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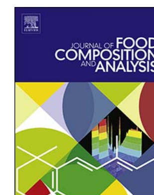

Original research article

Use of isoquinoline alkaloids as markers for identification of honey and pollen from *Macleaya cordata* (Willd.) R. Br

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ARTICLE INFO

Chemical compounds studied in this article:
Protopine (PubChem CID: 4970)
Allocryptopine (PubChem CID: 98570)
Sanguinarine (PubChem CID: 5154)
Chelerythrine (PubChem CID: 2703)
Berberine chloride (PubChem CID: 12456)
Dihydrochelerythrine (PubChem CID: 485077)
Dihydrosanguinarine (PubChem CID: 124069)

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ABSTRACT

Macleaya cordata (Willd.) R. Br. (McRB) is utilized in traditional medicine and is mainly distributed in North America, Europe, Japan, and China. McRB honey known as "mad honey" produced by honey bees from the nectar of McRB contains isoquinoline alkaloids that give rise to potential consumer risk. In this study, a QuEChERS (Quick, Easy, Cheap, Effective, Rugged, Safe) extraction procedure followed by ultra-high performance liquid chromatography–quadrupole time-of-flight mass spectrometry (UHPLC–MS/MS) was developed and optimized for the determination of seven isoquinoline alkaloids in McRB bee pollen and honey. The results revealed the presence of seven alkaloids in McRB bee pollen, four of which were also detected in McRB honey. Protopine and allocryptopine were the two predominant alkaloids, with concentrations of 0.17–0.66 mg/kg and 0.068–0.19 mg/kg in McRB honey and 1.25*10^3–3.07*10^3 mg/kg and 1.12*10^3–2.52*10^3 mg/kg in McRB bee pollen, respectively. None of the seven alkaloids were detected in commercial honey (n = 130) or pollen samples (n = 30). This study shows that protopine and allocryptopine could serve as potential markers of honey and pollen specifically from McRB.

1. Introduction

Honey is a sweet natural product produced by honeybees from the nectar of flowers (blossom honey) or from the secretions of other living parts of the plants or the excretions of sucker insects (honeydew honey) (Siddiqui et al., 2017). The floral origin and production region directly affect the honey quality (Luo et al., 2004). Various sensory and compositional properties of honey depend primarily on the botanical origin of the nectar (Sabri and See, 2016). Honeybees collect pollen and nectar not only from a single plant species, but also from multiple floral plants (Kato et al., 2012), and may inevitably collect nectar from medicinal plants. The phenomenon of poisoning associated with the consumption of so-called "mad honey" containing plant toxins has occurred in Eurasia, North America, and New Zealand (Larsen et al., 2015). "Mad honey" was associated with *Rhododendron* nectar (Cagli et al., 2009; Dubey et al., 2009; Koca and Koca, 2007; Sibel et al., 2014). Several cases of death caused by honey intoxication due to the consumption of honey from *Tripterygium wilfordii* Hook F. (TwHf) were also reported in South China (Zhang et al., 2016). Therefore, it is crucial to be able to identify varieties of honey from the nectar of medicinal plants for further evaluation of their safety.

Most of the studies in the current literature have focused on the plant *Macleaya cordata* (Willd.) R. Br. (McRB) (Chen et al., 2009; Pi et al., 2008; Zhang et al., 2005), which is a traditional medicinal plant that contains isoquinoline alkaloids (Kosina et al., 2010; Pěnčíková et al., 2011) and is distributed in many countries (Psotova et al., 2006). Alkaloids are chemical compounds that exist in medicinal plants (Zhang et al., 2012) and may cause risks to human health (Luo et al., 2004; Valese et al., 2016). Increasing attention has been paid to the analysis of alkaloids in honey, whereas only a few studies have addressed the

