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Detection of Serotonin, Melatonin, and Their Metabolites in Honey

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Abstract

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Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsfoodscitech.1c00119.
Standard curves for melatonin and its derivatives used for quantification; limits of quantification (LOQ) for melatonin and its metabolites; detection of tryptophan in honey; recovery rates for melatonin and its metabolites in methylene chloride or ethyl acetate extraction (PDF)

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Melatonin and serotonin, products of tryptophan metabolism, are endogenous neurotransmitters and hormones. We have identified and quantified these metabolites in natural honey from Australia, USA, and Poland using a Xevo G2 XS qTof LC–MS. To help ensure correct product identification, some samples were prepurified by RP-HPLC based on the retention times of standards, prior to LC–MS. The concentrations of the metabolites of interest depended on the source of the honey. For Australian honey, levels for melatonin and 2-hydroxymelatonin were 0.91 and 0.68 ng/g, respectively. Melatonin was detected in one brand of US commercial honey at 0.48 ng/g, while a second brand contained serotonin at 88.2 ng/g. In Polish natural honey, 20.6 ng/g of serotonin and 40.8 ng/g of *N*-acetylserotonin (NAS) were detected, while in Polish commercial honey 25.9 ng/g of serotonin and 7.30 ng/g of NAS were present. We suggest that addictive and health-related properties of honey may be in part dependent on the presence of serotonin, melatonin, and their metabolites, and that these compounds may play a role in the colony activities of bees.

Keywords

honey; bees; melatonin; 2-hydroxymelatonin; serotonin; N-acetylserotonin

INTRODUCTION

Melatonin is an ancient molecule, present since the evolution of life as indicated by its detection in alphaproteobacteria and cyanobacteria.[1,2] This pluripotent molecule with diverse actions is widely produced in nature by bacteria, eucarya, unicellular and multicellular fungi, plants, and animals including simple and complex vertebrates and invertebrates.[3-8] In the animal kingdom it is produced from serotonin following its acetylation to produce *N*-acetylserotonin (NAS) with subsequent methylation generating the final product, melatonin.[9-11] Melatonin is metabolized through hydroxylations at C2 and C6, through indolic and kynuric pathways as well as by nonenzymatic reactions initiated by UVR (predominantly UVB with possible action of UVA) or ROS.[12,13]

Serotonin, also an ancient molecule with pluripotent and diverse activities, is a product of sequential metabolic transformations of L-tryptophan which occur across different species such as vertebrates, invertebrates, including worms and insects, fungi, plants, and unicellular organisms.[14-22] The rate-limiting step in serotonin synthesis in vertebrates is tryptophan hydroxylation catalyzed by tryptophan hydroxylase.[23-25] Hydroxytryptophan can also be generated through nonenzymatic transformation of tryptophan by ultraviolet radiation (UVR) and reactive oxygen species (ROS).[26] 5-Hydroxytryptophan is decarboxylated by aromatic amino acid decarboxylase to produce serotonin.[22] Similar enzymatic reactions of the serotogenic/melatogenic pathway are catalyzed by homologues or paralogs of the above enzymes in different organisms, including plants. In plants, tryptophan is synthesized via the shikimic acid pathway, which is then decarboxylated to tryptamine by tryptophan decarboxylase and further converted to serotonin by tryptamine 5-hydroxylase.[3,6,7] Serotonin can also be metabolized to 5-mehoxytryptamine in plants with its further methylation to melatonin.[3,8] In insects, serotonin and melatonin are synthesized by same routes as in vertebrates.[27-30]

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Serotonin and melatonin function as pleiotropic signaling molecules regulating many physiological processes and counteracting pathology or environmental stresses across different species.[4,6-8,10,11,18,22,31] In insects including honey bees, they play important roles as neurotransmitters, hormones, and bioregulators, regulating different endocrine, behavioral (including social behavior), immune, developmental, and protective functions, as well as biological rhythms.[18,29,32-38] Serotonin regulates a wide range of physiological and pathological processes in humans by acting as a neurotransmitter, neurohormone, hormone, and biological modifier.[15-17,22,39,40] Melatonin, in addition to regulating the circadian rhythm, regulates a variety of endocrine, immune, neural, metabolic, and protective functions.[5,10,41] Many studies support that melatonin can be used as a health supplement due to its many biological functions such as its antioxidant,[42-45] anticancer,[46-49] and antiaging effects.[50,51]

