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ORIGINAL RESEARCH ARTICLE

Analysis of Swiss honeys for pyrrolizidine alkaloids

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Summary

Various studies have shown that honey may contain pyrrolizidine alkaloids (PAs) and thus can pose a potential health risk for consumers. It seems that the level of contamination depends on the geographical and botanical origin of the honey. The geographically central location of Switzerland within Europe accounts for various different climate zones, and therefore can serve as a model to study PA contamination in honeys. We analysed 71 honeys between 2009 and 2011. Honeys from various botanical origins were collected from regions north and south of the Alps as well as the alpine regions. The PA concentration of the honeys was determined by target analysis using an HPLC-MS/MS-system, allowing the detection of 18 different PAs and PA-N-oxides found in the genera *Echium*, *Eupatorium* and *Senecio*. 54% of the honeys contained PAs, while in 46% of the honeys PA concentrations were below the limit of quantitation (LOQ). The LOQs ranged from 1 µg/kg to 3 µg/kg, depending on the PA. The mean PA concentration of the positive samples was 6.7 µg/kg. The highest concentration of PAs (55 µg/kg) was found in a honey from Ticino, an area of the southern flank of the Alps. All the other positive honeys contained PAs at concentrations below 18 µg/kg. Therefore Swiss honey usually does not pose a risk for consumers. In the investigated honeys, honeys from the Swiss alpine regions (29 out of 37) tested more frequently positive for PAs compared to honeys from areas north of the Alps (9 out of 34). This probably reflects the different botanical settings of the central European and the alpine climate regions.

SPANISH ABSTRACT TO FOLLOW

Resumen

Various studies have shown that honey may contain pyrrolizidine alkaloids (PAs) and thus can pose a potential health risk for consumers. It seems that the level of contamination depends on the geographical and botanical origin of the honey. The geographically central location of Switzerland within Europe accounts for various different climate zones, and therefore can serve as a model to study PA contamination in honeys. We analysed 71 honeys between 2009 and 2011. Honeys from various botanical origins were collected from regions north and south of the Alps as well as the alpine regions. The PA concentration of the honeys was determined by target analysis using an HPLC-MS/MS-system, allowing the detection of 18 different PAs and PA-N-oxides found in the genera *Echium*, *Eupatorium* and *Senecio*. 54% of the honeys contained PAs, while in 46% of the honeys PA concentrations were below the limit of quantitation (LOQ). The LOQs ranged from 1 µg/kg to 3 µg/kg, depending on the PA. The mean PA concentration of the positive samples was 6.7 µg/kg. The highest concentration of PAs (55 µg/kg) was found in a honey from Ticino, an area of the southern flank of the Alps. All the other positive honeys contained PAs at concentrations below 18 µg/kg. Therefore Swiss honey usually does not pose a risk for consumers. In the investigated honeys, honeys from the Swiss alpine regions (29 out of 37) tested more frequently positive for PAs compared to honeys from areas north of the Alps (9 out of 34). This probably reflects the different botanical settings of the central European and the alpine climate regions.

