A case of human poisoning by grayanotoxins following honey ingestion: elucidation of the toxin profile by mass spectrometry

Anja These*, Dorina Bodi*, Samuel Uecker*, Katharina Reimers*, Stefan Ronczka*, Angelika Preiss-Weigert* and Monika Lahrsen-Wiederholt*

*Federal Institute for Risk Assessment, Department Safety in the Food Chain, Berlin, Germany; **Marien Hospital Herne, Klinikum der Ruhr-Universität Bochum, Herne, Germany

(Received 4 February 2015; accepted 12 April 2015)

High-resolution mass spectrometry (HRMS) was applied for the detection of grayanotoxins (GrTx) in a contaminated honey sample. This sample was provided by a hospital due to a suspicion of intoxication after a patient had shown the typical symptoms of GrTx poisoning. Subsequent analysis proved the contamination with high amounts of GrTx and other toxins belonging to grayanate-type diterpenoids. This group of natural toxins is synthesised by the plant family Ericaceae and comprises more than 60 individual toxins, but only one compound is available as a reference standard. We applied a screening approach that easily confirms the presence or absence of GrTx without access to standards. By searching for predictable mass spectrometric fragment ions, including typical in-source fragments arising from collision-induced dissociation during electrospray ionisation, the complete toxin profile was screened and allowed the mass spectrometric identification of 15 individual GrTx. The potential of this approach is especially demonstrated by the fact that at least two of these toxins have not been previously described in the literature. A semi-quantitative estimation indicated a total toxin concentration of 358 mg kg⁻¹. An investigation of 49 honeys from the German retail market did not reveal the presence of GrTx.

Keywords: mass spectrometry; grayanotoxins; intoxication; honey; grayanane; Rhododendron

Introduction

Grayanotoxins (GrTx) are formed by the plant family Ericaceae, for instance by genera of Rhododendron, Pieris (Andromeda), Leucothoe, Craibiodendron, Lyonia or Kalmia (Scott et al. 1971; Burke & Doskotch 1990; Teuscher & Lindequist 1994; Kron et al. 2002; Qiang et al. 2011; Li et al. 2013). These secondary metabolites are diterpenes with a tetracyclic A-nor-B-homo-kaurane skeleton called grayanate or andromedane, a polyhydroxylated cyclic hydrocarbon with a 5/7/6/5 ring structure (Wood et al. 1954; Tallent et al. 1957; Narayanan et al. 1970; Burke & Doskotch 1990; Li et al. 2013) (Figure 1).

In addition to the GrTx, related toxins such as asebotoxins, rhodojaponins, rhodomolleins, kalmitoxins, lyonatotoxins and craiibiotoxins are formed by different substitution of the skeleton (Figure 1 and Table 1). Up to now the occurrence of approximately 60 grayanate-type diterpenoids has been reported (Wood et al. 1954; Tallent et al. 1957; Hikino et al. 1976; Sakakibara et al. 1979; El-Naggar et al. 1980; Burke & Doskotch 1990; Klocke et al. 1991; Wang et al. 1998; Zhang et al. 2005, 2008; Li et al. 2013). Due to common structural features of grayanate-type toxins, their nomenclature is often confusing because derivatives are also designated as asebotoxins, rhodojaponine, androme-doto-, andromedol-, lyonia-, kalmia- and pieristoxins (Teuscher & Lindequist 1994). In particular, as several studies were published within a short period either investigating agents of similar activity or even the same agent (Wood et al. 1954; Tallent et al. 1957).

GrTx have acute toxic effects and the dose–effect relationships of some of them have been intensively investigated in mice (Hikino et al. 1976). Further data were received from studies in skeletal muscle of frogs (Deguchi & Sakai 1967), rats (Fukuda et al. 1974; Nishikawa et al. 1989; Onat et al. 1991) and brine shrimp larvae (Kinghorn et al. 1978). The harmful effects of GrTx result from their binding to sodium channels of excitable membranes and the resulting inactivation of action potential. This leads to continuous depolarisation and enhancement of the calcium cation influx (Narahashi & Seyama 1974; Teuscher & Lindequist 1994; Jansen et al. 2012; Li et al. 2013). In human cases of high ingested amounts of GrTx, symptoms began within 30 min up to 4 h of latency at the most. The most striking symptoms and signs were nausea, hypotension, bradycardia and impairment of consciousness (Gunduz et al. 2008; Jansen et al. 2012).