Honey is a widely available and highly consumed natural product with nutritional value and may impart significant health benefits and be used as a therapeutic.[52-59] While the health benefits of honey may not be fully grasped, many reports have indicated that honey contains numerous natural antioxidants such as polyphenols, flavonoids, phytochemicals, minerals, and vitamins.[53,56,57,59-61] Previously, we reported the presence of vitamin D3 and its biologically active hydroxyderivatives in honey.[62] In this study using highly sensitive analytical methods, we report the presence of serotonin, melatonin, and their derivatives in honey samples from Australia, the U.S.A, and Poland.

MATERIALS AND METHODS

Chemicals.

To extract honey, HPLC grade methylene chloride and ethyl acetate (Fisher Scientific, Hampton, NH) were used. LC–MS grade acetonitrile, water, and formic acid were purchased from Fisher Scientific (Hampton, NH) for HPLC or LC–MS. Melatonin, serotonin, and *N*-acetylserotonin were purchased from Sigma-Aldrich (St. Louis, MO) for standards. 2-Hydroxymelatonin was obtained from Santa Cruz Biotech.

Extraction of Honey and Prepurification.

Australian honey, collected in spring and summer and obtained directly from a local producer in Perth, was diluted with an equal volume of water and extracted with 2 volumes of CH_2Cl_2, three times. The combined extracts were dried under a stream of N_2 gas at 30 °C. Honey from Nature Nate's Corporate (McKinney, TX) was purchased in Walmart and named commercial honey sample 1. Commercial honey sample 2 was from Kirkland Signature, purchased at Costco. The natural Polish honey was collected during the summertime near Warsaw, while the commercial Polish one was purchased in Carrefour market. We extracted honey by two different methods using methylene chloride or ethyl acetate. For the methylene chloride extraction method, the honey was dissolved in a 3.5 volume of water and transferred to a 1 L extraction funnel. The aqueous layer was extracted with CH_2Cl_2 (3 × 1.5 volume of honey). The combined organic layers were washed with distilled water (3 × 1 volume of honey), brine (2 × 1 volume of honey), and dried over sodium sulfate. It was then filtered using a sintered glass Buchner funnel, and the solvent

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was evaporated using a rotary evaporator while maintaining the water bath temperature below 35 °C to give a semisolid (200 mg). For the ethyl acetate method, the honey was dissolved in distilled water (6× volume of honey) and transferred to a 1 L extraction funnel. The aqueous layer was extracted with ethyl acetate (3 × 6 volume of honey). The combined organic layers were dried over sodium sulfate, filtered, and evaporated using a rotary evaporator to give a semisolid (120 mg). For quantification, we selected the more efficient of the two extraction methods: methylene chloride for the extraction of melatonin and 2-hydroxymelatonin and ethyl acetate for serotonin and NAS, as shown in Table 1.

Liquid Chromatography–Mass Spectrometry (LC–MS).

Extracted samples were dissolved in acetonitrile and in some cases subjected to a HPLC prepurification step to isolate fractions of interest. This was carried out using a 1260 Infinity II HPLC system with a C18 column (250 mm × 4.6 mm, 5 μm particle size) (Waters, Milford, MA), using a gradient of acetonitrile in water (40–100%) at a flow rate of 0.5 mL/min for 15 min, then with 100% acetonitrile at a flow rate of 0.5 mL/min for 30 min followed by a flow rate of 1.5 mL/min for 20 min. All standards of samples of interest were well separated and displayed different retention times with this mobile phase (data not shown). Fractions with these retention times were then collected from the extracted honey and these samples, and in some cases the crude extracts as well, were analyzed using a Xevo G2 XS qTof LC–MS equipped with a Waters ACQUITY UPLC I-Class System (Waters, Milford, MA). LC–MS was performed using a Zorbax Eclipse Plus C18 column (2.1 × 50 mm, 1.8 μm) (Agilent Technology, Santa Clara, CA). Elution was achieved with a gradient of acetonitrile in water (all containing 0.1% formic acid), 15% acetonitrile for 1.5 min, 15–30% for 0.1 min, 30% isocratically for 0.9 min, 30–100% for 0.5 min, 100% isocratically for 3 min, 100%–15% for 0.1 min, and 15% for 0.9 min at a flow rate of 0.3 mL/min. Positive mode masses were scanned from 100 to 1000 Da using the continuum mode with a scan time of 1 s. The capillary voltage was 1.7 kV with 40 V as cone voltage. The desolvation gas flow rate was set as 800 L/hour with source temperature of 120 °C. As the lockspray reference compound, Leucine enkephalin (200 ng/mL, m/z = 556.2771) was used at a flow rate of 10 μL/min with the lockspray interval being 10 s and a scan time of 1 s. Extracted ion chromatograms (EICs) were obtained using m/z = 233.1 $[M + H]^+$, 255.1 $[M + Na]^+$, and 174.1 $[M + H - NH_2CH_3CO]^+$ for melatonin; 249.1 $[M + H]^+$ for 2-hydroxymelatonin; 160.1 $[M + H - NH_3]^+$ for serotonin and 160.1 $[M + H - NH_2CH_3CO]^+$ for NAS with Waters MassLynx 4.1 software.