Keywords: pyrrolizidine alkaloids; honey; *Echium*; *Senecio*; *Eupatorium*

Introduction

Pyrrolizidine alkaloids (PAs) are toxic compounds that are produced by plants as a defence mechanism against herbivores. More than 350 naturally occurring PAs have been described in plants so far (European Food Safety Authority, 2007). Approximately half of these compounds are toxic to animals and humans, causing liver and lung damage. Acute food poisoning has been caused by grains of PA containing plants contaminating flour used to produce bread, or by using herbal medicine, while chronic exposure to low levels of PAs in food can produce liver cirrhosis, pulmonary hypertension and probably also cancer, as shown in animal models (Edgar *et al.*, 2011). Due to the frequent occurrence of PA-containing plants, occasional contamination of food products by PAs can be expected. PA containing plant species are widespread all over the world and mainly belong to the families Asteraceae, Boraginaceae, and Fabaceae. Examples of frequently encountered PA containing plants in Switzerland belong to the family Asteraceae and include *Senecio* spp., *Eupatorium* spp., *Petasites* spp. and *Tussilago farfara*. For a number of years, *Senecio jacobaea* and *Senecio aquaticus* have been expanding into cultivated Swiss farmland. Both plant species are toxic for horses and cattle. Other examples of PA containing plants commonly found in Switzerland belong to the family Boraginaceae and include *Echium vulgare*, *Borago officinalis*, *Symphytum* spp., *Cynoglossum officinale* as well as *Myosotis* spp. If bees collect nectar and pollen from PA containing plants, PAs could be transferred into the honey and therefore present a food safety concern for the consumer of the honey. High concentrations of PAs up to 2850 µg/kg have been found in monofloral honeys of *Echium vulgare* or *plantagineum* (Culvenor *et al.*, 1981; Beales *et al.*, 2004; Betteridge *et al.*, 2005; Kempf *et al.*, 2011), and up to 3900 µg/kg in monofloral honey of *Senecio jacobaea* (Deinzer *et al.*, 1977; Crews *et al.*, 1997). Kempf *et al.* (2011) even found up to 13,019 µg/kg PAs in honey derived from hives which were placed intentionally in the vicinity of a patch of *Senecio jacobaea*; that value refers to retronecine equivalents. Assuming that senecionine and similar alkaloids were the main alkaloids in that honey (roughly twice the molecular mass of retronecine), the actual PA concentration was > 26,000 µg/kg. However, polyfloral honeys and honeys of floral sources other than *Echium* and *Senecio* may also contain PAs at concentrations that are critical for food safety, as shown in studies with large sample numbers of raw honeys or commercially available honeys from supermarkets (Kempf *et al.,* 2008, 2011; Dübecke *et al.*, 2011). Spread and growth rate of various plant species is affected by climatic conditions. Hence, contamination of honeys with PAs varies depending on their geographical origin. Further studies are required to correlate the occurrence of PAs with geographical and botanical origin (EFSA, 2011).

As a consequence of the geographically central location within Europe and the climatic influence of the Alps, Switzerland has a very species-rich flora, reflecting the different floras of the adjacent countries. It has both central and southern European types of climate zones, as well as the alpine type of climate zone. Therefore Switzerland can serve as an interesting model for studying PA contamination in European honey. The aim of our study was to assess the PA levels found in Swiss honeys originating from different climatic regions. Furthermore, previous studies mostly studied bulk honeys often with imprecise botanical or geographical information or honeys available in supermarkets which are often blended. In our study, we obtained honeys directly from beekeepers, which allowed us to obtain additional information about the floristic environment from where the honeys were collected. We also determined the botanical origin of the honeys and analysed all honeys with significant PA contamination by quantitative pollen analysis to determine the plant species that contributed to the PA contamination of the honeys.

Material and methods

Reagents

PA references were purchased from a range of distributors. Seneciphylline (#6414.1) was obtained from Carl Roth (Karlsruhe, Germany), echimidine (#520-68-3) and lycopsamine (#10285-07-1) from Cfm Oskar Tropitzsch (Marktredwitz, Germany), heliotrine (L6007) from Latoxan (Valence, France), senkirkine (#89274) from Phytolab (Vestenbergsgreuth, Germany), monocrotaline (C2401), retrorsine (R0382) and senecionine (17806) from Sigma-Aldrich (Steinheim, Germany). The solvents used were of analytical grade for residue analysis. *Lycopodium* spores (Batch 124961; one tablet containing 12542 spores) were obtained from Lund University, Department of Quaternary Geology, Lund, Sweden.

Honeys

In total, seventy-one honeys were collected, thirty-seven in 2009, and thirty-two in 2010. Each production year included honeys from the five different biogeographical regions (Wohlgemuth, 1996; Gonseth *et al.*, 2001; the two regions "East Central Alps" and "West Central Alps" were summarized as "Central Alps"). Furthermore two honeys of 2011 were added. In total, we analysed 11 honeys from the Jura Mountains, 23 honeys from the Swiss plateau and 37 honeys from the three alpine regions. From a botanical perspective, polyfloral, honeydew or mixtures of both origins are typical for the Swiss honey production, at times unifloral honeys, such as chestnut and robinia honeys are produced in the southern part of Switzerland. Hence, our study comprised 47 floral honeys of which 29 were polyfloral, 5 mixtures of blossom/ honeydew honeys and 13 unifloral honeys: 2 *Brassica* (rape), 4 *Tilia*, 4 *Robinia* and 3 *Castanea* (chestnut). Furthermore, we included 14 alpine mountain flower honeys and grouped them separately. As honeydew honeys we selected 3 fir, 3 *Latifoliae* as well as 4 honeydew honeys of mixed origin.

