result of cardioprotective, neuroprotective, antileukemic and antioxidant effects [12,13], resveratrol and piceid have gained much attention as functional food ingredients [14]. In nature, resveratrol and piceid exist in both *trans*- and *cis*-isomeric forms, which may have different biological effects [15–17]. Due to its stability, the *trans*-isomer is the most commonly used and the *cis*-isomer is unavailable commercially. *Trans*-forms can be converted into the *cis*-forms on exposure to UV irradiation [18].

Several different methods have been developed to determine serotonin, melatonin, *trans*- and *cis*-piceid, and *trans*- and *cis*-resveratrol in edible plants. The most commonly used are high performance liquid chromatography with UV [19–21], electrochemical [22,23], or fluorescence detectors [24–26]. Capillary electrophoresis (CE) techniques have also been reported and the sensitivity is similar to the HPLC methods [27–29]. Because of the low concentration of serotonin, melatonin, *trans*- and *cis*-piceid, and *trans*- and *cis*-resveratrol in most edible plants, sensitive and specific techniques are needed. Although the use of gas chromatography coupled to mass spectrometry (GC–MS) has been reported and has been shown to have improved sensitivity and selectivity, derivatization is required prior to analysis [30,31]. Recently, with the development of MS interface technology, high performance liquid chromatography coupled to mass spectrometry (HPLC–MS) has increased in popularity and been shown to be a powerful tool for complex sample analysis. It has already been applied to individual determinations of serotonin, melatonin, *trans*- and *cis*-piceid, or *trans*- and *cis*-resveratrol in edible plants [32–37], but not to their simultaneous determination.

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This study presents the development of an HPLC–MS method to determine, efficiently and simultaneously, serotonin, melatonin, *trans*- and *cis*-piceid, and *trans*- and *cis*-resveratrol. Gradient elution in HPLC and optimization of MS detection parameters were adjusted to obtain clear resolution of the peaks and maximum signal in the MS detector. To prepare samples, methanol extraction and further ethyl acetate extraction were carried out and compared in order to assess the accuracy of the extraction methods. Finally, we applied the analytical method and extraction procedure we developed to 44 samples from 24 different kinds of fruit. The results demonstrated that the analytical method presented here is rapid and reliable for the identification and quantification of serotonin, melatonin, *trans*- and *cis*-piceid, and *trans*- and *cis*-resveratrol in complex edible plant materials.

2. Experimental

2.1. Chemicals and reagents

Serotonin was obtained from Alfa Aesar (Ward Hill, MA, USA), melatonin from Sigma–Aldrich (Oakville, ON, Canada), and *trans*-piceid and *trans*-resveratrol from AvaChem Scientific (San Antonio, TX, USA).

HPLC grade acetonitrile was obtained from Fisher Scientific (Ottawa, ON, Canada), HPLC grade methanol and HPLC–MS grade water from EMD chemicals (Gibbstown, NJ, USA), HPLC–MS grade formic acid from Sigma–Aldrich, and HPLC grade ethyl acetate from Honeywell Burdick & Jackson (Morristown, NJ, USA).

2.2. Instrumentation

All determinations were carried out using an HPLC system (Ultimate 3000, Dionex, Bannockburn, IL, USA) which consisted of a pump, an autosampler, a column compartment, and a photodiode array detector, coupled with Finnigan LTQ mass spectrometer (Thermo Scientific, West Palm Beach, FL, USA) and controlled with Xcalibur 2.0 data system software.

The separations were performed using an Onyx Monolithic C_{18} HPLC column (100 mm × 3.0 mm; Phenomenex, Torrance, CA, USA) and an Onyx Monolithic C_{18} guard column (5 mm × 4.6 mm; Phenomenex) at 35 °C column temperature. The temperature of the autosampler was set at 15 °C and the injection volume was 5 μL.

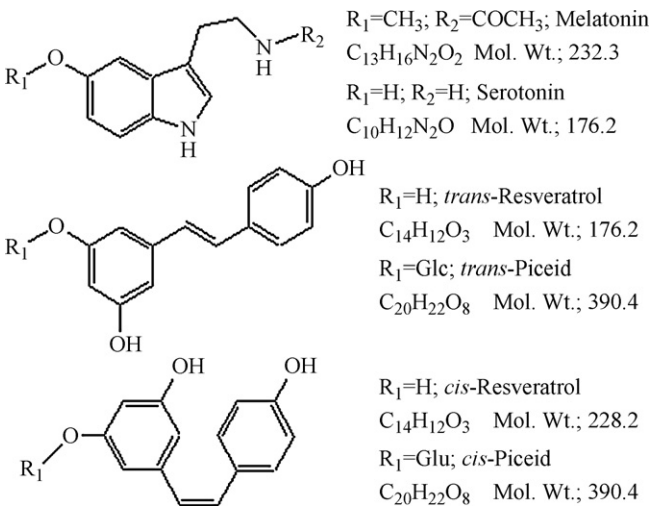

Fig. 1. Structures of serotonin, melatonin, *trans*- and *cis*-piceid, and *trans*- and *cis*-resveratrol.

The mass spectrometry was carried out using electrospray ionization (ESI). Selected reaction monitoring (SRM) was applied for HPLC–MS quantification of melatonin, serotonin, *trans*- and *cis*-piceid, and *trans*- and *cis*-resveratrol. The mass range and scan rate were set at normal and the data were scanned in centroid mode. The MS conditions are presented in Table 1.

2.3. Optimization of mass spectrometry and chromatography conditions

For all the analytes investigated, in order to gain maximum sensitivity, the mass spectrometry parameters were optimized by tuning each standard while directly infusing individual standard solutions at 5 μL/min. While other parameters were set manually, the spray voltage was studied in the range 3.0–4.5 kV and the capillary temperature was checked by varying it from 200 to 275 °C to find the best experimental conditions. Among other parameters,

Table 1
MS conditions in LC–MS detection of serotonin, melatonin, *trans*- and *cis*-piceid, and *trans*- and *cis*-resveratrol.