Due to beekeeping in regions, where GrTx producing species grow, honey can be contaminated and its ingestion can result in acute toxic effects (Carey et al. 1959; White & Riethof 1959; Scott et al. 1971; Sütlüpmar et al. 1993;
This paper describes the case of a 56-year-old man who caused a traffic accident after eating two tablespoons of wild Turkish honey. Three hours after consumption the man lost consciousness due to development of severe bradycardia (slow heart rate), which was diagnosed during physical examination in hospital. The hospital physician suspected GrTx intoxication due to the patient’s symptoms and, therefore, a sample of the honey was provided for toxin analysis using MS. Currently, only GrTx III can be unambiguously identified in suspect samples since it is the only compound commercially available as a reference standard. However, using high-resolution mass spectrometry (HRMS) it should be possible to match individual accurate mass measurement information to known structural formulae, thus enabling identification of other GrTx and grayanane toxins that might be present in the sample. The common core structure of these toxins (Figure 1 and Table 1) results in a similar and thus predictable mass spectrometric fragmentation. All toxins exclusively exhibit successive losses of water arising from collision-induced dissociation during electrospray ionisation (Holstege et al. 2001). Therefore, toxins were easily identified by exact mass determination of several typical in-source fragments resulting from repeated losses of water. For result confirmation, all analytes detected as supposed toxins were further investigated by recording product ion spectra. As criteria for the identification of an analyte as GrTx or grayanane-type toxin, the following had to be fulfilled: the detection of exact masses of the molecular ion and several diagnostic ions as well as the exhibition of a typical fragmentation pattern. As all putative toxins were subsequently confirmed by their product ion spectra, it could be demonstrated that the screening for in-source fragments resulting from losses of water is a successful approach for GrTx analysis in the absence of standards for the complete compound class.

For the quantification of detected toxins with no available reference standards, a semi-quantitative approach was used on the assumption that the mass spectrometric response of GrTx III is comparable with all other toxins.

Materials and methods
Solvents, standards and reagents
Formic acid (analytical grade), methanol (MeOH; hyper-grade), acetonitrile (ACN) and sulfuric acid were obtained from Merck (Darmstadt, Germany). Grayanotoxin III-standard was purchased from Sigma Aldrich (Steinheim, Germany).

Sample
The wild honey sample originated from the Turkish Black Sea region.
Table 1. Naturally occurring Ericaceous toxins with a grayanane-type structure summarised by their common carbon core structure which leads to identical exact masses for molecular ions or in-source fragments.

| Group | Carbon core structure C_{20}H_{2n+4}O_{4} + n H_2O |
|-------|--------------------------------------------------|
| Group 1: | Pieristoxin G C_{20}H_{30}O_8 |
| Further toxins: | |
| Group 2: | Grayanotoxin XVII C_{20}H_{32}O_8 Pieristoxin H C_{20}H_{26}O_7 |
| Further toxins: | |
| Group 3: | Grayanotoxin XI C_{20}H_{30}O_8 Rhodojaponin III C_{20}H_{32}O_7 Rhodomollein I C_{20}H_{32}O_6 |
| Further toxins: | C_{20}H_{30}O_6: rhodomollein XIX; C_{20}H_{32}O_6: grayanotoxin V, XII, rhodomollein IX, X, kalmintoxin II, deacylyoniatoxin, lyoniol B; C_{20}H_{34}O_7: rhodojaponin VI, rhodomollein XVIII, kalmintoxin I |
| Group 4: | Grayanotoxin II C_{20}H_{30}O_4 Grayanotoxin III C_{20}H_{32}O_5 Craiobiotoxin I C_{20}H_{32}O_5 |
| Further toxins: | C_{20}H_{30}O_4: grayanotoxin VII, VIII, XIX; C_{20}H_{32}O_5: grayanotoxin VI, craiobiotoxin II, V, VI, VII, VIII, |
| Group 5: | Grayanotoxin XVIII C_{20}H_{32}O_4 |
| Further toxins: | |
| Group 6: | Grayanotoxin II C_{21}H_{34}O_6 |
Table 1. Continued.

**Group 7: Carbon core structure C_{22}H_{26}O_{4} + n H_{2}O**

- Craiobiotoxin IV  
  C_{22}H_{4}O_{5}
- Pieristoxin J  
  C_{22}H_{4}O_{5}

Further toxins:

**Group 8: Carbon core structure C_{22}H_{28}O_{4} + n H_{2}O**

- Rhodojaponin II  
  C_{22}H_{34}O_{2}
- Rhodomollein III  
  C_{22}H_{34}O_{2}
- Lyoniol A/lyoniotoxin  
  C_{22}H_{34}O_{2}

Further toxins: C_{22}H_{34}O_{2}: rhodojaponin V, rhodomoleins B, rhodomollein XII; C_{22}H_{36}O_{8}: rhodomollein XVI, kalmitoxin III, lyoniol D

**Group 9: Carbon core structure C_{22}H_{30}O_{4} + n H_{2}O**

- Grayanotoxin I  
  C_{22}H_{3}O_{5}
- Grayanotoxin XIII  
  C_{22}H_{3}O_{5}
- Rhodomollein XIII  
  C_{22}H_{3}O_{5}