Sample preparation for LC-MS/MS analysis

Honey samples were prepared as described by Dübecke *et al.*, 2011. Heliotrine was used as an internal standard. Briefly, honey was dissolved in 0.05 M sulphuric acid, filtered (2 µm mesh) and subjected to solid-phase extraction (SPE). The eluted sample was dried, reconstituted in deionised water and filtered using a 0.45 µm syringe filter.

Quantification of PAs with LC-MS/MS

The PA concentration was determined by target analysis using an HPLC-MS/MS-system, allowing the detection of 18 different PAs and PA-N-oxides that have been found mainly in the genera *Echium*, *Eupatorium* and *Senecio.* The total PA concentration of a honey was calculated as the sum of the 18 different PAs. LC-MS/MS analysis was performed as described by Dübecke *et al.*, 2011, using an HTC PAL autosampler of CTC Analytics AG, a Shimadzu LC-system with a Thermo Hypersil Gold C18 column (50 x 2.1 mm, 1.9 µm particle size) and an Applied Biosystems API 4000 QTRAP triple quadrupole mass spectrometer. External calibrations were used for quantification. Concentrations were corrected against the recovery of the internal standard. The limit of quantitation (LOQ) was 1 µg/kg for echimidine and senkirkine, 2 µg/kg for heliotrine and 3 µg/kg for monocrotaline, lycopsamine, retrorsine, senecionine and seneciphylline. As only a limited number of reference standards were available, a number of other PAs and PA-N-oxides commonly found in *Echium* (echimidine-N-oxide, acetyl-echimidine (-N-oxide), echiumine (-N-oxide), acetyl-echiumine-N-oxide, echiuplatine (-N -oxide) and echivulgarine (-N-oxide)) and *Eupatorium* species (lycopsamine-N-oxide and isomers) were indirectly quantified by using the calibration of echimidine and lycopsamine, respectively, and assuming the same LOQs (Dübecke *et al.*, 2011).

Pollen analysis

Qualitative pollen analysis was performed according to DIN 10760: 2002-05. For the quantification of pollen grains in honeys, a tablet of *Lycopodium* spores was added to the honey prior to the isolation of the pollen sediment as previously described for quantification of pollen in royal jelly (Piana *et al.*, 2006). In general, a minimum of 500 *Lycopodium* spores, and the corresponding pollen grains, were counted. In honeys with a very high number of *Myosotis* pollen grains, a minimum of 250 *Lycopodium* spores and 1000 *Myosotis* pollen grains were counted. The absolute number of pollen grains in 1 g of honey was calculated using the following formula: pollen grains in 1 g honey = (pollen grains counted x *Lycopodium* total) / (*Lycopodium* counted x weight of honey).

Analysis of the botanical origin of the honeys

The determination of the botanical origin of the honeys was based on sensorial analysis (Gonnet and Vache, 1985), as well as chemical characteristics. Glucose and fructose levels as well as the electrical conductivity were determined by Mid-Infrared Spectroscopy (Ruoff *et al.*, 2006) using a Bruker Tensor 27 instrument. Our main criteria to distinguish honeydew from floral honeys or mixtures of blossom/honeydew honeys was the electrical conductivity that was required to be higher than 0.8 mS/cm for honeydew honeys (Persano Oddo and Piro, 2004). The electrical conductivities of honeys close to 0.8 mS/cm were confirmed with a conductivity meter according to the harmonized methods of the International Honey Commission (IHC). Alpine mountain flower honeys were classified according to their characteristic taste. Additionally, pollen analysis was performed for all honeys that contained PAs at a total concentration of 3 µg/kg or more.

Results

PA analysis of honeys

We based our study on a model with five floristic different geographical regions and collected honeys accordingly (Fig. 1). 54% of the honeys contained PAs, while the PA concentrations were below the LOQs in 46% of the honeys. The mean (arithmetic average) PA concentration of the positive samples was 6.7 µg/kg and the mean concentration of PAs in all honeys was 3.6 µg/kg (Table 1). 55 µg/kg was the highest PA concentration, measured in a honey from the region southern flank of the Alps. All the other positive honeys contained PAs at concentrations below 18 µg/kg.

Table 1. Pyrrolizidine alkaloids in Swiss honey. Target analysis using an HPLC-MS/MS-system allowed the detection of 18 different PAs and PA-N-oxides found in the genera *Echium*, *Eupatorium* and *Senecio*.