Conditions	Serotonin	*trans*-Piceid	Melatonin	*cis*-Piceid	*trans*-Resveratrol	*cis*-Resveratrol
ESI source						
Sheath gas flow rate (arb)	30	30	30	30	30	30
Aux gas flow rate (arb)	5	5	5	5	5	5
Sweep gas flow rate (arb)	0	0	0	0	0	0
Spray voltage (kV)	3.50	3.50	4.00	3.50	3.50	3.50
Capillary temp. (°C)	250.00	250.00	250.00	250.00	250.00	250.00
Capillary voltage (V)	7.00	−47.00	9.00	−47.00	−50.00	−50.00
Tube lens (V)	50.00	−121.50	55.00	−121.50	−96.50	−96.50
Ion optics						
Multipole 00 offset (V)	−3.25	3.00	−1.50	3.00	3.00	3.00
Lens 0 voltage (V)	−3.50	3.50	−4.50	3.50	4.50	4.50
Multipole 0 offset (V)	−5.00	5.75	−4.75	5.75	5.75	5.75
Lens 1 voltage (V)	−11.00	37.00	−10.00	37.00	39.00	39.00
Gate lens voltage (V)	−74.00	10.00	−68.00	10.00	10.00	10.00
Multipole 1 offset (V)	−16.50	7.00	−13.50	7.00	7.00	7.00
Multipole RF amplitude (V p–p)	400.00	400.00	400.00	400.00	400.00	400.00
Front lens	−5.25	6.25	−5.00	6.25	6.50	6.50
Define scan						
Ionization mode	Positive	Negative	Positive	Negative	Negative	Negative
Time range (min)	3.00–15.00	15.00–25.00	25.00–27.30	27.30–28.80	28.80–32.00	32.00–45.00
SRM transition (m/z)	177.00→160.00	389.20→227.20	233.00→174.00	389.20→227.20	227.00→185.30	227.00→185.30
Normalized collision energy	20.0	20.0	20.0	20.0	25.0	25.0
Scan ranges	159.25–160.75	226.45–227.95	173.25–174.75	226.45–227.95	184.55–186.05	184.55–186.05

the normalized collision energy was chosen to achieve the highest signal intensity of SRM detection.

To check the ionization mode of the standards, direct injection of each solution was analyzed under the ESI ion source in both positive and negative ion modes. For melatonin and serotonin, under the positive ion mode, the protonated molecular ion $[M+H]^+$ was the major ion in the MS spectra. While in the negative ion mode, the deprotonated molecular ion $[M-H]^-$ showed very low abundance. For piceid and resveratrol, higher sensitivity was achieved in the negative ion mode. Thus, HPLC–MS experiments were carried out in positive ion mode for melatonin and serotonin and in negative ion mode for *trans*- and *cis*-piceid, and *trans*- and *cis*-resveratrol.

To select the SRM transitions of the detected compounds, an MS^2 experiment was performed by infusing standard solutions directly. For all the compounds investigated, normalized collision energy experiments were chosen in order to reduce the relative abundance of the precursor ion to about 20%.

After direct MS analysis, the MS instrument was coupled to the HPLC system. First, the individual standard solutions were analyzed separately to determine their individual behaviour in the on-line system. Then the chromatographic conditions were optimized through several trials using the combined standards solution to ensure the appropriate resolution. For this, the various mobile phases and chromatographic gradients were studied. Adequate separation was achieved in 60 min by a linear gradient elution and a mobile phase consisting of 0.1% (v/v) formic acid in acetonitrile (solvent A) and 0.1% (v/v) formic acid in LC–MS grade water (solvent B). The gradient elution program was applied at a flow rate of 0.2 mL/min as follows: initial conditions of 5% (v/v) A held for 5 min, increased linearly to 35% (v/v) A in 30 min, increased linearly to 100% (v/v) A in 5 min, held at 100% (v/v) A for 5 min, returned to initial conditions in 5 min and maintained for 10 min.

2.4. Standard solution preparation

Individual standard solutions of serotonin, melatonin, *trans*-piceid and *trans*-resveratrol at 0.1 mg/mL were prepared with 1 mg of each compound dissolved in 10 mL of 80% (v/v) methanol–water. *Cis*-piceid and *cis*-resveratrol are not commercially available, because of their instability in solid form. Thus the *cis*-isomers were produced from the *trans*-isomers in solution by exposure to UV light (365 nm) at room temperature for up to 120 min and used as *cis*-standards in HPLC–MS analysis. Because previous studies have reported that the *trans*-form to *cis*-form isomerisation rate is less than 100% [38,39], even after a long exposure to UV light, the concentrations of the *cis*-isomers were calculated from the difference between the concentrations before and after UV light exposure of the *trans*-isomers.

Composite standard solutions were prepared by combining aliquots of each of the individual standard solutions and diluting them with 80% (v/v) methanol–water to obtain the final concentrations appropriate for the different samples. The standard solutions were prepared fresh every day and protected from light or kept under dim light. Calibration curves of serotonin, melatonin, *trans*- and *cis*-piceid, and *trans*- and *cis*-resveratrol were obtained using six dilutions of the initial standard mixture solution. These calibration curves were used to determine the amounts of serotonin, melatonin, *trans*- and *cis*-piceid, and *trans*- and *cis*-resveratrol in different fruit samples.

2.5. Sample preparation

The fruits used were obtained from local markets or orchards at different times. The following 24 fruits were studied: green and red seedless grapes, plantain, banana, cranberry, strawberry, blackberry, raspberry, Saskatoon berry, Lapins and Sweetheart sweet cherries, green, orange and red bell peppers, grape tomato, plum, peach, nectarine, Bartlett and D'Anjou pears, and Gala, Golden Delicious, Granny Smith, and Spartan apples. The fresh edible tissues of each fruit were washed with water, cut using standard kitchen tools, and put in a freezer. The frozen products were freeze-dried, then powdered using a coffee grinder, and maintained at −20 °C until extraction.

Extraction of serotonin, melatonin, *trans*- and *cis*-piceid, and *trans*- and *cis*-resveratrol from the fruits was performed using methanol extraction and ethyl acetate concentrations. This sample preparation procedure was optimized using published methods for serotonin and melatonin [21,22,36,40], and piceid and resveratrol [41,42], with some modifications.

For each extraction, a 2.5 g aliquot of the freeze-dried powdered sample was placed into a centrifuge tube and mixed with 25 mL HPLC grade methanol. The sample was homogenized for 60 s and then centrifuged at 10,000 × *g* for 10 min. The supernatant was filtered through a 0.22 μm PVDF syringe filter (Chromatographic Specialties Inc., Brockville, ON, Canada) and used for direct HPLC–MS analysis.

