Research Article

# Pyrrolizidine alkaloids in honey: Risk analysis by gas chromatography-mass spectrometry

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Recently, contamination of honey with pyrrolizidine alkaloids (PA) has been reported as potential health risk. Therefore, it was of interest to develop a reliable tool for selective and quantitative determination of PA in honey. Sample preparation of the novel method comprises strong cation exchange SPE (SCX-SPE), followed by two reduction steps using zinc and $LiAlH_4$, as well as subsequent silylation. During this procedure the separated PA are converted into the necin backbone, the common structural feature of PA toxicity, which is analyzed by GC-MS in the SIM mode. The procedure was validated using PA from extracts of *Senecio vernalis* as well as authentic PA standards including their corresponding *N*-oxides. The PA content of honey samples was quantified with heliotrine as internal standard. The method was applied to generate a dataset in order to evaluate the potential risk of PA contamination especially for retail honeys available on the German/European market. No selection criteria in terms of floral or geographical origin were applied on the samples before analysis. In total, 216 commercially available floral honey samples were analyzed. Among them 19 samples contained PA, in the range of 0.019 – 0.120 μg/g, calculated as retronecine equivalents. The reported method facilitates the selective determination of PA without the need to identify each individual PA independently. The PA contamination of honey is expressed in terms of a single sum parameter and no background information such as foraged plants and pollen analysis is necessary. The LOQ is 0.01 ppm with a S/N of 7:1.

**Keywords:** Food safety / GC-MS analysis / Honey / Pyrrolizidine alkaloids / Pyrrolizidine *N*-oxides

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## 1 Introduction

Pyrrolizidine alkaloids (PA) are a group of plant-derived compounds that consists of about 350 different structures. The occurrence of toxic PA structures is limited almost exclusively to only four unrelated families within the angiosperms, *i.e.*, the Asteraceae (tribes Senecioneae and Eupatorieae), the Boraginaceae, the Apocynaceae, the genus *Crotalaria* within the Fabaceae [1, 2]. PA are constitutive plant defense compounds. They are strong feeding deterrents for most insects, while grazing animals are most likely deterred by their taste. There are numerous examples worldwide, where plants containing 1,2-unsaturated PA esters caused severe intoxications to humans and livestock [2 – 4]. In plants, the PA esters occur in two major forms, the tertiary form and its corresponding *N*-oxide. Unspecific microbial reduction in the intestinal tract converts the PA *N*-oxides into the corresponding tertiary PA. This form can readily be resorbed and subsequently metabolically activated by hepatic P-450 enzymes to genotoxic pyrroles [4 – 6]. Besides acute toxic effects, the genotoxic and tumorigenicity potential of numerous PA was demonstrated in several eukaryotic model systems [3, 6, 7]. Humans are exposed to these toxins by direct consumption of herbal medicine, herbal teas, dietary supplements, or food contaminated with PA plant material. Secondary exposure was reported for food, where the upstream food chain was contaminated with PA containing plant material, such as milk [8], eggs [9], or honey [10, 11].

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**Abbreviations: PA,** pyrrolizidine alkaloids; **PANO,** pyrrolizidine *N*-oxides; **SCX,** strong cation exchange

Some European countries have passed regulations that govern the sale of herbal health products that contain PA. In 1992, the German Federal Health Bureau restricted the use of PA containing plants and preparations to only those with proven effectiveness. The use of these plants was further regulated to a total PA intake of 1 μg/day for a 6-wk period *per* year. In cases the period of application exceeds 6 wks, the level is just 0.1 μg total PA content *per* day with exclusion of pregnant or lactating women for which zero exposure is required [12]. Other countries such as Austria, The Netherlands, Switzerland, or Australia/New Zealand and their responsible authorities have established similar policies or are in the process of establishing regulations [13, 14].

Within the context of these regulations and the carcinogenic potential, the already proven PA transfer from plants to honey has drawn more attention [13]. Hence new methods based on LC-MS were developed. These methods were applied to honey from bees that had likely foraged on plants containing known PA structures [14 – 16].