Further toxins: C_{22}H_{3}O_{5}: grayanotoxin XIV

**Group 10: Carbon core structure C_{22}H_{32}O_{4} + n H_{2}O**

- Grayanotoxin IV  
  C_{22}H_{4}O_{5}
- Grayanotoxin IX  
  C_{22}H_{4}O_{6}
- Grayanotoxin X  
  C_{22}H_{3}O_{5}

Further toxins: C_{22}H_{32}O_{4}: grayanotoxin XVI

**Group 11: Carbon core structure C_{23}H_{26}O_{4} + n H_{2}O**

- Asebotoxin VII  
  C_{23}H_{3}O_{8}

Further toxins:

**Group 12: Carbon core structure C_{23}H_{28}O_{4} + n H_{2}O**

- Asebotoxin III  
  C_{23}H_{3}O_{8}

Further toxins:
| Group 13: Carbon core structure $C_{23}H_{30}O_4 + n\ H_2O$ |  |
|---|---|---|
| **Pierisformosin C** | **Asebotoxin VI** | **Asebotoxin X'**
$C_{23}H_{38}O_8$ | $C_{23}H_{38}O_8$ | $C_{23}H_{38}O_8$
| **Further toxins:** |  |

| Group 14: Carbon core structure $C_{23}H_{32}O_4 + n\ H_2O$ |  |
|---|---|---|
| **Asebotoxin I/Pierisformosin B** | **Asebotoxin II** |  
$C_{23}H_{38}O_8$ | $C_{23}H_{36}O_6$ |  |
| **Further toxins:** |  |

| Group 15: Carbon core structure $C_{24}H_{30}O_4 + n\ H_2O$ |  |
|---|---|---|
| **Rhodojaponin I** | **Rhodojaponin VII** |  
$C_{24}H_{38}O_8$ | $C_{24}H_{38}O_9$ |  |
| **Further toxins:** |  |

| Group 16: Carbon core structure $C_{24}H_{32}O_4 + n\ H_2O$ |  |
|---|---|---|
| **Rhodojaponin IV** | **Kalmitoxin V** | **Pierisformosin D**
$C_{24}H_{36}O_8$ | $C_{24}H_{36}O_7$ |  
| **Further toxins:** |  |

**Group 17: Carbon core structure $C_{25}H_{30}O_4 + n\ H_2O$**

| **Pieristoxin K** |  
$C_{25}H_{38}O_8$ |  |
| **Further toxins:** |  |

**Group 18: Carbon core structure $C_{25}H_{32}O_4 + n\ H_2O$**

| **Craibobiotoxin III** | **Asebotoxin VIII or Pieristoxin F** | **Asebotoxin IX**
$C_{25}H_{40}O_8$ | $C_{25}H_{36}O_8$ | $C_{25}H_{38}O_7$ |  |
| **Further toxins:** |  |
Sample preparation

The sample preparation method was developed for the simultaneous detection of pyrrolizidine alkaloids and GrTx in honey. A test portion of 3 g of honey was dissolved in 10 ml of aqueous sulfuric acid solution (50 mM) by shaking for 30 min on a rotary mixer. The sample solution was centrifuged at 3800g for 10 min. The entire supernatant was transferred into a new centrifuge tube by decanting and used for SPE clean-up. C-18 cartridges (500 mg, Discovery DSC-18 SPE, Supelco, Bellefonte, PA, USA) were conditioned with 5 ml of methanol and 5 ml of aqueous sulfuric acid solution (0.05 M) before loading. The sample was loaded onto the SPE cartridge at 35°C. The cartridges were then washed with 5 ml of water and dried under vacuum for 30 min. Elution of GrTx from the cartridges was accomplished with 10 ml (2 × 5 ml) of methanol. The eluate was dried under a nitrogen stream at 50 ± 5°C and the residue was dissolved in 1 ml of methanol/water (5/95, v/v) by shaking. The reconstituted sample extracts were filtered through a 0.2 µm membrane filter (VWR, Darmstadt, Germany).

Recovery for GrTx in honey

An excellent recovery of 97% ± 6.1% (n = 5) was obtained by spiking a blank honey with 167 µg kg⁻¹ of GrTx III.

HPLC

Analyses were performed using a Thermo Fisher Accela LC System in combination with an HTS PAL autosampler (PalSystem, Zwingen, Germany). Chromatography was carried out on a 150 × 2.1 mm, 1.9 µm particle size C18 Hypersil Gold column (Thermo Fisher, Runcorn, UK). Eluent A was prepared with 100% water containing 0.2% formic acid and eluent B with 95% ACN and 5% water containing 0.2% formic acid. A gradient elution was used as follows: 0–1 min A: 90%/B: 10%, 12.0 min A: 40%/B: 60%, 15 min A: 40%/B: 60%, 16 min A: 90%/B: 10% and 25 min A: 90%/B: 10%. The flow rate was 300 µl min⁻¹ and 10 µl of sample were injected.