To further concentrate melatonin, *trans*- and *cis*-piceid, and *trans*- and *cis*-resveratrol in the methanol extracts of samples, ethyl acetate extraction was used. A methanol extract (10 mL) was reduced to 1 mL under vacuum using a SpeedVac at ∼40 °C. To the concentrated methanol solution, 5 mL Milli-Q water and 6 mL ethyl acetate were added. The ethyl acetate extraction was achieved on a nutating mixer for 15 min. An aliquot of ethyl acetate extract (3 mL) was evaporated to dryness under vacuum using a SpeedVac at ∼40 °C. The residue was redissolved in 1 mL 80% (v/v) methanol–water and the supernatants were analyzed by HPLC–MS.

The entire extraction procedure was carried out protected from light or under dim light and at room temperature. The extracted samples were analyzed as soon as the extraction was completed. The extraction of each sample was repeated five times. Solvent samples extracted under the same procedure and used as blank samples were also analyzed.

2.6. Validation

The performance of the method was evaluated using the following figures of merit: retention time standard deviation (SD); linearity; linear range; intraday and interday repeatability; recovery; and limits of detection (LOD) and quantification (LOQ). Analytical curves were constructed to estimate the linear ranges, correlation coefficients, and detection and quantification limits for the proposed HPLC–MS method. The linearity of the method was evaluated by calculation of the regression line and expressed by the coefficient of correlation (R^2). The detectability of the method was evaluated by determining the LOD and LOQ, which were calculated as 3 and 10 times the signal-to-noise ratio (S/N), respectively. Intraday repeatability was assessed by five determinations in one day. Interday repeatability was assessed by five determinations on three separate days. The recovery was evaluated with separately prepared individual and mixed working solutions of serotonin, melatonin, *trans*-piceid, and *trans*-resveratrol over the linear dynamic range at different levels. The solutions with and without process under the extraction procedure were determined. The recoveries were calculated using the levels difference of serotonin, melatonin, *trans*-piceid, and *trans*-resveratrol in solutions with and without extraction. In methanol extracts, the levels were serotonin 1.2 ng/μL, melatonin 1.2 pg/μL, *trans*-piceid 120 pg/μL, and *trans*-resveratrol 60 pg/μL, respectively. The levels in ethyl acetate concentrates were serotonin 6.0 ng/μL, melatonin 6.0 pg/μL, *trans*-piceid 600 pg/μL, and *trans*-resveratrol 300 pg/μL, respectively. The recovery of *cis*-piceid and *cis*-resveratrol, was evaluated by separating prepared individual and mixed working

Table 2
Performance of the analytical method for serotonin, melatonin, *trans*- and *cis*-piceid, and *trans*- and *cis*-resveratrol.

Standards	Retention time SD (min)	LOD (pg/μL)	LOQ (pg/μL)	Intraday repeatability RSD (%)	Interday repeatability RSD (%)	Correlation coefficient (R^2)	Linear range (pg/μL)	Recovery (%)	
								Methanol	Ethyl acetate
Serotonin	0.02	50	150	0.2	0.8	0.9995	500–10,000	100.9 ± 0.2	–
Melatonin	0.02	0.01	0.03	0.2	0.5	0.9999	0.5–10	101.3 ± 0.2	92.6 ± 2.1
trans-Piceid	0.02	25	75	1.3	1.3	0.9998	50–1000	102.2 ± 1.8	72.7 ± 4.0
cis-Piceid	0.02	0.5	1.5	0.8	0.7	0.9999	25–500	100.1 ± 0.4	58.3 ± 0.8
trans-Resveratrol	0.02	7.5	25	0.6	2.0	0.9999	25–500	99.6 ± 1.5	99.2 ± 0.7
cis-Resveratrol	0.02	2.5	7.5	0.5	2.4	0.9998	15–300	99.9 ± 0.5	99.9 ± 0.5

"–": no recovery.

solutions of *trans*-piceid and *trans*-resveratrol after exposure to UV light for 120 min and then diluted over the linear dynamic range at different levels. The recoveries were calculated by the levels difference of *cis*-piceid and *cis*-resveratrol in solutions with and without extraction. In methanol extracts, the levels were *cis*-piceid 60 pg/μL and *cis*-resveratrol 36 pg/μL, respectively. The levels in ethyl acetate concentrates were *cis*-piceid 300 pg/μL and *cis*-resveratrol 180 pg/μL, respectively. Recovery was assessed by five repetitions.

3. Results and discussion

3.1. Development of HPLC–MS method

One aim of this work was to develop and validate an HPLC–MS method with high sensitivity and selectivity in order to simultaneously detect and quantify serotonin, melatonin, *trans*- and *cis*-piceid, and *trans*- and *cis*-resveratrol in different edible plant materials.

As serotonin and melatonin both have two nitrogen atoms in their structures (Fig. 1), it is easy to add a proton to their structures in the ion source and produce the protonated molecular ions; thus, the positive ion mode is more sensitive for the determination of serotonin and melatonin. For piceid and resveratrol, the deprotonated molecular ion $[M-H]^-$ presented higher sensitivity than the protonated molecular ion $[M+H]^+$ due to the phenolic hydroxyl group in their structures.

The MS^2 spectra we obtained for serotonin, melatonin, *trans*-piceid, and *trans*-resveratrol are presented in Fig. 2. From these spectra, the most abundant product ions reflected the selected SRM transitions of the detected compounds and their fragmentation pathways could be deduced. The precursor ion of serotonin was at *m*/*z* 177 and the most abundant ion at *m*/*z* 160, which involved the neutral loss of NH_3. For melatonin, the $[M+H]^+$ ion was at *m*/*z* 233 and it produced the major ion at *m*/*z* 174 through loss of the NH_2COCH_3 (59 Da) fragment. Serotonin and melatonin fragmented at the same position in their structures gave rise to the highest abundance fragment ions, which were monitored by SRM detection. By choosing the same fragmentation pattern it was possible to obtain homogeneous results that could be used for quantitative purposes [43].