RESULTS

Identification of Melatonin, 2-Hydroxymelatonin, Serotonin, and NAS in Honey.

Honey (from Australia) was extracted with methylene chloride, and fractions of interest were prepurified by HPLC using a C18 column (250 mm × 4.6 mm, 5 μm particle size). Both the crude and purified samples were then analyzed by LC–MS as described in the Materials and Methods. Analysis of purified samples resulted in melatonin, 2-hydroxymelatonin, and NAS being detected with m/z = 255.1 $[M + Na]^+$, 249.1 $[M + H]^+$, and 160.1 $[M + H - NH_2CH_3CO]_+$ with RTs corresponding to the standards (Figure 1A). Serotonin, 2-hydroxymelatonin, and NAS were also detected in crude honey extract

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with m/z = 160.1 $[M+H-NH_3]^+$, 249.1 $[M + H]^+$ and 160.1 $[M + H – NH_2CH_3CO]^+$, respectively, with RTs corresponding to the standards (Figure 1B). NAS-d7 was used as internal standard, having the same RT as NAS (Figure 1A). Thus, the identification of melatonin, 2-hydroxymelatonin, and NAS in the sample is based on the characteristic masses being observed for each one and also from the retention times being identical to the standards in two different LC systems. Serotonin was identified from its expected mass and its identical retention time to the standard by LC–MS.

We also investigated two different commercial brands of honey from the United States for the presence of serotonin and melatonin and its derivatives. After extraction of the commercial honey sample 1 with methylene chloride followed by LC–MS, we detected peaks of melatonin and serotonin with m/z = 233.1 $[M + H]^+$ and 160.1 $[M + H – NH_2CH_3CO]^+$, respectively, with RTs corresponding to melatonin and serotonin standards (Figure 2B). In the prepurified samples in which HPLC was used to collect a fraction at the same RT as standard melatonin and NAS, we detected peaks having m/z = 255.1 $[M + Na]^+$ and 160.1 $[M + H – NH_2CH_3CO]^+$ (Figure 2A) by subsequent LC–MS, again with retention times matching standards. A second commercial honey sample (sample 2) was extracted with ethyl acetate and analyzed by LC–MS before and after prepurifying the sample with HPLC. As shown in Figure 3A, melatonin was detected with m/z = 174.1 $[M + H – NH_2CH_3CO]^+$ having a RT corresponding to the standard. We also detected the peaks corresponding to melatonin and serotonin in a crude extract having the same RTs as standards, with m/z = 174.1 $[M + H – NH_2CH_3CO]^+$ and 160.1 $[M + H – NH_3]^+$, respectively (Figure 3B).

Quantification of Melatonin, 2-Hydroxymelatonin, Serotonin, and NAS in Honey.