Mass spectrometry

HRMS data were acquired on an Orbitrap Exactive™ system (Thermo Fisher Scientific, Bremen, Germany). The MS was operated at 3500 V using an S-lens voltage of 140 V. Two scan events were recorded in parallel by permanent switching. Firstly, a full-scan from m/z 160 to 1000, applying a resolution of 50 000; and secondly, fragments generated by an HDC 30 V experiment were acquired from m/z 90 to 800 using a resolution of 25 000.

Tandem mass spectrometry (ESI-MS/MS) data were acquired on a TSQ Vantage (Thermo Fisher Scientific, San Jose, CA, USA). GrTx were analysed using a spray voltage of 3500 V, an S-lens voltage of 140 V and a vaporiser temperature of 300°C. Q1 and Q3 were set at unit resolution (These et al. 2013).

Results and discussion

Screening for grayanotoxins and other grayanane-type toxins

Due to the geographic origin of the ingested honey and the observed patient symptoms, contamination with GrTx was considered by the treating physician. A sample of the honey was sent for toxin analysis. Numerous toxins with grayanane-type structure have been reported to cause acute toxicity (Hikino et al. 1976). Therefore, our analytical approach was not focused on a single toxin but on the whole group of toxins. Apart from GrTx III, no reference standards are available making targeted detection, for example, by MRM inapplicable. Therefore, full-scan HRMS was applied to screen the sample for grayanane-type toxins. The naturally occurring grayanane-type toxins produced by the Ericaceae family are summarised in Table 1 and grouped according to their common carbon core structures (see below).

Table 1: Screening for grayanane-type toxins

The first step was to screen the sample for the exact pseudo-molecular ion masses of all toxins listed in Table 1. However, the exact mass measurement of the pseudo-molecular ion for a toxin is an insufficient positive identification criterion in isolation: additional diagnostic ions must be identified. All the investigated toxins are polyhydroxylated compounds containing multiple saturated alkyl alcohol groups that are prone to in-source fragmentation resulting in successive losses of H₂O (~18 amu). This is exemplified by the HRMS full-scan spectrum of GrTx III which demonstrates the strong proneness to the losses of H₂O (Figure 2a). In order to demonstrate that the fragments observed in the full-scan spectrum originate from GrTx III a product-ion spectrum was acquired by tandem mass spectrometry. This enables the targeted fragmentation of a precisely selected specific precursor ion originating from the molecule of interest. Since no molecular ion ([C₂₀H₃₄O₅]⁺ or the respective adduct [C₂₀H₃₄O₆Na]⁺) was apparent on the tandem mass spectrometer, the in-source fragment m/z 353 [GrTx III – H₂O] was selected as a precursor ion for recording the product ion spectrum (Figure 2b). The congruence of both spectra confirms that the exact masses of in-source fragment ions formed by successive losses of H₂O can be used as diagnostic ions for the identification of grayanane-type toxins using HRMS. As the core structure of these toxins only contains saturated alcoholic groups, their product ion spectra solely consist of fragments originating from multiple losses of...
water and lack any other fragmentation. Both facts are important identification criteria as all other analytes than grayanane-type toxins that bear other functional groups than saturated alcohols would have to exhibit fragments or neutral losses which are specific for ethers, esters, sugar moieties etc. or other substance-specific fragmentations. Further, the application of HRMS scans allows the identification (and therefore exclusion) of analytes that contain nitrogen atoms or isotopes due to the presence of even molecular ions or specific isotopic masses respectively. The typical grayanane-type fragmentation pattern shown in Figure 2 leads to a grouping of these toxins according to their mass spectrometric fragmentation behaviour (Table 1). All toxins belonging to one group have either identical sum formulas or result in the same fragment ions and carbon cores due to losses of water in the MS source (Figure 2). In order to group individual toxins and to compare the carbon core structures of all grayanane-type toxins, their sum formulas were reduced to their smallest common representative structural unit. The minimum oxygen atom count is 4 (Table 1, refer to GrTx XVIII: C_{20}H_{32}O_{5} in group 5 and GrTx X: C_{22}H_{32}O_{5} in group 10). Therefore, all other sum formulas were theoretically recalculated to four oxygen atoms by successive the losses of water (-H_{2}O). This procedure results in carbon core sum formulas with four oxygen atoms allowing a direct prediction of which toxin will form identical fragment ions during in-source fragmentation. Consequently, each carbon core group in Table 1 summarises toxins that can be detected in the same extracted ion chromatogram (exact mass filter). For instance, the sum formula of pieristoxin G (C_{20}H_{32}O_{6}, group 1; Table 1) can be reduced to the four-oxygen atom core by a fourfold loss of H_{2}O, resulting in C_{20}H_{28}O_{4}. Obviously, no other grayanane-type toxin exhibits this core structure and, thus, no other known toxins will be detected using this exact mass filter (see below).
For the unambiguous identification of GrTx III in the honey sample, the elution profile for the exact mass ion chromatograms for GrTx III, the pseudo-molecular ion and specific fragment ions resulting from repeated losses of water were compared with those of the standard (Figure 3). When extracting these GrTx III-specific ion chromatograms ($R_t = 4.5$ min), additional signals at different retention times were detected (Figure 3). This can be explained by the fact that in addition to GrTx III (sum formula $C_{20}H_{34}O_{6}$), GrTx II, VI and craiobiotoxin II, V, VI, VII, VIII (sum formula of $C_{20}H_{32}O_{3}$), and GrTx VII, VIII, XIX (sum formula of $C_{20}H_{30}O_{4}$) share the common carbon core structure $C_{20}H_{16}O_{4}$ after successive losses of $H_2O$ (group 4; Table 1). Therefore, this screening approach extracts and screens concurrently all toxins belonging to the same group (Table 1) because, per group, the same diagnostic ions specific for a particular carbon core are formed. It can be concluded that the suspect honey sample contains five of the grayanane-type toxins summarised in group 4 (Table 1 and Table 2), including GrTx III, which was confirmed by comparison with the reference standard.