The tandem mass spectrum of the $[M-H]^-$ ion at *m*/*z* 389 for *trans*-piceid showed a fragment ion at *m*/*z* 227 (the same *m*/*z* value as the deprotonated molecular ion of *trans*-resveratrol), corresponding to the loss of the glucoside moiety. The fragmentation spectrum of *trans*-resveratrol was dominated by the product ion at *m*/*z* 185 representing the loss of a ketene molecule. The MS and MS^2 spectra of *cis*-isomers were similar to those of *trans*-isomers under the same MS conditions. The only difference between the structures of the *trans*- and *cis*-isomers is the geometry of the carbon–carbon double bond and the ionization efficiency of the *trans*- and *cis*-forms could be assumed to be the same [44]. The transition from precursor ion to characteristic fragment ion of each analyzed compound was useful for quantitative purposes.

In addition, the composition of HPLC mobile phases considerably affects the transference yield of the analyzed compounds from the liquid phase to the gas phase of MS detection. Formic acid (0.1%) in the mobile phase improved the analytes ionization efficiency leading to an important sensitivity enhancement of the signals. Fig. 3(a) and (b) shows the SRM mode total ion current chromatograms of HPLC–MS determination of serotonin, melatonin, *trans*-piceid, and *trans*-resveratrol standard solutions before and after exposure to UV light.

The elution times under the established conditions were serotonin 5.13 min, *trans*-piceid 23.82 min, melatonin 26.70 min, *cis*-piceid 27.98 min, *trans*-resveratrol 29.58 min, and *cis*-resveratrol 33.59 min. Peak identifications were made by comparing retention time and MS spectra of the chromatographic peaks with the individual standard solutions and this provided unambiguous results. The lower levels observed in the peaks corresponding to *trans*-isomers after UV light exposure, were proportional to the height of the new peaks corresponding to *cis*-isomers. *Trans*-isomers eluted from the column first, followed by *cis*-isomers. No other derivative peaks were detected under these conditions. Simultaneous monitoring of the six diagnostic product ions from each precursor ion could be performed without sacrificing specificity and sensitivity. The overlapping peaks could be resolved by using the appropriate chromatographic gradient and by measuring selected reaction ions characteristics through SRM detection. The use of SRM for quantitative analysis demonstrably improved the sensitivity and selectivity of the determinations and, in parallel, the signal-to-noise ratio was enhanced, a result consistent with a general improvement of the detection limits.

3.2. Performance of HPLC–MS detection and sample preparation methods

Table 2 shows the results for the validation parameters. The retention times were stable with SD around 0.02 min ($n = 10$). The LOD and LOQ were obtained. The relative standard deviation (RSD) was taken as a measure of intraday and interday repeatability ranged between 0.2% and 2.4%. The calibration curves were constructed by plotting the peak areas versus the concentration of each compound using the combined standards solution. The method developed showed very good linearity (R^2 higher than 0.999) in the range of concentrations studied. No isomeric conversion was observed during the extractions and HPLC–MS detection, based on a comparison of the differences between standards solutions with and without processing. In methanol extracts, the overall recovery ranged from 99.6 to 102.2%. In ethyl acetate concentrates, no serotonin was recovered, which would be expected due to the low solubility of this compound in ethyl acetate. The ethyl acetate recovery was an acceptable 72.7% for *trans*-piceid, and for *cis*-piceid, it was low (58.3%). These findings indicated that the

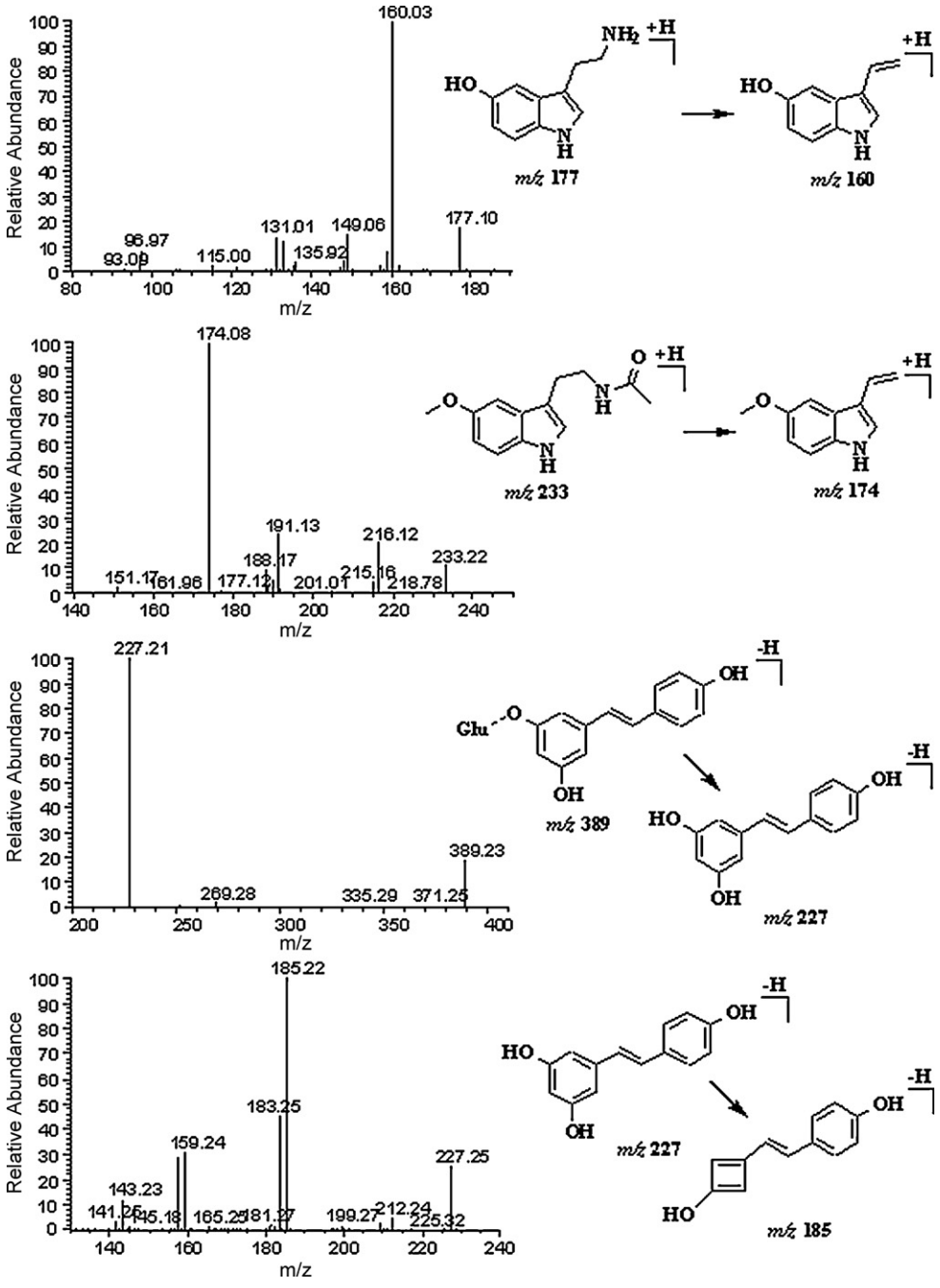

Fig. 2. MS^2 spectra of serotonin (*m*/*z* 177), melatonin (*m*/*z* 233), *trans*-piceid (*m*/*z* 389) and *trans*-resveratrol (*m*/*z* 227).