So far there is still limited data available to measure the potential risk for manufacturers and consumers of honey and honey products. Our newly developed GC-MS method reported in this paper is a general approach, aiming to survey honeys without any knowledge of their history. Since we wanted to generate a dataset on PA contamination especially for retail honeys available on the German/European market, we did not apply any selection criteria on the samples before analysis.

## 2 Materials and methods

### 2.1 Chemicals and solvents

All chemicals were of analytical reagent purity and purchased from Fluka (Buchs, Switzerland), Merck (Darmstadt, Germany), Roth (Karlsruhe, Germany), and Sigma – Aldrich (Steinheim, Germany). Solvents were of HPLC grade purity, other solvents were redistilled before use.

### 2.2 Reference materials

Five PA, monocrotaline (Sigma – Aldrich), senecionine (Roth), seneciphylline (Roth), senkirkine (Roth), and retrorsine (Sigma – Aldrich) were purchased. Furthermore, PA were extracted from flowers of *Senecio vernalis*.

Flower heads of *S. vernalis* were collected at their natural habitats, lyophilized, and powdered in a Warren Blender. This powder was stored at room temperature until use. Approximately 2.5 kg of the powder was extracted in batches of 350 g. Each batch of plant material was first treated in a soxhlet apparatus with petroleum ether to remove undesired lipophilic plant matter (20 solvent turns in the extraction chamber each). Subsequently, the tertiary PA and the corresponding *N*-oxides were extracted in a second soxhlet apparatus with methanol (20 solvent turns in the extraction chamber each). The crude methanol extracts were concentrated and redissolved in 1 N HCl. This mixture was reduced with Zn dust overnight. $NH_4OH_{conc}$ was used to raise the pH to 12 and the tertiary alkaloids were extracted five times with dichloromethane in a separator funnel. The organic phases were combined and concentrated. A pure mixture of tertiary alkaloids was obtained as a white powder through repeated precipitations in acetone at 4°C. The obtained extract gave a mixture of ten PA with the following composition: PA unknown (0.5%), senecivernine (8.4%), senecionine (43.6%), seneciphylline (22.0%), spartioidine (0.6%), integerrimine (9.1%), senkirkine (9.1%), retrorsine (6.3%), PA unknown (0.1%), PA unknown (0.4%). The purity of the PA mixture was checked by GC-FID, GC-NPD, and GC-MS.

### 2.3 Preparation of pyrrolizidine *N*-oxides (PANO)

The general procedure for preparation of tertiary amine *N*-oxides has been described previously [17]. An ice-cooled, stirred solution of 0.1 g purified *S. vernalis* PA mixture in chloroform (8.0 mL) was prepared. A solution of an equimolar amount of *m*-chloroperbenzoic acid (0.05 g) in chloroform (4 mL) was added gradually at 0 – 5°C. The mixture was stirred for 3 h at 0 – 5°C, during the last 15 min the temperature was gradually raised to room temperature. The resulting products were purified by column chromatography using 5 g basic aluminium oxide (0.063 – 0.200 mm). Unreacted PA and by-products were removed by washing with 10 mL chloroform. The PANO were eluted with 20 mL of methanol/chloroform (1:3 v/v) and further purified by crystallization in 5 mL warm acetone as well as addition of 5 mL of ice-cold hexane. The yield was 57.4 mg (54%) *S. vernalis* PANO mixture.

### 2.4 Sample preparation

Honeys ($n = 216$) were purchased from various supermarkets in Germany, other European countries (France, Turkey, *etc*.), and from internet stores. Since heliotrine, the internal standard we used, is a natural occurring PA, each honey analysis had to be performed *a priori* in duplicates (*cf*. Section 3.2). Duplicates (approx. 20 g each) of the honey samples were dissolved with 30 mL 0.05 M sulfuric acid, according to [16]. Only one sample received 50 μL of a heliotrine standard solution (40 ng/μL methanol). After the addition of ~500 mg zinc dust the mixtures were stirred at room temperature for 3 h. Samples were centrifuged (4000 U/min) for 5 – 10 min. The supernatant was kept warm on a water bath at 40°C as suggested by [16].