By means of the described approach, we screened the sample according to the toxin groups listed in Table 1. Signals were suspected to originate from grayanane-type toxins, if extracted ion chromatograms representing iterative water losses showed signals at the same retention time (Figure 3). For all peaks, which were identified as potential grayanane-type toxins by HRMS, product ion spectra were recorded. Those compounds displaying fragmentation patterns similar to the GrTx III standard (Figure 2) or as described in the literature (Holstge et al. 2001) were confirmed as grayanane-type toxins. Results are listed in Table 2.

As standards are not yet available, an unambiguous identification of individual toxins detected for each group is not possible, especially if several toxins share the same molecular formula. For a putative identification the highest observed $m/z$ precursor ion per detected toxin is listed in Table 2 together with all known toxins with matching sum formulas. For instance, for group 2: two analytes eluting at $R_t = 3.3$ and 4.5 (min) were detected and at both retention times the highest observed sum formula was $C_{20}H_{31}O_6$ ($[M + H]^+$ for positive mode). Assuming that $C_{20}H_{31}O_6$ is the pseudo-molecular ion, one of these two signals should originate from GrTx XVII and the other from a grayanane-type toxin not yet described in the literature. For group 3, three toxins were detected: two with the sum formula $C_{20}H_{32}O_5Na$, which can be theoretically assigned as rhodojaponin VI, rhodomollein XVIII or kalmitoxin I, and one with the sum formula $C_{20}H_{31}O_5$, which was identified as rhodomollein XIX (since no other toxin with that corresponding formula has been described in the literature). Most of the naturally occurring grayanane-type toxins belong to groups 3 and 4 and eight of the 15 toxins detected in the honey sample are summarised therein, with the most intensive signals belonging to toxins of group 4. Interestingly, two group 5 analytes were detected, although only GrTx XVIII is reported in the literature. Presumably, a further unreported grayanane-type toxin with the corresponding molecular formula was detected in the honey sample.

**Semiquantitative determination of grayanotoxins and other grayanane-type toxins to estimate toxin intake**

The concentration of GrTx III in the sample was determined by integrating the peak areas of selected ion chromatograms from in-source fragments obtained by HRMS (Figure 3) and applying an external calibration via the reference standard (Table 2). Amounts of all other toxins were estimated semiquantitatively by reference to the GrTx III standard, assuming equal response factors for all other toxins. For each unknown toxin, the concentration was estimated at 358 mg kg$^{-1}$, while the total ingested toxin amount was estimated at 8.9 mg, calculated based on the quantity of honey consumed (Table 2) (reported by the hospital to be two tablespoons, amounting to approximately 25 g).
Table 2. Results of the honey sample under investigation. Detected toxins per group (Table 1) and estimated concentrations. Product ion spectra for toxins of each carbon core group are published in the Supplementary data online.