extraction and HPLC–MS detection methods were sufficiently precise, accurate and sensitive enough for simultaneous quantitative evaluation of serotonin, melatonin, *trans*- and *cis*-piceid, and *trans*- and *cis*-resveratrol.

To demonstrate the applicability of this analytical method to various kinds of fruit, serotonin, melatonin, *trans*- and *cis*-piceid, and *trans*- and *cis*-resveratrol were analyzed and compared in both methanol extracts and ethyl acetate concentrates from 13 samples of five kinds of fruit (Table 3). The recovery values of different compounds were taken into account when the contents were calculated.

Green seedless grape showed high amounts of *trans*-resveratrol. Both *trans*- and *cis*-piceid were quantified and a relatively low amount of *cis*-resveratrol was found (Fig. 3(c)). No serotonin or melatonin was detected in the selected green seedless grape samples. In the literature, the contents of *trans*- and *cis*-piceid, and *trans*- and *cis*-resveratrol have been reported in various grape skin and berry samples and ranged from 2.8 to 187 μg/g [13,19,31,42,45], 0.1 to 151 μg/g [19,31,42,45], 0.11 to 2680 μg/g [13,19,31,34,42,45–49] and 20 to 250 μg/g [31,50] of dry weight, respectively, and were found at much higher levels in the grape skin than in the berry. The *trans*- and *cis*-piceid, and *trans*- and *cis*-resveratrol levels detected in this study are comparable to the published data. The variations in content of *trans*- and *cis*-piceid, and *trans*- and *cis*-resveratrol between the different samples indicate that these stilbene compounds act as phytoalexins in the plant and are synthesized in response to external stresses, such as pathogens, UV radiation, or lesions [51,52].

Table 3
Application of the analytical method developed to some fruit samples.

	Extracts	Serotonin (μg/g)	Melatonin (pg/g)	*trans*-Piceid (ng/g)	*cis*-Piceid (ng/g)	*trans*-Resveratrol (ng/g)	*cis*-Resveratrol (ng/g)
Green seedless grape[b]	Methanol	–	–	4,688.9 ± 29.5	1,891.7 ± 43.2	34,679.9 ± 284.9	463.6 ± 32.3
	Ethyl acetate	–	–	4,683.8 ± 15.9	1,894.2 ± 27.8	35,245.0 ± 82.9	462.0 ± 28.9
Green seedless grape[d]	Methanol	–	–	2,107.2 ± 24.3	2,389.1 ± 32.4	27,317.1 ± 87.4	251.4 ± 8.4
	Ethyl acetate	–	–	2,054.5 ± 20.9	2,355.2 ± 34.9	27,713.8 ± 77.5	264.2 ± 1.9
Green seedless grape[e]	Methanol	–	–	5,067.4 ± 27.6	1,006.2 ± 10.6	28,201.7 ± 155.2	179.7 ± 5.3
	Ethyl acetate	–	–	5,061.4 ± 18.7	996.4 ± 11.5	29,296.9 ± 28.3	164.2 ± 4.8
Plantain[b]	Methanol	39.3 ± 0.1	–	–	–	–	–
	Ethyl acetate	–	–	–	–	–	–
Plantain[d]	Methanol	42.2 ± 0.0	–	–	–	–	–
	Ethyl acetate	–	–	–	–	–	–
Plantain[f]	Methanol	51.3 ± 0.1	–	–	–	–	–
	Ethyl acetate	–	–	–	–	–	–
Cranberry frozen[a]	Methanol	–	–	BQ	273.1 ± 8.3	BQ	–
	Ethyl acetate	–	–	181.6 ± 6.4	274.6 ± 8.6	3.8 ± 0.1	–
Cranberry frozen[d]	Methanol	–	–	BQ	229.4 ± 5.6	BQ	–
	Ethyl acetate	–	–	182.7 ± 3.6	231.0 ± 3.3	8.7 ± 0.0	–
Strawberry[d]	Methanol	–	–	BQ	332.5 ± 9.4	–	BQ
	Ethyl acetate	–	–	160.3 ± 3.5	323.5 ± 4.8	–	2.4 ± 0.1
Strawberry frozen[d]	Methanol	–	–	BQ	334.2 ± 11.3	–	BQ
	Ethyl acetate	–	–	122.0 ± 3.5	322.7 ± 4.3	–	1.0 ± 0.0
Green bell pepper[c]	Methanol	–	521.4 ± 6.0	–	10.0 ± 0.0	–	–
	Ethyl acetate	–	510.3 ± 2.2	–	10.0 ± 0.0	–	–
Green bell pepper[d]	Methanol	–	BQ	–	BQ	–	–
	Ethyl acetate	–	25.5 ± 0.5	–	2.3 ± 0.0	–	–
Green bell pepper[e]	Methanol	–	BQ	–	BQ	–	–
	Ethyl acetate	–	31.2 ± 1.2	–	3.4 ± 0.0	–	–

Values are means ± SD ($n = 5$) of dry weight.
"–": not detected. BQ: below the limit of quantitation.
[a] Dec. 2009 from market A.
[b] Feb. 2010 from market A.
[c] Feb. 2010 from market B.
[d] Apr. 2010 from market A.
[e] Apr. 2010 from market B.
[f] Apr. 2010 from market C.