The samples were applied to preconditioned (6 mL methanol followed by 6 mL 0.05 M sulfuric acid) HF Bond Elut LRC (500 mg/10 mL), strong cation exchange (SCX) SPE colums (Varian, Palo Alto, CA, USA). The samples were applied on the SPE columns with ~1 mL/min using an SPE

 

vacuum manifold. Subsequently, the SPE columns were washed with 3 mL water and 3 mL methanol. PA elution was performed by using 6 mL ammoniated methanol (30 mL $NH_4OH_{conc}$- solution in 500 mL methanol). The eluate was immediately dried under a gentle flow of nitrogen in a heating block at 35°C.

The dried samples were reduced by the addition of 100 µL of a 1 M $LiAlH_4$ solution in THF to transfer the possibly structural diverse PA mixture into their common core structure, *i.e.*, retronecine. The sample vials were sealed airtight and the mixture was stored for 3 h at 4°C in the refrigerator. The reaction was stopped by the addition of 500 µl dichloromethane and five drops 10% NaOH solution and vigorously shaken. The organic phase was transferred to a small column packed with sodium sulfate (~250 mg) covered with celite (~250 mg) and the dichloromethane extraction was repeated two more times. The combined organic phases were dried under a gentle flow of nitrogen on a heating block at 35°C. The dry samples were mixed with 50 µL *N*-Methyl-*N*-(trimethylsilyl)trifluoroacetamide (MSTFA) and sealed airtight. The mixture was kept at 40°C for 30 min. The resulting solution was transferred into a microinsert, sealed, and analyzed by high resolution GC-MS (HRGC-MS).

### 2.5 HRGC-MS

A Fisons Instruments GC 8060 (Thermo Electron, Dreieich, Germany) gas chromatograph with split/splitless injection (220°C; 1:20) was directly coupled to a Fisons Instruments MD 800 mass spectrometer (Thermo Electron). Separation of volatiles was carried out on a J&W DB-1MS fused-silica capillary column (30 m × 0.32 mm), coated with a 0.25 µm film (J&W Scientific Products, Cologne, Germany). The following temperature program was used: 3 min isothermal at 60°C, then raised at 5°C/min up to 180°C, then at 22°C/min up to 310°C. MS operating values were as follows: ionization voltage, 70 eV (electron impact ionization); ion source and interface temperatures, 230 and 320°C, respectively. Identification was performed by comparison of linear retention indices and mass spectral data with that of authentic reference compound. Quantification was performed by splitless injection in the SIM mode for the analytical ions *m*/*z* 93, 183, and 299 [$M^+$]. The relative intensities of these analytical ions to each other were used as tool for the determination of the purity of the corresponding peak. These values were compared to values obtained from authentic reference compounds; variances of < 10% were tolerated.

### 2.6 Gas chromatography (HRGC-FID/NPD)

Separation was achieved using a Hewlett Packard 5890 Series II gas chromatograph (Hewlett Packard, Santa Clara, CA, USA) equipped with a J&W DB-1 fused-silica capillary column (15 m × 0.25 mm), coated with a 0.25 µm film (J&W Scientific Products). Conditions applied were: injection port temperature 250°C, split ratio 1:20, helium as carrier gas, and the temperature program: 100°C (3 min) to 300°C (3 min) at 6°C/min. The eluting compounds were detected simultaneously by using a fused-silica Y-splitter and FID and NPD detectors. The retention index (RI) was calculated by a set of hydrocarbons (even numbered from $C_{10}$ to $C_{28}$) by linear interpolation. Quantitative analyses were performed *via* the FID signal with heliotrine as internal standard.

### 2.7 Quantification

Standard controlled relative quantification with heliotridine (originated from 2 µg heliotrine *per* sample) as internal standard was performed by HRGC-MS under the above-mentioned conditions. Linear retention indices were 1600 and 1632 for di-TMS-retronecine and the standard di-TMS-heliotridine, respectively. No extraction/response factors ($F = 1.0$) were considered. Integration of peak area counts in the SIM (*m*/*z* 93, 183, and 299) EIMS mode was carried out. The data obtained were finally calculated into retronecine equivalents.