| Carbon core group | Rt (min) | Toxin concentration (mg kg\textsuperscript{-1}) | Ingested amount of toxin (mg)\textsuperscript{a} | Identified formula | Toxins with the corresponding molecular formula |
|-------------------|----------|-----------------------------------------|--------------------------------|-----------------|-----------------------------------------------------------------|
| Group 1: C\textsubscript{20}H\textsubscript{24}O\textsubscript{4} + n H\textsubscript{2}O | n.f. | n.f. | n.f. | n.f. | n.f. |
| Group 2: C\textsubscript{20}H\textsubscript{26}O\textsubscript{4} + n H\textsubscript{2}O | 3.3 | 6.2 | 0.15 | C\textsubscript{20}H\textsubscript{13}O\textsubscript{6} | Grayanotoxin XVII |
| | 4.5 | 12.1 | 0.30 | C\textsubscript{20}H\textsubscript{13}O\textsubscript{6} | |
| Group 3: C\textsubscript{20}H\textsubscript{28}O\textsubscript{4} + n H\textsubscript{2}O | 2.1 | 1.4 | 0.03 | C\textsubscript{20}H\textsubscript{14}O\textsubscript{2}Na | Rhodojaponin VI, rhodomollein XVIII, Kalmitoxin I |
| | 3.4 | 2.0 | 0.24 | C\textsubscript{20}H\textsubscript{14}O\textsubscript{2}Na | |
| Group 4: C\textsubscript{20}H\textsubscript{31}O\textsubscript{6} | 4.7 | 8.3 | 0.21 | C\textsubscript{20}H\textsubscript{13}O\textsubscript{3} | Rhodomollein XIX |
| | 5.3 | 116.3 | 2.91 | C\textsubscript{20}H\textsubscript{14}O\textsubscript{2}Na | Grayanotoxin IV, VII, VIII, XIX |
| | 5.6 | 5.8 | 0.14 | C\textsubscript{20}H\textsubscript{13}O\textsubscript{4} | |
| | 6.0 | 2.0 | 0.05 | C\textsubscript{20}H\textsubscript{13}O\textsubscript{3} | |
| | 7.2 | 18.6 | 0.46 | C\textsubscript{20}H\textsubscript{14}O\textsubscript{2}Na | Grayanotoxin II, VI, Craiobiotoxin I, II, V, VIII |
| Group 5: C\textsubscript{20}H\textsubscript{33}O\textsubscript{4} | 8.5 | 39.8 | 0.99 | C\textsubscript{20}H\textsubscript{13}O\textsubscript{4} | Grayanotoxin XVIII |
| | 9.2 | 3.2 | 0.08 | C\textsubscript{20}H\textsubscript{13}O\textsubscript{4} | |
| Group 6: C\textsubscript{21}H\textsubscript{26}O\textsubscript{4} + n H\textsubscript{2}O | n.f. | n.f. | n.f. | n.f. | n.f. |
| Group 7: C\textsubscript{22}H\textsubscript{28}O\textsubscript{4} + n H\textsubscript{2}O | n.f. | n.f. | n.f. | n.f. | n.f. |
| Group 8: C\textsubscript{24}H\textsubscript{30}O\textsubscript{4} + n H\textsubscript{2}O | n.f. | n.f. | n.f. | n.f. | n.f. |
| Group 9: C\textsubscript{24}H\textsubscript{32}O\textsubscript{5} + n H\textsubscript{2}O | 5.3 | 58.3 | 1.46 | C\textsubscript{22}H\textsubscript{30}O\textsubscript{2}Na | Grayanotoxin I, rhodomollein XIII |
| | 7.7 | 12.2 | 0.30 | C\textsubscript{22}H\textsubscript{30}O\textsubscript{2}Na | Grayanotoxin XIII, XIV |
| Group 10: C\textsubscript{22}H\textsubscript{35}O\textsubscript{5} | 7.2 | 10.5 | 0.26 | C\textsubscript{22}H\textsubscript{35}O\textsubscript{5} | Grayanotoxin IV, IX, X, XVI |
| Group 11: C\textsubscript{23}H\textsubscript{28}O\textsubscript{4} + n H\textsubscript{2}O | n.f. | n.f. | n.f. | n.f. | n.f. |
| Group 12: C\textsubscript{23}H\textsubscript{30}O\textsubscript{4} + n H\textsubscript{2}O | n.f. | n.f. | n.f. | n.f. | n.f. |
| Group 13: C\textsubscript{23}H\textsubscript{32}O\textsubscript{5} + n H\textsubscript{2}O | n.f. | n.f. | n.f. | n.f. | n.f. |
| Group 14: C\textsubscript{24}H\textsubscript{30}O\textsubscript{4} + n H\textsubscript{2}O | n.f. | n.f. | n.f. | n.f. | n.f. |
| Group 15: C\textsubscript{24}H\textsubscript{32}O\textsubscript{5} + n H\textsubscript{2}O | n.f. | n.f. | n.f. | n.f. | n.f. |
| Group 16: C\textsubscript{24}H\textsubscript{34}O\textsubscript{5} + n H\textsubscript{2}O | n.f. | n.f. | n.f. | n.f. | n.f. |
| Group 17: C\textsubscript{25}H\textsubscript{32}O\textsubscript{5} + n H\textsubscript{2}O | n.f. | n.f. | n.f. | n.f. | n.f. |
| Group 18: C\textsubscript{25}H\textsubscript{34}O\textsubscript{5} + n H\textsubscript{2}O | n.f. | n.f. | n.f. | n.f. | n.f. |
| Sum | 358 | 8.93 | | | |

Notes: \textsuperscript{a}Estimated ingested amount from the consumption of two table spoons (25 g).
\textsuperscript{b}Identification by cross-matching with the available standard.
n.f., Not found.
Investigation of honey from the German retail market

In order to assess the relevance of GrTx for human consumption, 49 honey samples of different geographic as well as botanical origin from retail markets were analysed. A full sample description is given in the Supplementary data online. No GrTx could be detected in any of the investigated samples.