Abbreviations: UHPLC–MS/MS, ultra-high performance liquid chromatography–quadrupole time-of-flight mass spectrometry; QuEChERS, quick easy cheap effective rugged safe; McRB, *Macleaya cordata* (Willd.) R. Br; TwHf, *Tripterygium wilfordii* Hook F; SEM, scanning electron microscope; MRM, multiple reaction monitoring; SD, standard deviation; PRO, protopine; ALL, allocryptopine; SA, sanguinarine; CHE, chelerythrine; BER, berberine chloride; DHCHE, dihydrochelerythrine; DHSA, dihydrosanguinarine

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alkaloids present in McRB pollen and little information has been obtained regarding the identification of honey or pollen from McRB. The isoquinoline alkaloids present in McRB roots, leaves, flowers, and fruits include sanguinarine (SA), chelerythrine (CHE), protopine (PRO), allocryptopine (ALL), berberine chloride (BER), dihydrochelerythrine (DHCHE) and dihydrosanguinarine (DHSA), according to high-performance liquid chromatography with diode array detection and electrospray ionization mass spectrometry (HPLC-DAD-ESI/MS) analyses (Chen et al., 2009). The isoquinoline alkaloids SA and CHE have been detected in honey contaminated with McRB by HPLC-ESI/MS (Luo et al., 2004). In general, pollen morphology and composition have been used to prove the botanical origin of honey (Gan et al., 2016). Some researchers detect mad honey by analyzing its pollen morphology. Cagli et al. (2009) diagnosed mad honey intoxication by confirming the presence of pollen from *Rhododendron ponticum*. Zhang et al. (2016) successfully used pollen morphology analysis to evaluate honey contaminated with TwHf pollen. When bee pollen is present in honey only at low concentrations, screening characteristic components can represent an alternative method. As such, pollen morphology and alkaloid analysis can be used to effectively identify the floral origin of honey.

The objectives of this study were to establish a simple and effective method of integrating QuEChERS (Quick, Easy, Cheap, Effective, Rugged, Safe) and ultra-high performance liquid chromatography–quadrupole time-of-flight mass spectrometry (UHPLC–MS/MS) for the determination of seven alkaloids in honey and pollen from McRB, alongside pollen morphology analysis and the screening of suitable potential marker compounds for honey from McRB.

2. Materials and methods

2.1. Chemicals and reagents

Analytical standards (all with purity ≥98%) of PRO (CAS Number 130-86-9), ALL (CAS Number 485-91-6), SA (CAS Number 2447-54-3), CHE (CAS Number 34316-15-9), BER (CAS Number 633-65-8), DHCHE (CAS Number 6880-91-7) and DHSA (CAS Number 3606-45-9) were obtained from Heyuan Biotechnology Co. (Shanghai, China). LC–MS-grade formic acid and HPLC-grade methanol and acetonitrile were purchased from Fisher Chemicals (FairLawn, NJ, USA). Deionized water was prepared using a Millipore Milli-Q Plus system (Millipore, Bedford, MA, USA). The Bond Elut QuEChERS kits (part no. 5682-5550) with 50 mL tubes and salt packets containing 4 g $MgSO_4$ and 1 g NaCl, and QuEChERS d-SPE EMR-lipid (part no. 5982-1010) were purchased from Agilent Technologies (Santa Clara, USA).

2.2. Preparation of standard solutions

A 1 mg/mL stock solution containing ALL, PRO, SA, CHE, DHSA, DHCHE and BER was prepared and then diluted with methanol and blank matrix extract (honey and pollen) to obtain working standards at six different concentrations. The analytical stock standards were stored at −20 °C and working standards were stored at 4 °C.

2.3. Sample collection

McRB honey samples were obtained from various provinces by placing bee hives close to areas with McRB plants. Seven honey samples were collected from seven apiaries in Xinxiang, Henan province (H1–H7), and four honey samples were collected from Hubei province (H8–H11). McRB pollen samples were also collected from apiaries in Henan (P1–P7) and Hubei (P8–P11). Several samples from Hubei (n = 4) and Henan (n = 3) potentially contaminated with McRB were provided by two apicultural cooperative and used for method validation.