Total No. of Honeys	Honeys containing PA	Honeys containing PA	mean conc. in positive Honeys	mean conc. in all Honeys
(n)	(n)	(%)	(µg/kg)	(µg/kg)
71	38	54	6.7	3.6

PAs and botanical origin

PA contamination of honey may be restricted to some types of honeys (e.g. floral honeys). Hence, we grouped the honeys as follows: alpine mountain flower honeys, floral honeys other than alpine mountain flowers and honeydew honeys. The frequency of PA contamination was highest in the group of the alpine mountain flower honeys (86%, mean concentration of positives 5.7 µg/kg), while 45% of the honeys of the floral group (mean concentration of positives 7.5 µg/kg) and 50% of the honeydew honeys (mean concentration of positives 5.4 µg/kg) were positive for PAs. The results listed in Table 2 demonstrate that PA contamination occurs in all types of honey including honeydew honeys, where the accompanying floral pollen probably contribute to the PA contamination. Thus PA contamination is not exclusively restricted to floral honeys.

Table 2. Comparison of the PA contamination in honeys of different botanical origin.

Origin of Honeys	Number of Honeys	Honeys containing PA	Honeys containing PA	mean conc. in PA-positive Honeys	mean conc. in all Honeys
	(n)	(n)	(%)	(µg/kg)	(µg/kg)
floral (other than alpine mountain flowers)	47	21	45	7.5	3.4
alpine mountain flower	14	12	86	5.7	4.9
honeydew	10	5	50	5.4	2.7

Table 3. Comparison of PA contents in honeys from different climatic regions of Switzerland. Honeys were grouped according to the biogeographical classification shown in Fig. 1. n.d. = not detectable.

Region	Honeys	Honeys containing PA	Honeys containing PA	mean conc. in PA-positive Honeys	mean conc. in all Honeys	PA group 1 (typical for *Echium* species)	PA group 2 (typical for *Eupatorium* species)	PA group 3 (typical for *Senecio* species)
	(n)	(n)	(%)	(µg/kg)	(µg/kg)	(µg/kg)	(µg/kg)	(µg/kg)
Jura Mountains	11	1	9	8.0	0.7	n.d.	n.d.	0.7
Swiss plateau	23	8	35	6.1	2.1	2.1	n.d.	n.d.
N flank of Alps	8	6	75	3.5	2.6	2.3	n.d.	0.4
Central Alps	12	11	92	5.9	5.4	4.0	n.d.	1.4
S flank of Alps	17	12	71	9.1	6.5	4.6	1.8	n.d.

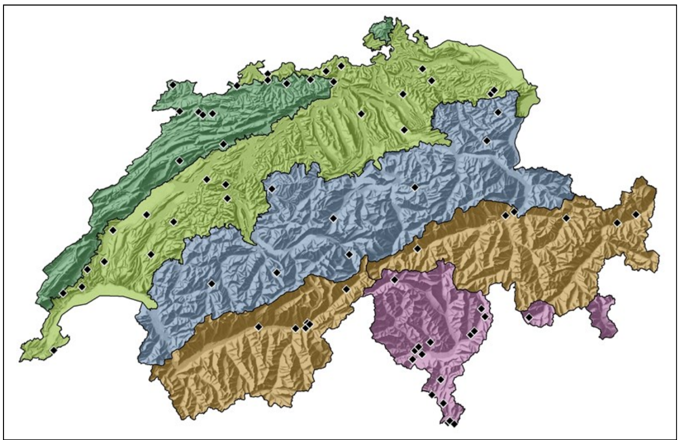

Fig. 1. Biogeographical subdivision of Switzerland based on floristic/ faunistic characteristics (Gonseth *et al.*, 2001). Jura Mountains: dark green; Swiss plateau: light green; northern flank of the Alps: blue; Central Alps: brown; southern flank of the Alps: purple. Locations of honey collection denoted by black diamonds.

PA and geographical origin

We were interested whether the occurrence of PAs in honeys varies according to the climatic regions of Switzerland. PAs were detected in the following proportions of the samples: 9% for the Jura Mountains, 35% for the Swiss plateau, 75% for the northern flank of the Alps, 92% for the Central Alps and 71% for the southern flank of the Alps (Table 3.). We observe, that the mean concentration of PAs of all honeys per region increased from north to south of Switzerland. Hence in the investigated Swiss honeys, products from north of the Alps contained less frequently PAs as compared to those from the Alpine regions, probably reflecting the different floristic pattern.

Considering the small number of positive honeys from the Jura Mountains and the high concentration measured in one of the honeys from the southern flank of the Alps, the average PA concentrations of positive honeys were in similar ranges for all regions.