Selected peaks in the mass chromatograms were quantified using standard curves for the relevant compounds (Figure S1). The limits of quantification (LOQ) of each compound using their respective standard curve are shown in Figure S2. NAS quantification was done using the standard curve and calculating the ratio of the peak area of NAS/NAS-d7. All standard curves were linear over the range of measurement with coefficients of variation of $r^2 \geq 0.996$ (Figure S1). The limits of quantification (LOQ) were 0.33 to 20 pg, depending on the compound, and the S/N (signal-to-noise) ratio was over 20, meaning the detection and quantification were convincing (Figure S2). The quantifications were corrected by dilution factors and are described in Table 1. Melatonin and 2-hydroxymelatonin were measured in CH_2Cl_2-extracted honey while serotonin and NAS were measured in ethyl acetate-extracted honey because the recovery using methylene chloride was better for melatonin while the ethyl acetate method was better for serotonin and NAS (Tables S1, S2, and S3). The recovery rates for melatonin using methylene chloride and ethyl acetate were 75–87% and 58–66%, respectively. In contrast, the recovery rates for serotonin using methylene chloride and ethyl acetate were 15% and 134%, respectively. For NAS the recovery rates using methylene chloride and ethyl acetate were 18% and 82%, respectively.

Melatonin and 2-hydroxymelatonin in Australian honey extracted with methylene chloride were quantified using m/z = 255.1 $[M + Na]^+$, and 249.1 $[M + H]^+$, respectively. Melatonin in commercial honey sample 1 extracted with methylene chloride was quantified using m/z

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= 233.1 $[M + H]^+$. Serotonin from commercial honey sample 2 extracted with ethyl acetate was quantified using *m/z* = 160.1 $[M + H – NH_3]^+$. For Polish honey extracted with ethyl acetate, *m/z* = 160.1 $[M + H – NH_3]^+$ was used for serotonin quantification while for NAS the ratio of NAS to NAS-d7 (160.1 $[M + H – NH_2CH_3CO]^+$/NAS-d7 (*m/z* = 164.1 $[M + H – NH_2CH_3CO]^+$) was used for quantification. Finally, L-tryptophan, the precursor to serotonin and melatonin, was clearly detected in honey (Figure S3).

DISCUSSION

Melatonin and serotonin are present in most if not all plants, vertebrates, and invertebrates including insects.[1,3-8,14-22,30,31] 2-Hydroxymelatonin is a predominant melatonin metabolite in plants.[63,64] Therefore, it is logical to assume that bees produce NAS and can convert it to melatonin and 2-hydroxymelatonin, using serotonin as a substrate, with some of these products being transferred to their honey. There is already evidence that serotonin is produced by bees.[32,35,65,66] However, despite some indications,[37,38] analytical evidence for its transformation to NAS, melatonin, and other metabolites is lacking.

Using analytical chemistry methods we have detected intermediates of the serotonigenic/melatonigenic pathway in honey for the first time. Tryptophan, serotonin, NAS, melatonin and/or 2-hydroxymelatoinin were present in Australian, two commercial US, one natural Polish and one commercial Polish honey samples. Using LC–MS, melatonin was detected in natural Australian and two commercial US honeys at levels of 0.91 and 0.48 ng/g for Australian and commercial US honey 1, respectively. Serotonin was detected in all honey samples with levels ranging from 20.6 to 88.2 ng/g for commercial US honey 2 and two Polish honeys (Figure 4), illustrating the variation between the sources of the honey employed for its analysis. Our data provide analytical evidence that bees can transform serotonin to melatonin. Interestingly, 2-hydroxymelatonin was detected only in Australian honey which had a content of 0.7 ng/g, but the related metabolite, 6-hydroxymelatonin, was not detected. It should be noted that 2-hydroxymelatonin is an important product of melatonin metabolism in plants with important medicinal properties in humans.[8,63,64] However, it has also been detected in skin samples and can be generated from melatonin by UVB irradiation,[12,13] and can also be generated in mitochondria.[67] In the case of NAS, it was detectable in all honey samples except commercial US honey 2 with levels of 40.8 and 7.3 ng/g for natural and commercial Polish honey, respectively. Although expected because it is an intermediate in the melatoninergic pathway, this is the first detection of NAS and of 2-hydroxymelatonin originating from insects in general.