### 2.8 Recovery

Analyzing the di-TMS – retronecine and the di-TMS – heliotridine, respectively, recovery control was achieved by determining directly reduced (with zinc and $LiAlH_4$ in THF) PA and PANO without extraction treatment. In these assays using HF Bond Elut LRC, SCX SPE columns (Varian) exhibited a mean recovery efficiency of 83 ± SD 3.0%, using tertiary PA or their *N*-oxides, respectively.

### 2.9 Pollen analysis

After analytical PA analysis, a set of randomly arranged PA honeys was submitted to a detailed pollen analysis performed according to DIN 10760 "Analysis of honey – Determination of the relative frequency of pollen" using an Olympus AX70 microscope. Special attention was turned to PA producing plants from families of Asteraceae (tribes Senecioneae and Eupatorieae), Boraginaceae, and the genus *Crotalaria* (Fabaceae); in any case 500 pollens were counted. Pollen analysis, particularly the underrepresented pollen grains were used to determine the geographic origin of the honey samples.

## 3 Results and discussion

### 3.1 Method development

In the beginning several methods to separate and enrich PA from honey were checked. Liquid – liquid extraction resulted in high recovery rates but its practical application was of limited use due to problems associated with repro-

 

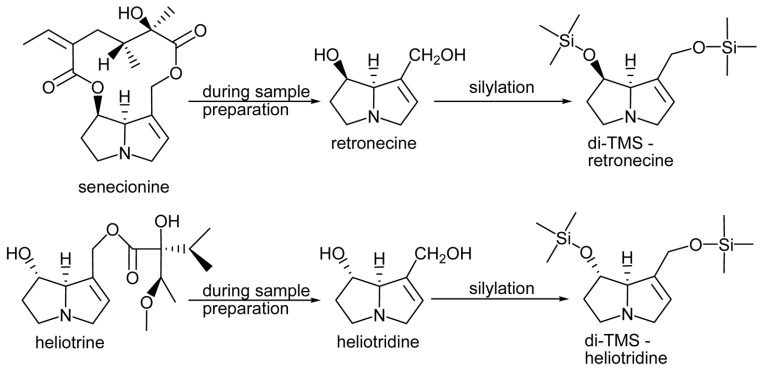


**Figure 1.** Chemical reactions (scheme) occurring in the course of PA analysis, as to, *e.g.*, senecionine (top) and the internal standard, heliotrine (bottom), leading to silylated diastereomeric necin backbones.

ducibility caused by the complex honey matrix. Therefore, we orientated our isolation strategy to the recently published method of Betteridge *et al*. [16]. We screened 14 different types of SCX SPE cartridges using water or honey samples spiked with PA and PANO mixtures that contained up to 11 different PA structures. The obtained results were evaluated in terms of flow rate, handling, and blockage of the SPE column caused by the viscosity of the honey matrix, interferences, and recovery, respectively. These pilot tests showed a very broad range of results, *e.g.*, some SPE cartridges proven to be unsuitable due to complete blockades caused by the sticky honey matrix. During this screening a second important result became apparent. The more complex the PA mixture added to the SPE, the more chromatographic interferences caused by the complexity of the matrix occurred. Therefore, we revised our strategy to simplify this complex analysis.

Toxic PA share the common structural features of a 1,2-double bond of the necine base in combination with an allylic ester bond (Fig. 1; for example senecionine). These attributes are required for the bioactivation by the hepatic P-450 enzymes to genotoxic pyrroles [4 – 6]. Therefore, retronecine/heliotridine itself are nontoxic. But, free necine bases are usually not very common in plants. In general the vast majority of natural occurring toxic PA structures are monoesters and diesters of retronecine and, to a much lower extend, toxic heliotrine- or supinidine-type PA esters [1]. Besides, the restrictions of phyto-pharmaceuticals by the German authorities is based on the existence of this 1,2-double bond [12]. Hence, we decided to convert all retronecine/heliotridine-based PA monoesters and diesters as well as their corresponding *N*-oxides into the core structures, *i.e.*, retronecine and/or heliotridine, to generate a single sum parameter. Since all different 1,2-unsaturated PA of one sample get converted into one single compound, this strategy was also helpful to achieve a reasonable sensitivity.