Conclusions

A combination of MS tools allowed a detailed characterisation of the toxin profile in a suspect honey sample. The exact mass measurement of pseudo-molecular and fragment ions proved the presence of 15 grayanane-type toxins comprising 12 containing 20 carbon atoms and three containing 22. At least two of these have, to the best of our knowledge, not been previously described in the literature. Of the two compounds detected with molecular formulas corresponding to $C_{20}H_{30}O_6$ (group 2) and the two with $C_{20}H_{32}O_4$ (group 5), only one matching toxin has been reported in each case, namely GrTx XVII and XVIII, respectively. Grayanane-type toxins containing 21, 23, 24 or 25 carbon atoms, which have been reported in the literature, were not detected. For most of the compounds detected, molecular sum formulas were assigned, corresponding to toxins already reported in the literature. GrTx III was unambiguously quantified in the sample at a concentration of 54 mg kg$^{-1}$ and the semiquantitative determination of other toxins revealed an estimated total toxin concentration of 358 mg kg$^{-1}$. Coupled with the reported ingested amount, a grayanane-type toxin intake of 8.9 mg has been estimated. However, effects of the individual toxins in humans are not known. The current knowledge on relative toxicities of different toxins is limited and information is derived from only a few animal experiments. There is some evidence from mice that GrTx I and III exhibit comparable toxicities, while GrTx II is of minor toxicity (Scott et al. 1971; Hikino et al. 1976). We cannot exclude that toxins were quantified that are not relevant for risk assessment and, therefore, conclusions concerning individual toxin concentrations and the extent of their effects on the patient regarding the reported symptoms cannot be drawn.

The analytical investigation of German retail honey samples did not confirm the presence of GrTx or grayanane-type toxins and it can therefore be assumed that GrTx intoxication is a potential risk limited to honey of a certain botanical origin and does not constitute a global problem. Intoxications by honey seem to be limited to regions where plants with respective producing capacities are densely growing and honey is produced on a small scale without any further dilution with honey from other production areas.

Acknowledgements

The authors thank Angelika Hiller for her careful laboratory work, especially the skilful preparation and analysis of samples.

Disclosure statement

No potential conflict of interest was reported by the authors.

Supplemental data

Supplemental data for this article can be accessed here: http://dx.doi.org/10.1080/19440049.2015.1042410.

References

BfR. Scientific Opinion. 2010. Cases of poisoning through grayanotoxins in rhododendron honey originating from the Turkish Black Sea Region [Internet]. [cited 2010 Sep 3]. Available from: http://www.bfr.bund.de/cm/349/cases_of_poisoning_through_grayanotoxins_in_rhododendron_honey_originating_from_the_turkish_black_sea_region.pdf

Burke JW, Doskotch RW. 1990. High-Field H-1-NMR and C-13-NMR assignments of grayanotoxins I, IV, and XIV isolated from Kalmia-Angustifolia. J Nat Prod. 53:131–137.

Carey FM, Lewis JJ, Macgregor JL, Martinsmith M. 1959. Pharmacological and chemical observations on some toxic nectars. J Pharm Pharmacol. 11:T269–T274.

Deguchi T, Sakai Y. 1967. Sustained after-depolarization in grayanotoxin-treated muscle cell membrane. J Physiol Soc Jpn. 29:172–173.

El-Naggar SF, Doskotch RW, Odell TM, Girard L. 1980. Antifeedant diterpenes for the gypsy moth larvae from Kalmia latifolia: isolation and characterization of ten grayanoids. J Nat Prod. 43:617–631.

Fukuda H, Kudo Y, Ono H, Yasue M, Sakakibara J, Kato T. 1974. Structure-activity relationship of Lyoniol-A and related compounds in association with the excitatory effect on muscle spindle afferents. Chem Pharm Bull. 22:884–888.

Gunduz A, Turedi S, Russell RM, Ayaz FA. 2008. Clinical review of grayanotoxin-mad honey poisoning past and present. Clin Toxicol. 46:437–442.

Gunduz A, Turedi S, Uzun H, Topbas M. 2006. Mad honey poisoning. Am J Emerg Med. 24:595–598.

Hikino H, Ohta T, Ogura M, Ohizumi Y, Konno C, Takemoto T. 1976. Structure-activity relationship of ericaceous toxins on acute toxicity in mice. Toxicol Appl Pharmacol. 35:303–310.