Melatonin and serotonin are beneficial to human health with properties which include enhancing sleep quality, regulating the circadian rhythm, regulating behavior, and modifying immune activity including anti-inflammatory actions. They also display endocrine, metabolic, anticancer, antiviral, antioxidative, photoprotective, and antiaging properties.[4,5,11,15,16,21,22,41,50,68] Therefore, one may suggest that some of the beneficial health properties of Honey[53,54,56-58,60,69-73] may be due, at least in part, to the presence of serotonin and melatonin. NAS and 2-hydroxymelatonin may also play a role from their antioxidative and other beneficial properties, although these have not been thoroughly investigated to date.[8,21,63,64,74-76] Interestingly, the reported beneficial effects of honey

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on bones,[55] may be secondary to not only serotonin action, but also to the presence of active forms of vitamin D in this product.[62] The well recognized behavioral functions of serotonin[22] may contribute to our inclusion of honey in our diet. In the case of melatonin, it is unclear whether the amount obtained from moderate honey consumption is sufficient to have beneficial effects on humans that are assigned to the honey.[70,73,77-79] Importantly, the presence of serotonin and melatonin in honey may play an important developmental role in bees, affecting well-being of individual bees and their colony functions. The role of serotonin in these functions is well established,[32-35,61,65,66,80-83] which includes learning and cognitive responses.[34,84] The importance of melatonin in these functions remains to be investigated, but there are some indications for such a role.[29,37,38]

In summary, we report for the first time the detection of serotonin, NAS, melatonin, and 2-hydroxymelatonin in honey with considerable variation in levels of these bioactive compounds depending on the source of the honey. We suggest that the presence of these compounds may contribute to some health benefits assigned to the honey, and that they play a role in bee development, health, and colony functions.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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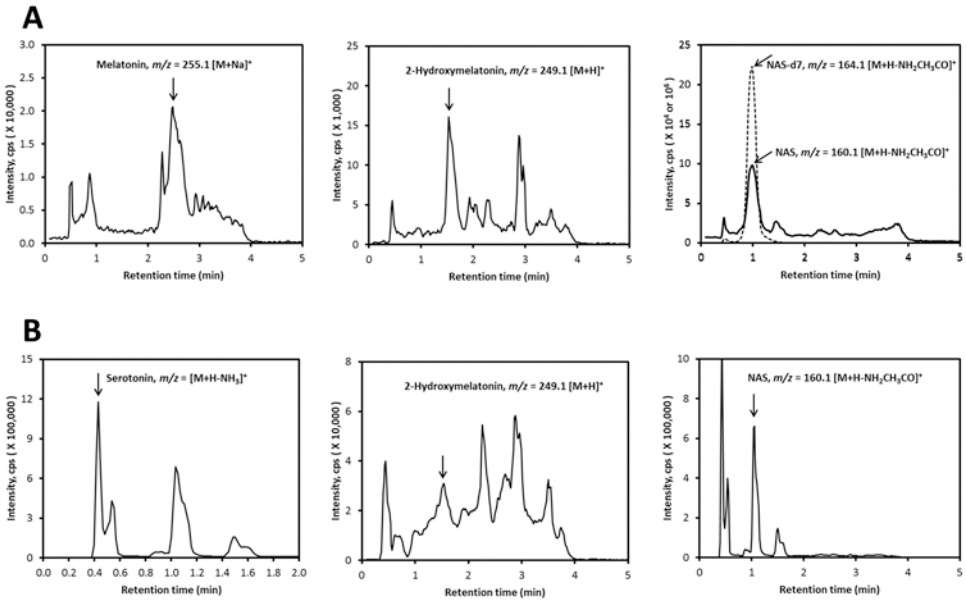

Figure 1.

Detection of melatonin, 2-hydroxymelatonin, serotonin, and NAS in Australian honey. The honey was extracted with methylene chloride and analyzed after being prepurified on a C18 column (A) or directly (B) using a Zorbax Eclipse Plus C18 column connected to a Xevo G2 XS equipped with an ACQUITY UPLC I-Class System (Waters, Milford, MA). The extracted ion chromatograms (EICs) were obtained using m/z = 255.1 $[M + Na]^+$, 249.1 $[M + H]^+$, 160.1 $[M + H - NH_3]^+$ and 160.1 $[M + H - NH_2CH_3CO]^+$ for melatonin, 2-hydroxymelatonin, serotonin, and NAS, respectively.