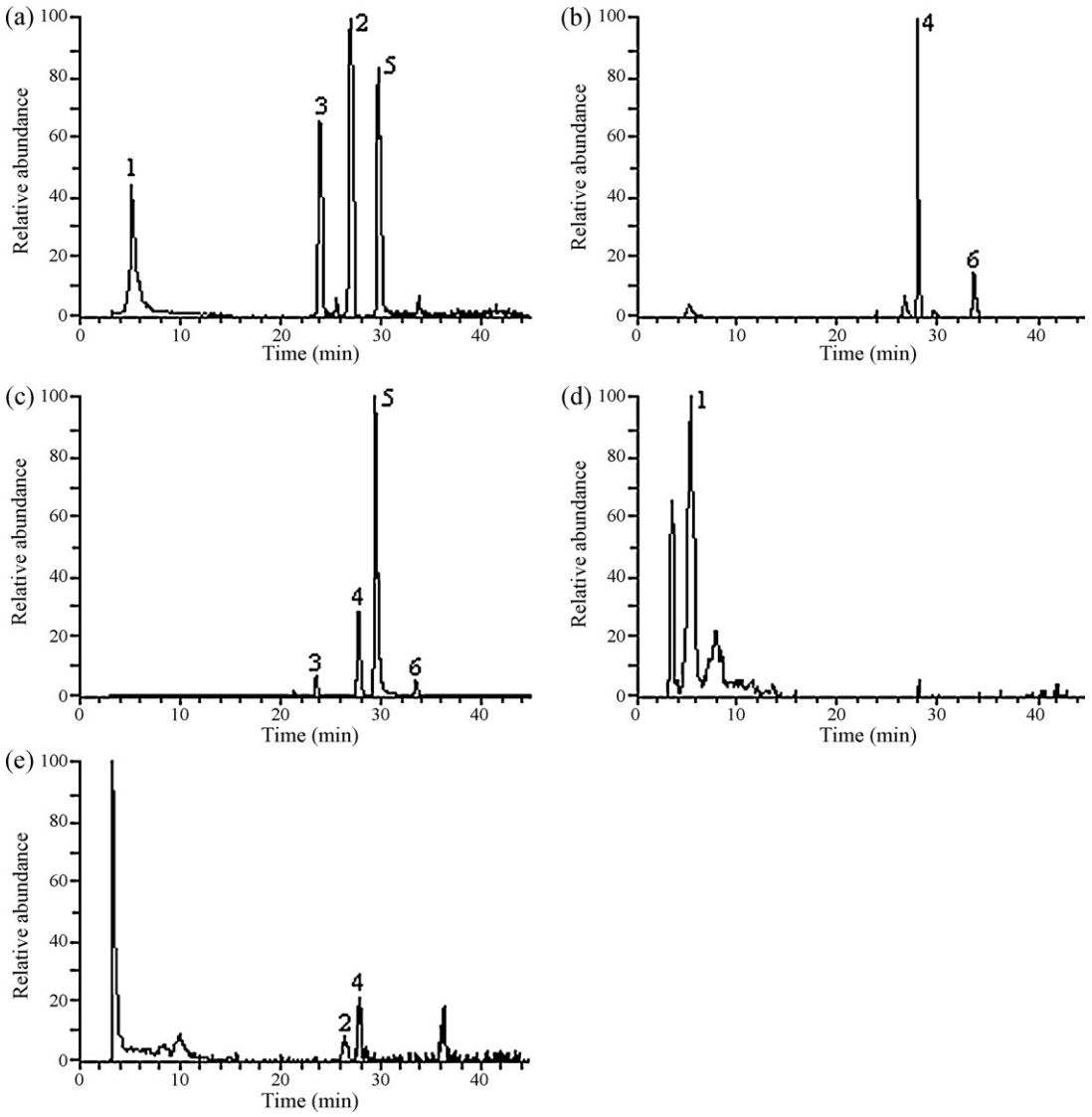

Fig. 3. Total ion current chromatogram of LC–MS determination of serotonin (peak 1), melatonin (peak 2), *trans*- and *cis*-piceid (peaks 3 and 4), and *trans*- and *cis*-resveratrol (peaks 5 and 6). (a) Standards before exposure to UV light; (b) standards after exposure to UV light; (c) green seedless grape sample; (d) plantain sample; (e) green bell pepper sample.

Samples of plantain from three different stores were found to contain significant amounts of serotonin, at 13.2 ± 0.04, 15.1 ± 0.01 and 19.3 ± 0.04 μg/g (fresh weight), respectively. These results are in agreement with fresh weight values reported in the literature; i.e., 12.0–56.7 μg/g [53] and 30 ± 7.5 μg/g [54]. Serotonin was present at a high level in the methanol extracts, but was not detected in the ethyl acetate extracts. There was no significant difference between samples from different stores at different times. Melatonin, *trans*- and *cis*-piceid, and *trans*- and *cis*-resveratrol were not detected in the plantain samples we examined (Fig. 3(d)).

The two frozen cranberry samples contained *trans*- and *cis*-piceid and low amounts of *trans*-resveratrol. In previous studies of cranberry, Rimando et al. [55] and Borowska et al. [56] found that *trans*-resveratrol contents were 900 ng/g of dry weight and 533.4–712.3 ng/g of fresh weight, respectively, which are about 100–200-fold higher than the levels we detected in this study. Wang et al. [44] reported the total resveratrol (including free resveratrol and resveratrol from piceid) in cranberry raw juice as *trans*-resveratrol 212.04 ng/g and *cis*-resveratrol 31.92 ng/g.

For strawberry, the fresh fruit contained *trans*-piceid 160.3 ± 3.5 ng/g, *cis*-piceid 323.5 ± 4.8 ng/g, and *cis*-resveratrol 2.4 ± 0.1 ng/g of dry weight while the frozen fruit was found to contain similar levels of these compounds: *trans*-piceid 122.0 ± 3.5 ng/g, *cis*-piceid 322.7 ± 4.3 ng/g, and *cis*-resveratrol 1.0 ± 0.0 ng/g of dry weight. No *trans*-resveratrol was detected in either the fresh or frozen fruit and, since the levels of *trans*-piceid and *cis*-resveratrol in the methanol extracts were lower than the quantitation limits, further concentration with ethyl acetate extraction was necessary to accurately determine the content of these compounds. Wang et al. [57], in their study, detected resveratrol in strawberry achenes (seeds) and pulp (receptacle tissue). The *trans*-resveratrol content (dry weight) in pulp was 830.5 ± 20.6 ng/g and in achenes, 1640 ± 80.0 ng/g. The *cis*-resveratrol content (dry weight) in pulp was 227.3 ± 11.4 ng/g, and in achenes 2030 ± 90.0 ng/g. More resveratrol was found in the achenes than in the fruit pulp.