The 18 PAs analysed with our analytical method are PAs typically present in *Echium* spp. (PA group 1), *Eupatorium* spp. (PA group 2) and *Senecio* spp. (PA group 3). In this study, group 1 PAs typical for *Echium* spp. were found in honeys originating from the Swiss plateau as well as from all three alpine regions. In contrast, group 2 PAs typical of the *Eupatorium* spp. were only found in honeys of the southern flank of the Alps and group 3 PAs typical for *Senecio spp.* were only detected in the Jura Mountains, the northern flank of the Alps and the Central Alps (Table 3.).

PA and pollen analysis

In addition to the chemical PA assays, we analysed all positive honeys containing PAs at concentrations 3 µg/kg or higher by qualitative and quantitative pollen analysis as described above (Table 4.). We confirmed the presence of *Echium* pollen in the majority of honeys (19 out of 23) that tested positive for group 1 PAs, thus further supporting the concept of *Echium* as the source for contamination of these honeys. Honey No. 20 contained the highest amount of *Echium* pollen grains (91 pollen grains/g) and also the highest concentration of group 1 PAs (49 µg/kg). However, the amount of *Echium* pollen grains did not always quantitatively correlate with the concentration of group 1 PAs in the honeys, e.g. honey No. 27 contained 44 *Echium* pollen grains/g honey and group 1 PAs at 3 µg/kg, while we found only 3 *Echium* pollen grains/g in honey No. 3 with a concentration of 16 µg/kg group 1 PAs (Table 4.). The PA concentrations in plants can vary due to environ-

Table 4. Examples of honeys analysed for the content of PAs typical for *Echium*, *Eupatorium* and *Senecio spp.*, respectively. The presence of *Echium* and *Asteracea* HA pollen was confirmed by pollen analysis. n.d. = not detectable.

Honey	**Honey Type**	**Regional Provenance**	**total PA**	**PA group 1**	***Echium***	**PA group 2**	**PA group 3**	***Asteracea* HA**
			(µg/kg)	(µg/kg)	(pollen/g honey)	(µg/kg)	(µg/kg)	(pollen/g honey)
				typical for *Echium sp.*		typical for *Eupatorium sp.*	typical for *Senecio sp.*	
1	fir honeydew	Jura Mountains	8	n.d.	n.d.	n.d.	8	6
2	polyfloral with honeydew	Swiss plateau	17	17	24	n.d.	n.d.	n.d.
3	polyfloral	Swiss plateau	16	16	3	n.d.	n.d.	n.d.
4	polyfloral with honeydew	Swiss plateau	5	5	8	n.d.	n.d.	2
5	polyfloral	Swiss plateau	4	4	1	n.d.	n.d.	2
6	polyfloral with honeydew	Swiss plateau	3	3	26	n.d.	n.d.	n.d.
7	polyfloral	N flank of Alps	7	7	1	n.d.	n.d.	n.d.
8	polyfloral	N flank of Alps	3	3	n.d.	n.d.	n.d.	n.d.
9	alpine mountain flower	N flank of Alps	3	3	n.d.	n.d.	n.d.	n.d.
10	alpine mountain flower	N flank of Alps	3	3	n.d.	n.d.	n.d.	2
11	alpine mountain flower	N flank of Alps	3	n.d.	n.d.	n.d.	3	2
12	alpine mountain flower	Central Alps	12	12	n.d.	n.d.	n.d.	2
13	alpine mountain flower	Central Alps	11	11	12	n.d.	n.d.	2
14	fir honeydew	Central Alps	10	10	23	n.d.	n.d.	1
15	alpine mountain flower with honeydew	Central Alps	8	1	2	n.d.	7	3
16	alpine mountain flower	Central Alps	7	3	14	n.d.	4	n.d.
17	honeydew	Central Alps	6	n.d.	n.d.	n.d.	6	2
18	alpine mountain flower with honeydew	Central Alps	4	4	8	n.d.	n.d.	1
19	alpine mountain flower	Central Alps	3	3	9	n.d.	n.d.	n.d.
20	polyfloral with chestnut	S flank of Alps	55	49	91	6	n.d.	2
21	alpine mountain flower	S flank of Alps	12	12	28	n.d.	n.d.	n.d.
22	chestnut	S flank of Alps	11	n.d.	1	11	n.d.	n.d.
23	chestnut	S flank of Alps	8	2	13	6	n.d.	5
24	chestnut/tilia	S flank of Alps	7	2	3	5	n.d.	2
25	tilia	S flank of Alps	3	3	8	n.d.	n.d.	6
26	tilia	S flank of Alps	3	3	10	n.d.	n.d.	12
27	tilia	S flank of Alps	3	3	44	n.d.	n.d.	11
28	chestnut/tilia/ raspberry	S flank of Alps	3	n.d.	n.d.	3	n.d.	n.d.