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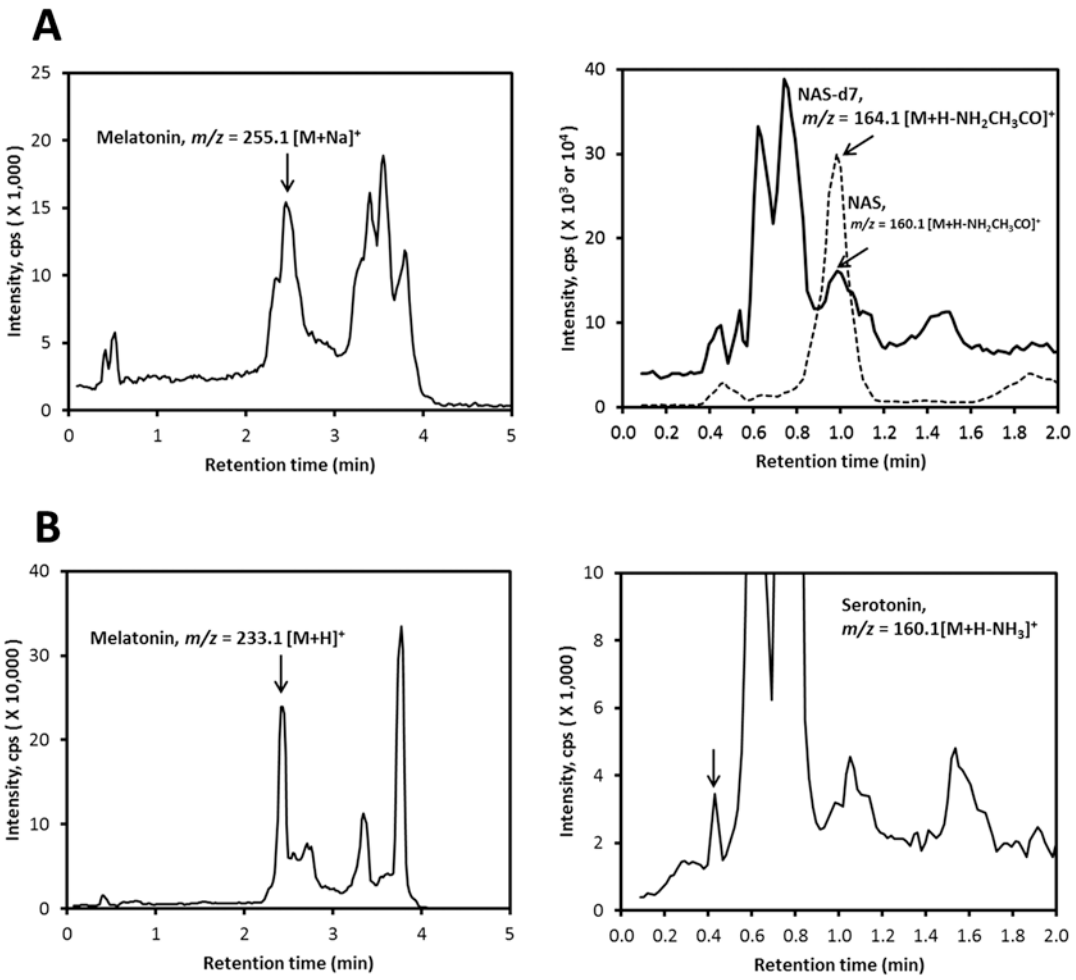

Figure 2.

Detection of melatonin, serotonin, and NAS in commercial honey sample 1. The honey was extracted with methylene chloride and analyzed after prepurification of fractions of interest in a C18 column (A) or directly (B) using a Zorbax Eclipse Plus C18 column connected to a Xevo G2 XS equipped with an ACQUITY UPLC I-Class System (Waters, Milford, MA). The EICs were obtained using m/z = 255.1 $[M + Na]^+$ or 233.1 $[M + H]^+$, 160.1 $[M + H – NH_3]^+$ and 160.1 $[M + H – NH_2CH_3CO]^+$ for melatonin, serotonin, and NAS, respectively.

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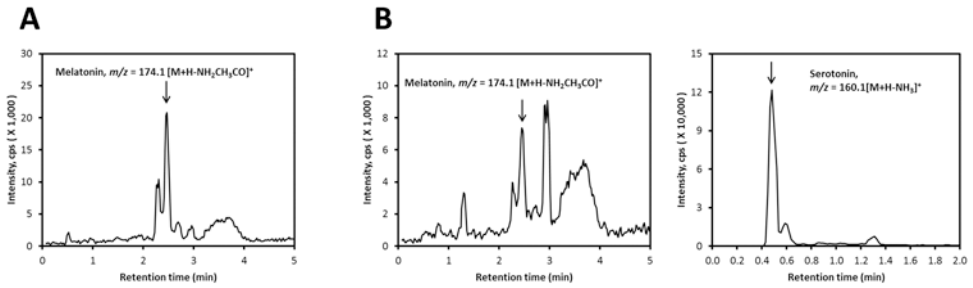

Figure 3.