Sixty-one honey samples obtained from 28 beekeepers in Beijing, Hebei, Henan, Fujian, Sichuan, Xinjiang, Jilin, Shaanxi, and Hubei were used in this study. The samples included acacia honey (n = 10), rape honey (n = 10), linden honey (n = 6), jujube honey (n = 7), chestnut honey (n = 5), and multi-floral honey (n = 23). The honey samples were stored at 2–8 °C.

Thirty pollen types (rape, lotus, camellia, and multi-flower pollen) and 130 commercial honey samples were obtained from Chinese markets in Beijing, Hebei, Henan, Hubei, Fujian, Shaanxi, Jilin, and Sichuan.

Honey samples were stored at room temperature prior to analysis, and pollen samples were stored at −20 °C.

2.4. Pollen and honey preparation

2.4.1. Microscopic examination of bee pollen

To ensure sample authenticity, scanning electron microscope (SEM) analysis was performed to identify bee pollen stems from McRB plants. Bee pollen samples obtained from the McRB apiaries were fixed using glutaraldehyde (Wallace et al., 2015). A Hitachi S-750 SEM (Hitachi Company, Japan) was used to determine the McRB pollen morphology.

2.4.2. Sample preparation

The extraction procedure was based on a modified QuEChERS method (Anastassiades et al., 2003). Briefly, the homogenized sample (5.0 g of honey or 0.2 g of pollen) was weighed into a 50-mL polypropylene tube and extracted with 0.2% aqueous formic acid (10 mL) and acetonitrile (10 mL) using end-over-end shaking for 10 min. $MgSO_4$ (4 g) and NaCl (1 g) were added to the extraction mixture, and the tube was shaken for a further 1 min then centrifuged at 5000 rpm for 5 min. The following clean up step for the pollen using dispersive solid-phase extraction was necessary: the upper acetonitrile extract was collected and transferred to an EMR-Lipid d-SPE 15 mL tube already containing the adsorbent and 5 mL of water. Then the mixture was vortexed for 30 s and centrifuged at 5000 rpm for 5 min. Aliquots (0.5 mL) of the upper phase of the honey or cleaned pollen extract were diluted with deionized water (0.5 mL), mixed, and passed through a 0.2-μm nylon membrane filter prior to UHPLC–MS/MS analysis.

2.5. Instrumentation and UHPLC–MS/MS conditions

An Agilent 6495 triple quadrupole mass spectrometer (iFunnel technology; Agilent, Palo Alto, CA, USA) coupled to an Agilent 1290 liquid chromatograph and autosampler system was used. An electrospray ionization ion source was used with Agilent Jet Stream Technology (AJS ESI) in the positive-ion mode. Data analysis was performed with MassHunter software (version B07). Chromatography was carried out using an Agilent Poroshell 120 EC C18 column (2.1 mm × 100 mm, 2.7 μm). Because of the ionic interactions between the alkaloids, the mobile phases chosen were water with 0.1% formic acid (A) and methanol with 0.1% formic acid (B). Gradient elution was performed with 30% B (0–2 min), 30%–70% B (2–8 min), 70%–90% B (8–10 min), 90% B (10–13 min), and 90% − 30% B (13–14 min). The column was then conditioned with 30% B for 7 min. The total run time was 21 min, the flow rate was 0.4 mL/min, the injection volume was 2 μL, and the column temperature was 35 °C.

The optimum ESI operating conditions included a gas temperature of 150 °C, a gas flow of 15 L/min, a nebulizer pressure of 40 psi, a sheath gas temperature of 320 °C, and a sheath gas flow of 12 L/min. Capillary voltages were optimized to 4000 V in positive mode with equal nozzle voltages (0 V). The iFunnel parameters were optimized in positive mode at 60 V for low-pressure radio frequency (RF) and at 110 V for high-pressure RF. The multiple reaction monitoring (MRM) m/z transitions, collision energy (CE), and collision voltage presented in Table 1 were used in the qualitative and quantitative measurements of PRO, ALL, SA, BER, CHE, DHCHE, and DHSA.

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