mental factors such as soil conditions or flowering stage of the plant and thus the PA concentration may also deviate accordingly in the pollen grains. Hence, the amount of *Echium* pollen by itself does not allow an accurate determination of the PA content at the observed PA levels in the investigated Swiss honeys; further chemical analysis is necessary for accurate determination of the PAs at such low levels.

We also aimed to correlate the chemical data for the *Senecio* and *Eupatorium* PAs with the occurrence of the corresponding plant pollen in the honeys. Both *Senecio* and *Eupatorium* pollen belong to the *Asteraceae* HA-type of pollen. Microscopically, they are difficult to be distinguished from each other and from pollen of other plants with *Asteraceae* HA-type pollen; such examples are *Arnica montana*, *Adenostyles* spp., *Bellis perennis*, *Doronicum* spp. or *Petasites* spp. We found *Asteraceae* HA-type pollen in three of the five honeys positive for group 2 PAs typical for *Eupatorium spp.* as well as in four of the five honeys positive for group 3 PAs typical for *Senecio spp.* In honeys No.s 16, 22 and 28 (Table 4.), we could not find the corresponding *Asteraceae* HA-type pollen.

Despite extensive microscopic analysis we could not find *Echium* pollen grains in the entire pollen preparations of honeys No.s 8 -10 and 12 containing group 1 PAs (Table 4). Honeys No.s 8 and 10 contained *Cynoglossum* pollen (2 pollen grains/g) together with a high number of *Myosotis* pollen; honeys No.s 9 and 12 also contained a high number of *Myosotis* pollen. We might have missed the *Echium* pollen grains in our microscopic analysis or else other plant species, such as *Myosotis* or *Cynoglossum* may contribute to the PA contamination of these honeys. It also has to be kept in mind that the observed PA concentrations in those samples are mostly near the LOQ. While the 18 PAs analysed with our methods are typically PAs present in *Echium* spp., *Senecio* spp. or *Eupatorium* spp., they are not exclusively found in these plant species. Other plant species may also contribute to the PA pool of these honeys.

While we rarely observe pollen of *Borago*, *Symphytum*, *Cynoglossum* in Swiss honey, we often encounter pollen of *Myosotis* at high concentrations, especially in alpine mountain flower honeys. In this study, we analysed 13 honeys from the northern flank of the Alps and the Central Alps by melissopalynology (honeys No.s 7-19, Table 4). 12 of these honeys contained *Myosotis* pollen from 24 to 2,921 pollen grains/g honey (data not shown). Our own preliminary experiments on plant material of *Myosotis* spp. suggest that these plant species may contain PAs related to echimidine and lycopsamine (data not shown). However, further analytical tools have to be developed in order to be able to identify and quantify these PAs, thus allowing an estimation of the risk of these PAs for the contamination of alpine honeys.

Discussion

About half of the honeys tested in this study contained PAs, all but one at relative low concentrations. Therefore, the average PA concentrations in Swiss honey are also low, and they are comparable to those of other Central European countries. Consequently, Swiss honey usually does not pose a risk for consumers. An exception was one honey from the southern flank of the Alps, which contained a higher concentration of PAs and a significant amount of *Echium* pollen. The beekeeper who produced this honey confirmed the presence of many *Echium* plants around his apiary.

Dübecke *et al*. (2011) observed substantial differences in the amount of PAs found in honeys depending of their country of origin. The mean PA concentration in 2839 honeys from Central and South America was higher (46 µg/kg) compared to the mean PA concentration in 381 European honeys (17 µg/kg). Within Europe, honeys from Germany, Bulgaria and Romania showed lower levels of PAs (1-43 µg/kg) compared to honeys from Italy and Spain (concentrations up to 225 µg/kg). Melisso-palynological analysis of Spanish and Portuguese honeys often reveal a high number of *Echium* pollen grains (Sanz *et al.,* 2004; Pires *et al.,* 2009).