Holstege DM, Puschner B, Le T. 2001. Determination of grayanotoxins in biological samples by LC-MS/MS. J Agric Food Chem. 49:1648–1651.

Jansen SA, Kleerekooper I, Hofman ZLM, Kappen IFPM, Stary-Weinzing A, Van Der Heyden MAG. 2012. Grayanotoxin poisoning: ‘Mad honey disease’ and beyond. Cardiovasc Toxicol. 12:208–215.

Kaplan M, Olgun EO, Karaoğlu O. 2014. Determination of grayanotoxins in honey by liquid chromatography tandem mass spectrometry using dilute-and-shoot sample preparation approach. J Agric Food Chem. 62:5485–5491.

Kinghorn AD, Jawad FH, Doorenbos NJ. 1978. Structure-activity relationship of grayanotoxin derivatives using a tetrodotoxin-antagonized spasmodic response of brine shrimp larvae (Artemia-Salina). Toxicon. 16:227–234.

Klocke JA, Mei-Ying H, Shin-Foon C, Kubo I. 1991. Grayanoid diterpene insect antifeedants and insecticides from Rhododendron molle. Phytochemistry. 30:1797–1800.

Koca I, Koca AF. 2007. Poisoning by mad honey: a brief review. Food Chem Toxicol. 45:1315–1318.

Kron KA, Judd WS, Stevens PF, Crayn DM, Anderberg AA, Gadek PA, Quinn CJ, Luteyn JL. 2002. Phylogenetic classification of Ericaceae: molecular and morphological evidence. Bot Rev. 68:335–423.
Li Y, Liu Y-B, Yu S-S. 2013. Grayanoids from the Ericaceae family: structures, biological activities and mechanism of action. Phytochem Rev. 12:305–325.

Narahashi T, Seyama I. 1974. Mechanism of nerve membrane depolarization caused by grayanotoxin I. J Physiol. 242:471–487.

Narayanan P, Röhr M, Zechmeister K, Hoppe W. 1970. Crystal and molecular structure of grayanotoxin - I, C_{22}H_{36}O_{7}. Tetrahedron Lett. 11:3943–3944.

Nishikawa Y, Fukumoto K, Tetsumi T, Katai M, Meguri H. 1989. Effects of grayanotoxin-III on liver-function and renal-function in rats. Yakugaku Zasshi-J Pharm Soc Jpn. 109:340–343.

Onat F, Yegen BC, Lawrence R, Oktay A, Oktay S. 1991. Site of action of grayanotoxins in mad honey in rats. J Appl Toxicol. 11:199–201.

Qiang Y, Zhou B, Gao K. 2011. Chemical constituents of plants from the genus rhododendron. Chem Biodivers. 8:792–815.

Sakakibara J, Shirai N, Kaiya T, Nakata H. 1979. Grayanotoxin-XVIII and grayanoside B, a new a-nor-b-homo-ent-kaurene and its glucoside from Leucothoe grayana. Phytochemistry. 18:135–137.

Scott PM, Coldwell BB, Wiberg GS. 1971. Grayanotoxins - occurrence and analysis in honey and a comparison of toxicities in mice. Food Cosmet Toxicol. 9:179–184.

Sütlüpmar N, Mat A, Satganoglu Y. 1993. Poisoning by toxic honey in Turkey. Arch Toxicol. 67:148–150.

Tallent WH, Riethof ML, Horning EC. 1957. Studies on the occurrence and structure of Acetylandromedol (Andromedotoxin). J Am Chem Soc. 79:4548–4554.

Teuscher E, Lindequist U. 1994. Biogene Gifte. Stuttgart: Gustav Fischer Verlag.

These A, Bodi D, Ronczka S, Lahrsen-Wiederholt M, Preiss-Weigert A. 2013. Structural screening by multiple reaction monitoring as a new approach for tandem mass spectrometry: presented for the determination of pyrrolizidine alkaloids in plants. Anal Bioanal Chem. 405:9375–9383.

Wang L-Q, Ding B-Y, Qin G-W, Lin G, Kin-Fai C. 1998. Grayanoids from Pieris formosa. Phytochemistry. 49:2045–2048.

White Jr JW, Riethof ML. 1959. The composition of honey. III. Detection of acetylandromedol in toxic honeys. Arch Biochem Biophys. 79:165–167.

Wood HB, Stromberg VL, Keresztesy JC, Horning EC. 1954. Andromedotoxin - a potent hypotensive agent from rhododendron-maximum. J Am Chem Soc. 76:5689–5692.

Zhang HP, Wang LQ, Qin GW. 2005. Grayanane diterpenoids from the leaves of Craiobiodendron yunnanense. Bioorg Med Chem. 13:5289–5298.

Zhang WD, Jin HZ, Chen G, Li XF, Yan SK, Zhang L, Shen YH, Yang M. 2008. A new grayanane diterpenoid from Rhododendron decorum. Fitoterapia. 79:602–604.