We are aware of the fact that otonecine-type PA which are also toxic will not be detected by this method. But according to our knowledge there are no examples so far where the otonecine-type PA are the only type of PA found in one plant species. Since retronecine ester PA are the natural precursors of the otonecine-type PA [18], it is usually the other way around, *i.e.*, that retronecine ester PA are accompanied by otonecine-type PA, hence a potential PA contamination can still be registered by detection of the core structure of the retronecine ester PA.

We checked several methods to convert the ester PA into their corresponding necine bases. Basic hydrolysis with sodium hydroxide or potassium hydroxide failed due to reproducibility problems. The method of choice proved to be reduction of the ester bond with $LiAlH_4$ in THF. The reagent is readily available, easy to handle, and reliable results were obtained in the test series.

Based on this new strategy, we checked all promising SPE materials again with the available PA and their *N*-oxides (*cf*. Sections 2.2 and 2.3) in spiked honeys samples. The best results were obtained using the HF Bond Elut LRC (500 mg/10 mL) SCX SPE column (Varian). In a test series each of ten replicates, the HF Bond Elut LRC SPE column exhibited a mean recovery efficiency of 83 ± SD 3.0%, using tertiary PA or their *N*-oxides, respectively. Identical experiments with the Strata SCX SPE cartridges (Phenomenex, Torrance, CA, USA), recently used by Betteridge *et al*. [16], showed in our experiments a mean recovery of 65 ± SD 6.0%. Plugging of the SPE column could be minimized by keeping the supernatant at 40°C [16] and using the HF Bond Elut LRC SPE column. This product is equipped with a wider pore size and is therefore predestinated for sticky viscous matrices such as honey.

### 3.2 Quantification

The method was standardized by the addition of 2 µg of the natural PA heliotrine. The $LiAlH_4$, reduction of heliotrine results in the formation of heliotridine, the diastereomer of retronecine (Fig. 1). After silylation with MSTFA, both naturally appearing toxic necine bases, retronecine and heliotridine, could be readily separated and detected by HRGC-MS (Fig. 2). The linear retention indices were 1600 and 1632 for di-TMS-retronecine and the standard di-TMS – heliotridine, respectively. Since the variety of possible PA structures is too diverse we did not consider extraction/response factors ($F = 1.0$) and recovery of the standard heliotrine. Integration of peak area counts in the SIM

 

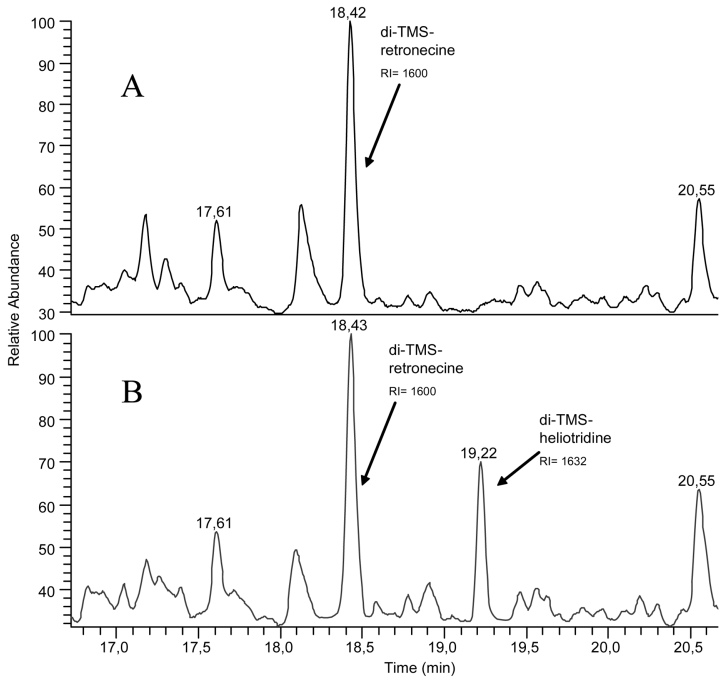


**Figure 2.** SIM mode chromatogram (*m*/*z* 93, 183, and 299) of a PA positive honey sample, (no. 21). (A) Without internal standard heliotrine and (B) after the addition of heliotrine (shown as di-TMS–heliotridine; RI [DB1] = 1632).