Similarly, our study showed that honeys from the northern part of Switzerland, (i.e. the Jura Mountains and the Swiss plateau) were less frequently contaminated with PAs compared to honeys from the alpine regions. Distribution of the plant species and the climate of the north of Switzerland resembles that of the south of Germany, while the climatic and botanical setting in the southern part of Switzerland is similar to the northern part of Italy. Therefore, similar PA contamination of honeys of these regions as adjacent areas in the immediate neighbour is in agreement with other studies.

Although not detected at high concentrations, the most abundant PAs in the honeys of this study are group 1 PAs typical for *Echium* spp. This supports data previously reported for European honeys (Dübecke *et al*., 2011). PAs typical for *Echium* spp. were found in honeys of all regions of Switzerland, except from the Jura Mountains. Thus, *Echium* represents a major source for PA contamination of Swiss honeys. Therefore, beekeepers are advised to avoid this plant genus around the apiary as much as possible to reduce PA contamination in the honey.

Group 2 PAs were reported in *Eupatorium* spp. as well as other plants, such as *Borago officinalis* or *Symphytum* spp. (Röder, 1995). We detected group 2 PAs in five of 17 honeys from the southern part of Switzerland. While three of the honeys contained *Asteraceae* HA type pollen, two did not contain *Asteraceae* HA type nor *Borago* or *Symphytum* pollen, suggesting that additional plants may contribute to the PAs of this group.

Group 3 PAs typical for *Senecio* spp. (and also *Adenostyles* spp.) were found in honeys of the colder mountain regions of Switzerland, the Jura Mountains, the northern flank of the Alps and the Central Alps. *Senecio* spp. are toxic for horses and cattle and eradication programmes in Switzerland for *Senecio* spp. are directed towards reducing the spreading of these plants. Nevertheless, beekeepers are advised to remove such plants near apiaries to avoid contamination of their honey.

The structural diversity of more than 350 known PAs presents an analytical challenge. Up to now, no standardized method has been established to determine the PA content in honey. Two main analytical methods are commonly used: a sum parameter GC-MS method (Kempf *et al.,* 2008, 2011) and a multiparameter LC-MS method (Betteridge *et al*., 2005). The GC-MS method covers most PAs, except the otonecine-type PAs, but the structural information of the original PAs is lost. The LC-MS method allows the identification of each individual PA, but unknown PAs are not covered and some PAs are difficult to determine due to the lack of standard materials. The chromatographic separation of the many isomers (e.g. lycopsamine, intermedine, indicine, rinderine and echinatine) is also not easily obtained. Conversely, the PA spectrum as determined by the LC-MS method often allows one to deduce the probable plant species that contributed to the PA contamination of the honey. Furthermore, it is preferable to know the type of PA present in honey for toxicological consideration, since toxicity of the different PAs varies to a great extent, e.g. lycopsamine is about 10 times less toxic than echimidine.

We were interested in which type of plants contribute to the PA contamination of the honeys. Hence we analysed our honeys using an LC-MS method, covering a wide spectrum of PAs from *Echium*, *Eupatorium* spp*.* as well as *Senecio* spp. For comparison, we also analysed 40 of the previously analysed honeys with a GC-MS method where the necine bases (retronecine, heliotridine, supinidine) were flash methylated in the injector and converted into their corresponding dimethylethers previous to the separation procedure (Böhlen *et al*., 2011). The PA levels in the honeys analysed by LC-MS ranged from 1 – 55 µg/kg and the honeys analysed by GC-MS ranged from 1- 16 µg/kg. Especially honeys containing group 1 PAs typical for *Echium* spp. showed approximately three times lower PA values when determined by GC-MS as compared to the results obtained by the LC-MS method, probably due to the low recovery of echimidine (Böhlen *et al.,* 2011). None of the honeys showed substantially higher PA values when analysed by the sum parameter GC-MS method as compared to the single parameter LC-MS method, suggesting that probably no major additional PAs in the honeys were missed using the LC-MS method.

Recently, the GC-MS and LC-MS methods were compared in a study on various sets of honeys (Kempf *et al*., 2011). Qualitative and quantitative results of the two analytical methods correlated well for *Echium* honeys and honeys originating from *Eupatorium* spp. For honeys originating from *Senecio* spp. both analytical methods agreed on qualitative results, such as "PA negative" or "PA positive", however the total amount of PAs was much higher with the GC-MS method than with the LC-MS method. This is probably due to incomplete coverage of the spectrum of PAs from *Senecio* spp. by the LC-MS method (Kempf *et al*., 2011).