The green bell peppers contained melatonin in both the methanol extracts and the ethyl acetate concentrates. The February sample showed relatively higher amounts of melatonin (510.3 ± 2.2 pg/g, dry weight) than the April samples (25.5 ± 0.5 and 31.2 ± 1.2 pg/g, dry weight) while *cis*-piceid was found at similar levels in the February and April samples, 10.0, 2.3 and 3.4 ng/g of

Table 4
Contents of serotonin, melatonin, *trans*- and *cis*-piceid, and *trans*- and *cis*-resveratrol in 31 fruit samples.

	Extracts	Serotonin (μg/g)	Melatonin (pg/g)	*trans*-Piceid (ng/g)	*cis*-Piceid (ng/g)	*trans*-Resveratrol (ng/g)	*cis*-Resveratrol (ng/g)
Red seedless grape[b]	Methanol	–	–	2,621.2 ± 13.9	2,988.9 ± 13.4	13,733.5 ± 177.2	95.7 ± 1.2
Red seedless grape[d]	Methanol	–	–	4,126.7 ± 3.7	2,512.4 ± 10.1	25,524.4 ± 203.3	111.8 ± 1.8
Red seedless grape[e]	Methanol	–	–	3,377.5 ± 5.8	3,380.7 ± 8.7	13,204.2 ± 3.2	84.8 ± 1.5
Banana[b]	Methanol	25.7 ± 0.2	–	–	–	–	–
Banana[d]	Methanol	25.8 ± 0.1	–	–	–	–	–
Banana[e]	Methanol	25.5 ± 0.1	–	–	–	–	–
Blackberry[d]	Ethyl acetate	–	–	201.2 ± 5.0	101.3 ± 1.7	–	120.6 ± 2.2
Raspberry frozen[b]	Ethyl acetate	–	–	211.5 ± 2.3	93.8 ± 3.3	58.6 ± 0.9	8.0 ± 0.4
Raspberry[g]	Ethyl acetate	–	–	234.1 ± 8.2	38.4 ± 3.8	37.7 ± 0.5	1.2 ± 0.0
Saskatoon berry frozen[a]	Ethyl acetate	–	33.9 ± 3.3	345.4 ± 4.9	104.7 ± 3.0	20.7 ± 0.0	–
Lapins sweet cherry[g]	Methanol	–	–	609.2 ± 17.4	47.2 ± 1.5	–	–
Sweetheart sweet cherry[g]	Methanol	–	–	510.3 ± 3.6	85.6 ± 4.9	–	–
Orange bell pepper[c]	Methanol	–	581.1 ± 19.4	–	–	–	–
Orange bell pepper[d]	Ethyl acetate	–	49.5 ± 3.7	–	–	–	–
Orange bell pepper[f]	Ethyl acetate	–	45.0 ± 0.8	–	–	–	–
Red bell pepper[c]	Methanol	–	179.5 ± 2.4	–	–	–	–
Red bell pepper[d]	Ethyl acetate	–	24.3 ± 1.2	–	–	–	–
Red bell pepper[e]	Ethyl acetate	–	66.4 ± 1.0	–	–	–	–
Grape tomato[a]	Methanol	–	–	–	–	175.0 ± 0.3	–
Grape tomato[b]	Methanol	–	–	–	–	169.8 ± 8.4	–
Grape tomato[e]	Methanol	–	–	–	–	168.0 ± 0.1	–
Plum[c]	Methanol	–	–	340.8 ± 0.9	137.2 ± 2.7	20.0 ± 0.0	–
Plum[d]	Ethyl acetate	–	–	117.2 ± 2.2	59.3 ± 3.8	13.1 ± 0.0	–
Peach[c]	Methanol	–	–	446.5 ± 7.5	163.9 ± 4.2	–	–
Nectarine[d]	Methanol	–	–	688.0 ± 1.5	93.7 ± 0.3	–	–
Bartlett pear[d]	Ethyl acetate	–	–	245.6 ± 1.5	48.4 ± 2.3	–	–
D'Anjou pear[c]	Methanol	–	–	454.1 ± 8.2	28.8 ± 2.2	–	–
Gala apple[g]	Methanol	–	–	473.9 ± 2.0	110.1 ± 2.9	–	–
Golden delicious apple[g]	Methanol	–	–	294.6 ± 2.9	100.1 ± 2.1	–	–
Granny Smith[d]	Methanol	–	–	573.7 ± 2.1	251.0 ± 1.0	–	–
Spartan apple[g]	Methanol	–	–	704.7 ± 2.4	110.0 ± 2.1	–	–

Values are means ± SD ($n = 5$) of dry weight.
"–": not detected.
[a] Dec. 2009 from market A.
[b] Feb. 2010 from market A.
[c] Feb. 2010 from market B.
[d] Apr. 2010 from market A.
[e] Apr. 2010 from market B.
[f] Apr. 2010 from market C.
[g] 2010 from local orchard.

dry weight, respectively. For the April green pepper samples, melatonin and *cis*-piceid were present at above the detection limit but below the quantitation limit in the methanol extracts; thus, it was necessary to use the ethyl acetate extracts for detection. No significant difference was detected between the two April samples purchased from different stores. No detectable serotonin, *trans*-piceid, or *trans*- and *cis*-resveratrol was found in the green bell pepper samples (Fig. 3(e)).

The results demonstrated that the sample preparation method used could extract serotonin, melatonin, *trans*- and *cis*-piceid, and *trans*- and *cis*-resveratrol simultaneously from the materials. Because the amount of some compounds in methanol extracts of some materials was not sufficiently high for accurate quantitative determination using HPLC–MS, a further concentration and cleaning of the sample using ethyl acetate extraction was required. For compounds that could be quantified in both methanol extracts and ethyl acetate concentrates, the final contents were similar in the two extracts, which indicates that the matrix did not significantly affect the HPLC–MS detection and confirms that the recoveries obtained were applicable to the samples.