(*m*/*z* 93, 183, and 299) EIMS mode was carried out. Data were calculated as retronecine equivalents.

Since we used the natural occurring PA heliotrine as an internal standard for quantification all honey analysis had to be performed *a priori* in duplicates. Sample A underwent a regular work-up procedure, while sample B was spiked with 2 μg of heliotrine in 50 μL methanol, beforehand. Sample A served as a control and was analyzed for the presence/absence of heliotridine. If sample A was devoid of heliotridine but retronecine was present, we used the duplicate sample B to quantify the retronecine content with the internal standard peak of heliotridine (*cf*. Fig. 2). In the case of a positive tested honey sample, sample B was repeated in triplicates (*cf*. Table 1). If there was no retronecine detected in samples A and B but the internal standard heliotridine was detected in sample B, the sample was designated "PA negative". Thus, sample B served always as a control for correct sample preparation. The internal standard heliotrine/heliotridine with its rather unusual *S*-configuration at C-7 turned out to be very helpful. In the course of the 216 honey analyses it never occurred that heliotridine was detected in sample A. Nevertheless, the synthesis of a stable isotope labeled reference compound is in progress to reduce the time of sample preparation. The achieved LOQ was 0.01 ppm with a S/N of 7:1 (data not shown).

Our method represents a reliable tool to detect PA contamination with toxic 1,2-unsaturated PA structures using retronecine as sum parameter. The results were calculated as retronecine equivalents. Retronecine is one of smallest known PA, obeying the toxic principle of a 1,2-unsaturated necine structure (Fig. 1). Generally PA are found in nature as complex macrocyclic diesters or open chain mono- or diesters. Furthermore, the potentially occurring toxic otonecine and supinidine esters are not included in our quantitation as well. Due to these facts, all presented results are an "underestimate" of the actual toxic PA content and represent the lowest possible degree of contamination of a specific sample.

### 3.3 Honey samples

Our purpose was to generate a dataset on PA contamination especially for retail honeys available on the German/European market. We did not apply any selection criteria on the samples before analysis. Neither specific PA producing plant honeys were purchased, nor any information, such as apiarist interrogations about the habitats of the bee colonies or pollen analyses was collected. The honey samples were purchased from various supermarkets in Germany and other European countries, as well as from internet stores. The new developed method was applied to 216 commercially available floral honey samples. Among them 94 honeys were from Europe, 34 from Central- and South America, 6 from USA/Canada, and 22 from Australia and New Zealand. Another 60 samples had no regional identification or were of dubious origin. These 60 samples were mostly mixtures of different proveniences labeled as "mixture from honeys of non-EC-countries" or "mixture from honeys of EC-/non-EC-countries" (Fig. 3A). Within these 216 honeys under study 19 samples (9%) contained PA in the range of 0.019 – 0.120 μg/g (*cf*. Table 1). The average PA contamination was 0.056 μg/g.

These results are in good agreement with the previously reported PA contents of various honeys. Already Deinzer *et*

 

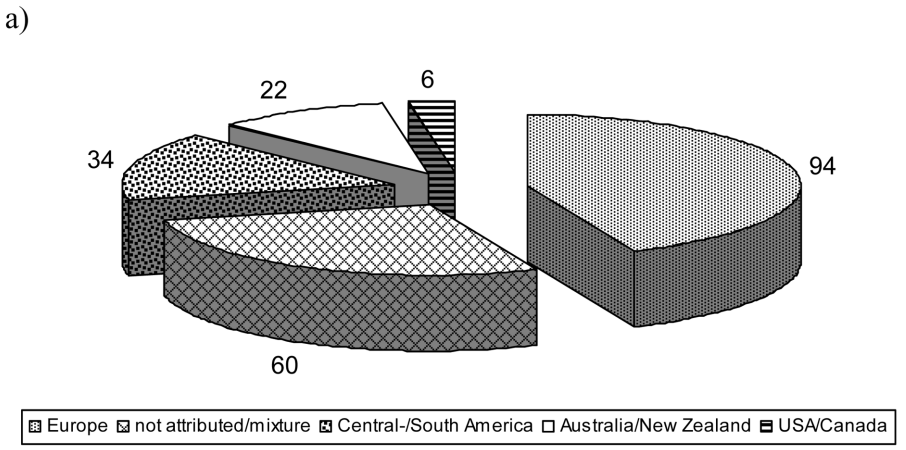


**Figure 3.** (a) Regional arrangement according to the label of the analyzed honey samples. (b) Regional arrangement according to the label of the PA positive tested samples.