The most frequent PAs in honeys of our study originated from *Echium spp.*; according to Kempf *et al*. (2011) both analytical methods provided comparable results. Together with our results of the pollen analysis, we conclude that we had good coverage of the PAs originating from *Echium* and most likely also *Eupatorium* spp., while honeys with PAs originating from *Senecio* spp. probably are correctly detected as PA positive honeys. The amount of PAs originating from this latter plant source might be underestimated.

At the moment, limits for PA concentrations in food are not established in Switzerland nor in the European Union. However, such limits have been set in several countries for phytopharmaceutical products. In Switzerland, PAs must be declared in herbal medicinal products and the daily intake of 1,2-unsaturated PAs should not exceed 0.1 µg per day (Verordnung des Schweizerischen Heilmittelinstituts über die vereinfachte Zulassung von Komplementär- und Phytoarzneimittel).

In several European countries, limits for PAs in food products are under discussion. Recently, the German Federal Institute for Risk Assessment (Bundesinstitut für Risikobewertung, 2011) published a statement on PAs in honey. They recommended to take up not more than 0.007 µg of 1,2-unsaturated PAs per day and kg bodyweight (b.w.). Considering a person of 60 kg b.w. consuming 20 g of honey (typical hotel serving), the maximum PA concentration of that honey should not exceed 21 µg/kg. In this respect, only one of the tested honeys exceeded the concentration of 21 µg/kg, while the PA concentrations in the other 70 honeys were below this concentration. Consequently, Swiss honey usually does not pose a risk for the consumers. Recently, the German Federal Institute for Risk Assessment (Bundesinstitut für Risikobewertung, 2011), as well as the European Food Safety Authority (EFSA, 2011), also stated, that the average consumer of honey would experience little risk to develop negative effects.

Acute or chronic poisoning with 1,2-unsaturated PAs in humans typically leads to hepatic venoocclusive disease (HVOD). The lowest known dose associated with chronic development of HVOD in humans is reported to be 15 µg/kg b.w. per day for a period of 6 months (EFSA, 2011). Although foetuses and small children seem to be more sensitive, it is nearly impossible to achieve such intakes by consumption of honey. The primary concern is therefore carcinogenicity. As the carcinogenicity is caused by a genotoxic mechanism, the ALARA (as low as reasonably achievable) approach should be chosen. Probably the German recommendation ensures a reasonable safety level.

However, 21 µg PAs/kg may not be an achievable limit for honeys, if the worldwide production of honey is considered. A higher limit of 50 or 100 µg PAs/kg may be acceptable from a safety management point of view, as there are some indications that the predominant PAs in honey may be somewhat less toxic than the compound used for the derivation of the maximum limit. In this respect, the content of PA in this single honey (55 µg/kg) from the southern flank of the Alps region of Switzerland is within the limit that may be acceptable. But since many details on toxicity are not available and there remains a considerable risk of underestimating the true concentrations in honey samples, it is difficult to estimate what risk one takes with such an approach. Another uncertainty is the possible widespread low level intake of PAs through staple foods. Therefore the ALARA principle should be applied as strictly as possible.

Target analysis used in this study allowed the detection of 18 different PAs typical for *Echium* spp., *Senecio* spp., as well as *Eupatorium* spp. However, there may be other plant species with further PAs that contribute to the PA content in Swiss honeys not considered in this study. Analysis of these PAs is difficult because the exact structure of these PAs is often not known or because of a lack of reference material for these PAs.

Such additional PAs may originate from plants of the family of the *Boraginaceae*, especially *Myosotis* spp., *Symphytum* spp. or *Cynoglossum officinale* that have been reported to contain several different types of PAs (Hartmann and Witte, 1995; Röder, 1995; Edgar *et al*., 2002). Pollen of these plants have been identified by pollen analysis of raw honeys imported to Germany (Kempf *et al*., 2011) as well as in honeys of our study (data not shown). *Myosotis* pollen (Maurizio, 1940) is frequently found in high numbers in alpine mountain flower honeys, the major type of honey collected in the alpine regions of Switzerland, while *Symphytum* and *Cynoglossum* pollen were only found occasionally in Swiss honeys. Therefore, the development of analytical methods allowing the elucidation of the structures and quantification of different PAs in *Myosotis* spp., especially *Myosotis alpestris,* may detect further PAs relevant for alpine mountain honeys.

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