Detection of melatonin and serotonin in commercial honey sample 2. The honey was extracted with ethyl acetate and analyzed after prepurification on a C18 column (A) or directly (B) using a Zorbax Eclipse Plus C18 column connected to a Xevo G2 XS equipped with an ACQUITY UPLC I-Class System (Waters, Milford, MA). The EICs were obtained using m/z = 174.1 $[M + H – NH_2CH_3CO]^+$ and 160.1 $[M + H – NH_3]^+$ for melatonin and serotonin, respectively.

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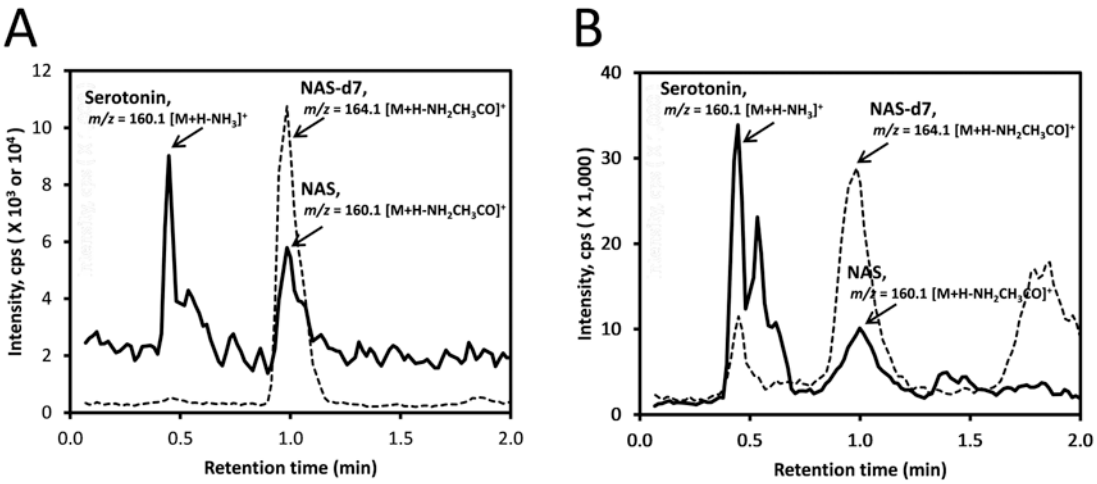

Figure 4.

Measurement of serotonin and NAS in Polish honey. The honey was extracted with ethyl acetate and analyzed directly using a Zorbax Eclipse Plus C18 column connected to a Xevo G2 XS equipped with an ACQUITY UPLC I-Class System (Waters, Milford, MA). The extracted ion chromatograms (EICs) were obtained using m/z = 160.1 $[M + H – NH_3]^+$ for serotonin and 160.1 $[M + H – NH_2CH_3CO]^+$ for NAS. (A) Polish natural honey; (B) Polish commercial honey.

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Table 1.

Quantification of Melatonin and Its Derivatives in Honey[a]

compounds detected	natural honey from Australia	commercial honey 1 from USA	commercial honey 2 from USA	natural honey from Poland	commercial honey from Poland
melatonin (ng/g)	0.91 ± 0.03[b]	0.481 ± 0.004	NQ[e]	-	-
2-hydroxymelatonin (ng/g)	0.68 ± 0.03	-	-	-	-
serotonin (ng/g)	NQ[d]	NQ[d]	88.2 ± 1.0	20.6 ± 7.9	25.9 ± 0.2
NAS (ng/g)	NQ[d]	NQ[d]	-	40.8 ± 9.2	7.3 ± 0.3

[a]Melatonin and 2-hydroxymelatonin were quantified using methylene chloride-extracted honey while serotonin NAS was quantified using ethyl acetate-extracted honey. NQ, Detected but not quantified. (−) Could not detect a clear separate peak by LC–MS.

[b]After purification using C18 column.

[d]Detected in honey extracted with methylene chloride.

[e]Detected in honey extracted with ethyl acetate.

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