*al*. [10] reported in 1977 that honeys ($n = 4$) of *Senecio jacobaea* L. contained PA in a range of 0.3 – 3.9 μg/g. Another study [15] dealing with PA of *S. jacobaea* revealed that 10 samples out of 23 implied PA in the range of 0.002 – 1.48 μg/g. The PA content of honey ($n = 5$) from another PA containing plant, *Echium plantagineum* (Boraginaceae) was reported as 0.27 – 0.95 μg/g [11]. Beales *et al*. [14] showed that samples (29 out of 29) assigned to PA producing plants contained 0.033 – 2.2 μg/g. Further samples (19 out of 39) exhibited 0.003 – 0.8 μg/g; the analysis was restricted to *Echium* spp. and *Heliotropium* spp. In 2005, Betteridge *et al*. [16] reported that seven samples (out of nine) contained 0.017 – 2.85 μg/g PA of *Echium vulgare*. In these nine honeys under study five were attributed, by the apiarist, to be *E. vulgare* honey; the remaining four were unassigned.

Seven out of the 19 positive PA honeys found in our study were labeled as "non-EC-countries," five were labeled as "EC-/non-EC-countries," three from New Zealand, three from Central-/South America, and one sample from Canada, respectively (Table 1 and Fig. 3B). Two honeys, including the sample with the highest amount of PA in our study (no. 142), were labeled as borage honey (New Zealand). *Borago officinalis* is a common PA containing plant, therefore a PA contamination can be easily understood. Nevertheless, a pollen analysis of the sample no. 142 (borage honey, New Zealand) showed that 62% of the counted pollen came from *Echium* spp. The other 17 PA containing samples were without any conspicuous declaration. From the 94 samples which were labeled as European origin no honey was tested PA positive (Fig. 3B).

Among the 19 PA positive samples random arranged pollen analyses with special attention to PA producing plants were performed. The pollen analysis showed that honeys with a high PA content do not necessarily correlate with the amount of PA plant pollen found (*cf*. Table 1). In general, there was a good correlation between high PA contents and great amount of PA plant pollen. For example, honey no. 145 with the highest PA content – 0.120 μg/g – also showed the highest degree of PA plant pollen (62% *Echium* spp.). However, there were some samples with relatively high amounts of PA but only trace amounts of PA plant pollen (sample no. 36 and 39: PA content 0.069 and 0.060 μg/g high and 2% *Echium* spp. and 2% *Eupatorium* spp. pollen, respectively). These results show, that even in cases in which the attention is focused on PA plant pollen, the pollen analyses are only of limited use in predicting the extend of PA contamination.

 

**Table 1.** Amounts of PA (μg/g) determined by using heliotrine/heliotridine as internal standard in commercial honey ($n = 216$); only the honeys containing PA are listed. SDs ($n = 3$) are given. Results of pollen analysis are also represented.

