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Characterisation and fate of grayanatoxins in mad honey produced from *Rhododendron ponticum* nectar

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ABSTRACT

Mad honey from *Rhododendron ponticum* nectar is produced in a large quantity in the western Black Sea region of Turkey and causes poisoning due to consumption of grayanatoxins (GTX I and III). There are a few studies about characterisation of GTXs in mad honey produced from *R. ponticum*. This study quantified basic properties including concentrations of GTX I and GTX III in mad honey samples collected in three consecutive years. Although the chemical composition of mad honey varied annually depending on the production year, mean GTX I and GTX III contents were estimated at 20.4 ± 1.69 and 8.20 ± 1.93 mg/kg, respectively. The concentrations of GTXs did not change significantly during storage of 6 months.

1. Introduction

Consumption of grayanatoxins (GTXs), also known as andromedotoxin, acetylandromedol, or rhodotoxin, in mad honey produced from *Rhododendron ponticum* nectar is reported to cause mad-honey poisoning (Constantine, Sheth, & Catalfomo, 1967; Gunduz, Turedi, Russell, & Ayaz, 2008; Onat, Yegen, Lawrence, Oktay, & Oktay, 1991; Yilmaz, Eser, Sahiner, Altintop, & Yesildag, 2006). Various other products, such as Labrador tea, cigarettes, and decoctions used in alternative medicine can also contain the toxin (Onat et al., 1991; Poon et al., 2008). The chemical structure of the toxin is defined as a diterpene, a polyhydroxylated cyclic hydrocarbon with a 5/7/6/5 ring structure that does not contain nitrogen (Tallent, Riethof, & Horning, 1957). GTXs cause dizziness, hypotension, and bradycardia when consumed in low doses, with impaired consciousness, syncope, atrioventricular block, and asystole due to vagal stimulation in high doses (Constantine et al., 1967; Gunduz et al., 2008; Onat et al., 1991; Poon et al., 2008; Tallent et al., 1957).

Japan, Nepal, Brazil, parts of North America, Europe, and eastern and western Black Sea regions of Turkey have a widespread and abundant distribution of *R. ponticum* plants (Cagli et al., 2009; Gunduz et al., 2008; Onat et al., 1991; Tallent et al., 1957). Poisoning effects of GTXs in mad honey across the world have been reflected in case reports from Turkey, Germany Austria, Japan, Nepal, North America, and Brazil (Aliyev et al., 2009; Demir, Denizbasi, & Onur, 2011; Koca & Koca, 2007). Owing to the rapid transport and growing interest of natural honey products, honey poisoning may become a worldwide problem (Erejuwa, Sulaiman, & Wahab, 2012; Hikino et al., 1976). Presence of GTXs in mad honey derived from *R. ponticum* and *Rhododendron luteum* nectars is one of the biggest issues for beekeepers across the Black Sea region of Turkey, since the final products obtained from a small area or even a single bee hive can contain a high concentration of GTXs. Restrictions on sales of mad honey produced from *R. ponticum* and *R. luteum* species in Turkey put mad honey production near extinction. A lack of detailed studies about the characterisation of GTXs in mad honey in response to storage conditions further adds uncertainties to a better understanding and addressing of how to solve food safety issues concerning mad honey production. Therefore, the present study was conducted to quantify concentrations of GTXs in mad honey in response to storage conditions.

2. Materials and methods

2.1. Mad honey samples

One hundred and seventy-eight mad honey samples from nectar of *R. ponticum* were collected in 2010, 2011 and 2012 from

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seven locations in Kastamonu, Duzce, Zonguldak, Karabuk and Bartin across the western Black Sea region (Turkey). The collected samples in each year were homogenised by mixing, and samples were kept in a dry place at 20 C for further analyses.

2.2. Moisture analysis

Moisture content of the samples was measured according to the Official Method 2010 (969.38) using a refractometer (Atoga, Italy).

2.3. Titratable acidity

The samples were analysed by a potentiometric titration method (AOAC, 2010-998.12) using 0.1 N NaOH, and results were expressed in meq/kg.

2.4. Conductivity

Conductivity measurement of the samples was realized by an IHC (2009) method and results were expressed in mS/cm.

2.5. Hydroxy methyl furfural (HMF) analysis

An IHC spectrophotometric (Shimadzu UV–Vis 1800; Kyoto, Japan) method (2009) at 550 nm was used, and results were given as mg/kg.

2.6. Proline analysis

An OAC 2010–979.20 method at 510 nm wavelength with proline calibration standard was used. The amount of proline was expressed in mg/kg.

2.7. Diastase number

An AOAC 2010-958.09 method at 660 nm was applied to determine diastase number.

2.8. Sugar analysis

Sugar content (% w/w), as fructose, glucose, sucrose and maltose, in addition to fructose + glucose and fructose/glucose ratio of the samples was determined by HPLC (Shimadzu, LC20AT) with a refractive index detector by the DIN 10758 method.