3.3. Contents of serotonin, melatonin, trans- and cis-piceid, and trans- and cis-resveratrol in 31 fruit samples

Using the analytical methods we developed, serotonin, melatonin, *trans*- and *cis*-piceid, and *trans*- and *cis*-resveratrol levels were determined for several kinds of fruit samples (Table 4). Both methanol extracts and ethyl acetate concentrates of 31 fruit samples were analyzed by HPLC–MS. For compounds that could be quantified in both methanol extracts and ethyl acetate concentrates, the final contents were similar in these two extracts. Thus, when the detected level of each compound in the methanol extracts was sufficiently high for accurate quantitative analysis, only the results from the methanol extracts are shown. Otherwise, the results from the further ethyl acetate concentrates are shown. The values (mean ± SD, $n = 5$) are expressed on a dry weight basis.

Red seedless grapes had a high *trans*-resveratrol content (13.2–25.5 μg/g) and a low level of *cis*-resveratrol (84.8–111.8 ng/g). *Trans*- and *cis*-piceid were 2.6–4.1 μg/g and 2.5–3.4 μg/g, respectively. No serotonin or melatonin was detected in the red seedless grape samples. These results were similar to those obtained for the green seedless grapes (Table 3). Ragab [31] reported the *trans*- and *cis*-resveratrol, and *trans*- and *cis*-piceid levels in the skin of red seedless grape to be 2680, 20, 50 and 30 μg/g of dry weight, respectively. The levels of *trans*- and *cis*-piceid, and *trans*- and *cis*-resveratrol were found to be much higher in the grape skin than in the grape berry.

Three banana samples showed serotonin contents of 25.7 ± 0.2, 25.8 ± 0.1, and 25.5 ± 0.1 μg/g which is equivalent to 6.7 ± 0.05, 6.6 ± 0.03, and 6.3 ± 0.01 μg/g fresh weight, respectively, levels which are comparable to data reported in the literature: 11.5 ± 0.4 [24], 15 ± 2.4 [54], and 7.5–22 μg/g [58] of fresh weight, respectively. No melatonin, *trans*- and *cis*-piceid, or *trans*- and *cis*-resveratrol was detected in the banana samples.

Table 4 also shows that all the berry and cherry samples had *trans*- and *cis*-piceid in moderate to low concentrations, which ranged from 201.2 to 609.2 and 38.4 to 104.7 ng/g, respectively. Among the berry and cherry samples, melatonin was found at a low level (33.9 ± 3.3 pg/g) in frozen Saskatoon berry. Raspberry (frozen and fresh) and Saskatoon berry (frozen) contained low amounts of *trans*-resveratrol (58.6 ± 0.9, 37.7 ± 0.5 and 20.7 ± 0.0 ng/g). The content of *cis*-resveratrol was comparable in blackberry (120.6 ± 2.2 ng/g) and red seedless grape (84.8–111.8 ng/g), but much lower in frozen and fresh raspberry (8.0 ± 0.4 and 1.2 ± 0.0 ng/g).

For the orange and red bell peppers, the melatonin content was 3–12 times higher in the February samples than that in the April samples. None of the other compounds was found in these three orange and three red bell pepper samples purchased from different stores at different times. Further research is needed to determine the source of this variation which may, inter alia, reflect differences in region of origin, harvest time, climate, production system, genetics or environment.

Trans-resveratrol was detected as the main compound in the grape tomatoes, with no significant difference found between samples obtained from different stores at different times.

The plum sample from February showed relatively higher amounts of *trans*- and *cis*-piceid (340.8 ± 0.9 and 137.2 ± 2.7 ng/g) than the April sample (117.2 ± 2.2 and 59.3 ± 3.8 ng/g) and both plum samples had detectable levels of *trans*-resveratrol (20.0 ± 0.0 and 13.1 ± 0.0 ng/g). No serotonin, melatonin or *cis*-resveratrol was detected in the selected plum samples.

Peach, nectarine, Bartlett and D'Anjou pears, and Gala, Golden Delicious, Granny Smith, and Spartan apples had moderate *trans*- and *cis*-piceid contents. The highest levels of *trans*- and *cis*-piceid were detected in Spartan and Granny Smith apples.

This is the first report of the screening of fruits for serotonin, melatonin, *trans*- and *cis*-piceid, and *trans*- and *cis*-resveratrol simultaneously using advanced HPLC–MS. We found serotonin, melatonin, *trans*- and *cis*-piceid, and *trans*- and *cis*-resveratrol over a very wide range of concentrations. *Trans*- and *cis*-piceid were detected in most of the samples. The variability of these six compounds in the fruits investigated in this study is probably due to many factors, including species and variety differences, growing conditions, ripening degree, storage conditions, etc. Our results not only confirm some published findings [13,19,24,31,34,42,45–50,53,54,58] but also provide evidence that many fruits contain detectable amounts of melatonin (green, orange and red bell peppers and Saskatoon berry), *trans*- and *cis*-piceid (cranberry, strawberry, green bell pepper, blackberry, raspberry, Saskatoon berry, Lapins and Sweetheart sweet cherries, plum, peach, nectarine, Bartlett and D'Anjou pears, and Gala, Golden Delicious, Granny Smith, and Spartan apples), and *trans*- and *cis*-resveratrol (blackberry, raspberry, Saskatoon berry, grape tomato and plum).

The six compounds we investigated have two basic chemical structures; therefore, there are factors that need to be taken into account for their simultaneous analysis by HPLC–MS. An important factor is the extraction of the compounds from samples of diverse origin since plant tissues contain a variety of primary and secondary metabolites [59,60]. The sample preparation we used in this study was able to extract serotonin, melatonin, *trans*- and *cis*-piceid, and *trans*- and *cis*-resveratrol simultaneously from plants of diverse types. HPLC separation is another important factor. Gradient elution is necessary and 0.1% formic acid as a modifier in the mobile phase provides good MS detection sensitivity. The third important consideration is the ionization mode. Finnigan LTQ mass spectrometry allows rapid switching between positive and negative ion detection in a single HPLC–MS run under stable conditions; therefore, each analyte can be scanned under its optimum ionization mode during the HPLC–MS determination. In addition, SRM mode in MS monitoring with higher selectivity permits simpler and cleaner chromatograms to be obtained and this facilitates quantitation.

4. Conclusions

The new analytical method we developed proved to be accurate, sensitive, and rapid and has the potential for application in further research on the occurrence of trace amounts of serotonin, mela-

tonin, *trans*- and *cis*-piceid, and *trans*- and *cis*-resveratrol in many types of plant materials. It combines a simple sample extraction procedure with HPLC–MS detection and was successfully applied to simultaneously quantify these six compounds in a total of 44 samples from 24 kinds of fruit.

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