| Total PA (μg/g)[a] | | | Pollen analysis | Geographic origin | |
|---|---|---|---|---|---|
| Sample no. | Mean[b] (μg/g) | SD (μg/g) | | By pollen analysis | As labeled |
| 21 | 0.084 | 0.016 | 48% *Echium* spp., sporadic *Borago* | n.p.[c] | New Zealand |
| 32 | 0.035 | 0.011 | n.p.[c] | n.p.[c] | Non-EC-Countries |
| 34 | 0.027 | 0.014 | n.p.[c] | n.p.[c] | EC-/non-EC-Countries |
| 35 | 0.053 | 0.034 | 16% *Echium* spp. | South America (Argentina, Uruguay) | EC-/non-EC-Countries |
| 36 | 0.069 | 0.008 | 2% *Echium* spp. | South America(Chile, Brazil) | Central-/South America |
| 39 | 0.060 | 0.013 | 2% *Eupatorium* spp. | Central-/South America (Chile, Cuba) | EC-/non-EC-Countries |
| 41 | 0.040 | 0.007 | n.p.[c] | n.p.[c] | Canada |
| 43 | 0.019 | 0.003 | n.p.[c] | n.p.[c] | EC-/non-EC-Countries |
| 50 | 0.091 | 0.012 | 11% *Echium* spp. 2% *Eupatorium* spp. | Central-/South America | Non-EC-Countries |
| 93 | 0.044 | 0.008 | n.p.[c] | n.p.[c] | Non-EC-Countries |
| 119 | 0.026 | 0.005 | n.p.[c] | n.p.[c] | EC-/non-EC-Countries |
| 145 | 0.120 | 0.047 | 62% *Echium* spp. | New Zealand | New Zealand |
| 153 | 0.053 | 0.014 | n.p.[c] | n.p.[c] | New Zealand |
| 155 | 0.040 | 0.005 | n.p.[c] | n.p.[c] | Argentina |
| 161 | 0.052 | 0.008 | n.p.[c] | n.p.[c] | Chile |
| 176 | 0.090 | 0.007 | 20% *Echium* spp., <1% *Eupatorium* spp. | Prevalent South America | Non-EC-Countries |
| 179 | 0.038 | 0.003 | n.p.[c] | n.p.[c] | Non-EC-Countries |
| 188 | 0.078 | 0.029 | 5% *Echium* spp. | Prevalent South America | Non-EC-Countries |
| 212 | 0.048 | 0.001 | 9% *Echium* spp. | Prevalent South America | Non-EC-Countries |

a) Data given as retronecine equivalents.
b) mean ($n = 3$).
c) not performed.

In these cases pollen types, which are considered to be extremely over-represented such as *Eucryphia* (Eucryphiaceae) or *Mimosa* spp. (Mimosaceae), pushed PA plant pollen into the background. On the other hand, pollen grains from PA plants belonging to Asteraceae are most of the time underrepresented in honey.

## 4 Concluding remarks

The reported per-capita consumption of honey in Europe is considered to be 1.3 g/day [19] and hence one of the highest amounts in the world. Correcting the value by subtracting the "nonhoney eaters," this level increases to 3.9 g/day [19, 20]. In particular cases maximum values from 32 to 93 g/day for British infants and adults, respectively [20], or 28.6 up to 64.2 g/day for Australian infants (2 – 4 years) and teenagers (13 – 10 years) [21] have been reported. In our opinion, if we take into account the genotoxic potential of the PA, it would be reasonable to reconsider the results with respect to a usual amount of honey consumed by a single person *per* day. It seems not to immoderate to assume that a common "honey dose," for a spread or as sweetener in beverages, is usually around 1 or 2 table spoons *per* day (equals 10 – 20 g).

If we apply the current regulations of the German Federal Health Bureau for herbal pharmaceutical products, the German authorities have prohibited herbal products containing PA that have no demonstrated health effects [12]. Exemptions are made for a few specific plants where the authorities regulate no more than 1.0 μg PA/day, restricted to a maximum of 6 wks *per* year [12]. Under these conditions half of our PA positive tested samples exceed this limit. If we further consider that our results are calculated as retronecine equivalents, *i.e.*, comprising only approximately half of the PA molecule, a factor of 2 would most likely represent the amount of the original mono- to diester PA in the sample. In doing so, all but one (no. 43) PA positive samples in this study would exceed the 1 μg/day level of the German authorities for herbal pharmaceutical products. Although there are no existing regulations for food so far, the suggested maximum level of 0.1 μg PA *per* 100 g food (1 ppb) from the Netherlands [22] will be exceeded by all the 19 PA honeys. According to a position paper proposed by the German Bundesinstitut für Risikoforschung [23] there should be a zero tolerance for PA in foodstuff and animal feed.

 

These numbers are even more striking if we keep in mind that our calculations are retronecine equivalents without considering otonecine and supinidine-type PA and therefore represent the calculable lowest possible value.

The data presented here is meant to prepare the basis for discussion of the involved parties, *i.e.* consumers, beekeepers, distributors, and legislators, to respond to PA contamination of honey.

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