2.9. Total phenolic content

Total phenolic content (TPC) of the samples was measured by a Folin–Ciocalteu colorimetric method with some modifications. An aliquot of the samples (0.3 mL) was diluted with 15 mL water and 0.2 N Folin–Ciocalteu reagent (1.5 mL) was added before vortexing the mixture for 3 min. Three mL of Na_2CO_3 were added to the mixture and mixed thoroughly by vortexing. Before absorbance measurements of the samples at 724 nm, they were set at room temperature for one hour. Obtained absorbance values were calculated from the gallic acid standard curve prepared with 100, 200, 300, 400, and 500 mg/L gallic acid. The total phenolic content of the samples was calculated as mg/L gallic acid equivalents (Slinkard & Singleton, 1977; Spanos & Wrolstad, 1990).

2.10. Radical-scavenging activity (RSA)

The samples (0.5 mL) were mixed with 1 mM DPPH (0.5 mL) and 3 mL ethanol (99%). The mixture was shaken and left to stand for 30 min in the dark. Absorbance of the mixture was measured at 517 nm. Ascorbic acid at both 0.1 and 1.0 mM was used as a positive control (Liu, Ye, Lin, Wang, & Peng, 2013; Moon & Terao, 1998).

2.11. Metal ion concentration

Metal ion concentration of the samples was measured with an inductively coupled plasma mass spectrometer (ICP-MS) (XSERIES 2; Thermo Scientific, Schwerte, Germany). One millilitre of the sample was taken and 0.5 mL of hydrogen peroxide were added before 2.5 mL of 65% nitric acid. After complete digestion of the samples (30 min) at room temperature, samples were held for 20 min at 140 C in a microwave oven (CEM MARS 5, ABD). The samples were then filtered through a 0.45-μm filter (hydrophilic PVDF; Millipore Millex-HV), and the filtered samples were made up to 10 mL with distilled water. Standard metal solution was prepared daily from 1000 mg/L stock (Merck, Darmstadt, Germany) in 2% nitric acid (Suprapur grade; Merck). ICP-MS had a Babington-type nebuliser and Scott-type spray chamber, and an RF generator which has 10 MHz frequency and 1300 W power output. Argon flow rate (L/min) was 15 for plasma, 0.9 for auxiliary, and 1–1.1 for nebuliser. Solution uptake rate was 1.8 mL/min. Sampler cone and skimmer were nickel with i.d. of 1.1 and 0.9 mm, respectively. Pressures of interface and quadrupole were 4 and 2×10^{-6} torr, respectively. Data acquisition was provided by peak hopping, 200 ms replicate time, 200 ms dwell time, 3 sweeps/reading, and 3 readings/replicate. Analytical masses were ^{75}As, ^{9}Be, ^{40}Ca, ^{111}Cd, ^{59}Co, ^{52}Cr, ^{63}Cu, ^{56}Fe, ^{69}Ga, ^{39}K, ^{7}Li, ^{24}Mg, ^{55}Mn, ^{23}Na, ^{60}Ni, ^{208}Pb, ^{80}Se, ^{88}Sr, ^{48}Ti and ^{66}Zn (Cubadda et al., 2001).

2.12. Grayanatoxin analyses

Five milliltres of water were added to 5 g of samples and mixed for 60 s by vortexing. After the addition of 25 mL methanol, the mixture was vortexed for another 60 s, and then centrifuged for 5 min at 3000 rpm. The upper layer of the sample was filtered and the extraction process was repeated twice by addition of 25 mL ethanol each time. Undesired compounds in the extracted liquids were removed by using an Oasis HLB SPE cartridge. SPE cartridge was conditioned by passing 2×5 mL ethanol and 2×5 mL water. The filtered honey sample, 5 mL water and 5 mL 20/80 acetone/water were passed though the cartridge. The samples were eluded with 5 mL of methanol and were dried under nitrogen at 40 ± 5 °C. One millilitre of ethanol was added to dried extract placed in ultrasonic water bath for 1 min. The samples were centrifuged at 3000 rpm for 1 min, and the upper layer was removed by filtration using 0.45-μm filters. The extracts were analysed using LC-MS/MS (3200 QTrap, Applied Biosystems, Foster City, CA) for GTX I and III. The analyses were performed using a Symmetry C18 5 μm 2.1 × 150 mm column (Altmann Analytik GmbH & Co. KG Munich, Germany) with mobile phases A (water + 0.1% acetic acid) and B (methanol + 0.1% acetic acid), 10 μL injection volume, 40 °C oven temperature and 0.3 mL/min flow rate. Flow gradient of mobile phase B was 10% first 1 min, 80% for 10 min, 80% for 30 min, 10% for 31 min and 10% for 41 min. LC–MS/MS parameters were 0.7 s scan time, 1800 V detector voltage, 5000 V needle voltage, 600 V spray shield voltage, 50 °C drying gas temperature, 55 psi nebulising gas pressure and 35 °C drying gas temperature. MS ions determined as Q1 (main ion, *m/z*) and Q3 (fragmentation ion, *m/z*) for GTX I were 435 and 375, respectively. The selected Q1 and Q3 ions for GTX III were 335 and 317, respectively (Fig. 1).

Validation of GTX analysis was conducted according to the optimised method 2002/657/EC (Antignac, Le Bizec, Monteau, & Andre, 2003) summarised in Table 1. GTXs concentration was calculated by the calibration curve obtained by measuring the area calculated from the chromatogram. Acceptance of quantitative and qualitative ions for the confirmation of the compounds according to SANFO