RESEARCH ARTICLE

Pyrrolizidine alkaloids in pollen and pollen products

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Recently, 1,2-dehydropyrrolizidine alkaloid (PA) ester alkaloids, found predominantly as their *N*-oxides (PANOs, pyrrolizidine *N*-oxides), have been reported in both honey and in pollen obtained directly from PA plants and pollen loads collected by bees, raising the possibility of health risks for consumers of these products. We confirm these findings in regard to floral pollen, using pollen collected directly from flowers of the known PA plants *Senecio jacobaea*, *S. vernalis*, *Echium vulgare* and pollinia of *Phalaenopsis* hybrids, and we extend analyses of 1,2-unsaturated PAs and 1,2-unsaturated PANOs to include bee-pollen products currently being sold in supermarkets and on the Internet as food supplements. PA content of floral pollen ranged from 0.5 to 5 mg/g. The highest values were observed in pollen obtained from *Senecio* species. Up to 95% of the PAs are found as PANOs. Detailed studies with *S. vernalis* revealed unique PA patterns in pollen and flowers. While seneciphylline was the most prominent PA in *S. vernalis* pollen, the flowers were dominated by senecionine. To analyze trace amounts of 1,2-unsaturated PAs in pollen products, our previously elaborated method consisting of strong cation exchange-SPE, two reduction steps followed by silylation and subsequent capillary high-resolution GC-MS using SIM mode was applied. In total, 55 commercially available pollen products were analyzed. Seventeen (31%) samples contained 1,2-unsaturated PAs in the range from 1.08 to 16.35 μg/g, calculated as retronecine equivalents. The 1,2-unsaturated PA content of pollen products is expressed in terms of a single sum parameter and no background information such as foraged plants, pollen analysis, *etc.* was needed to analyze the samples. The detection limit of overall procedure and the reliable quantitation limit were 0.003 and 0.01 μg/g, respectively.

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

1 Introduction

Pyrrolizidine alkaloids (PAs) are constitutively expressed secondary plant defense compounds. They are mainly found in four angiosperm families: the tribes Senecioneae and Eupatorieae of the Asteraceae, many genera of the Boraginaceae, the Apocynaceae and the genus *Crotalaria* within the Fabaceae [1]. All structures that contain a 1,2-double bond are protoxins and are metabolically activated by the action of hepatic P-450 enzymes to toxic pyrroles (for review see [2–4]). In addition, PAs devoid of 1,2-unsaturation are common in Phalaenopsis [1]. To date far more than 350 different PA structures are known. Despite their diversity they all share a common theme; they are ester alkaloids composed of a necine base esterified to one or more necic acids. Most PAs occur in two forms, as tertiary form and the corresponding *N*-oxide (PANO, pyrrolizidine *N*-oxide) [1]. For an overview of the diversity of PA structures, occurrence and chemotaxonomy see [1].

Several studies on honey have shown PA contents up to 3.9 μg/g [5–7]. Two recent studies from Dutch authorities (http://www.vwa.nl/cdlpub/servlet/CDLServlet?p_file_id = 22703; accessed on May 20th, 2009)[8] and Kempf *et al.* [9] on retail honey have shown PA-positive cases in 27 and 9% of the investigated samples, respectively.

The knowledge on the PA content of pollen and/or pollen baskets of bees is rather limited and ranges from 6 to

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Abbreviations: EIMS, electron impact MS; **PAs,** pyrrolizidine alkaloids; **PANOs,** pyrrolizidine *N*-oxides

14 000 μg/g [10, 11]. It is obvious that pollen contains much higher PA levels than those recently reported for honey. Higher alkaloid levels in pollen than in nectar were also observed for other plant species containing other alkaloid types [12]. Against this background it still needs to be verified whether floral nectar of PA plants really contains PAs. The appearance of PAs in honey could also be caused by liberation of PAs from pollen, since usually there is a co-occurrence of PA plant pollen and PAs in honey [5, 9]. It has been noted previously that pollen contains much higher PA levels than those reported for honey and the suggestion has been made that the water-soluble PANOs and PAs found in honey might be primarily leached from pollen rather than coming from nectar [10, 11, 13].

It can be assumed that only relatively low amounts of pollen are consumed through honey or other foodstuff containing honey. But there are niche products that consist of almost pure unaltered pollen baskets from bees or products called ''Perga'' preparations. These products are mixtures of nectar, honey or honeydew and bee secretions and contain high amounts of plant pollen. If bees regularly forage on PA plants, these pollen loads should contain rather high amounts of PAs, including the protoxic 1,2-unsaturated forms.

The main constituents of pollen are amino acids, minerals, vitamins, flavanoids and phytoestrogens [14]. The normal daily serving recommended on the retail pack of these products ranges from 5–10 g (1–2 tablespoons). Because of the robust pollen exine, pollen cannot be digested completely. Whether pollen can be of substantial value as human food supplements remains rather vague under these circumstances and is not well documented. For bees on the other hand, pollen is essential since it presents the major source for amino acids in their diet.

To increase the knowledge of PA content in pollen products we analyzed 55 retail samples. To our knowledge this is the first study in this context. Furthermore, we investigated floral pollen of the known PA plants *Senecio jacobaea*, *S. vernalis*, *Echium vulgare* and pollinia of *Phalaenopsis* hybrids.

Both aspects should be helpful in evaluating the potential threat to human health as well as the impact of pollen PAs in the bee diet, especially since pollen plays an essential role in raising the larvae and maintaining the fitness through hibernation, two critical stages in the continuation of the colony.

2 Materials and methods

2.1 Chemicals and solvents

All chemicals were of analytical reagent purity and purchased from Acros Organics (Geel, Belgium), Fluka (Buchs, Switzerland) and Sigma-Aldrich (Steinheim, Germany). All solvents were of HPLC grade purity or redistilled before use.

2.2 Reference materials

To evaluate the analytical method six PA, monocrotaline (Sigma-Aldrich), senecionine (Roth, Karlsruhe, Germany), seneciphylline (Roth), senkirkine (Roth), heliotrine (Latoxan, Valence, France) and retrorsine (Sigma-Aldrich) were purchased. PAs and PANOs mixtures from *S. vernalis* were applied as described in Kempf *et al.* [9].

2.3 Plant material and sample preparation of floral pollen

Flowering specimens of *S. vernalis*, *S. jacobaea* and *Eupatorium cannabinum* were harvested with and without roots from wild populations in the vicinity of Braunschweig (Germany) and kept indoors in water bowls for 5 days. Daily, the flowers were slightly tapped to harvest the pollen that fell off the inflorescence. Pollen of each 5-day period were combined, air dried and stored in the dark at room temperature.

S. vernalis was used as model system for more detailed analyses, since the plant was readily available and pollen collection was highly efficient. Wild populations of flowering *S. vernalis* plants from four different habitats were harvested (40–60 individual plants *per* location) once a week. Since the biosynthesis of PAs in *Senecio* spp. is located in the roots [15], each sample was split in two subsets (clipped plants *versus* plants with intact roots). The pollen production of each set *per* week was pooled and analyzed for PA content applying method 1 (see Section 2.5). The study was conducted over a 3 wk period, which represented the main flowering season.

Before PA extraction the pollen samples were further purified by multiple washes with cyclohexane to assure that only pure pollen and no other plant material was analyzed. Purification by cyclohexane washings was possible, because pollen showed a different settling behavior than concomitant plant debris, which settled much faster. Pure pollen fractions were combined, the organic solvent was decanted and the pollen purity (absence of other plant material) was verified by light microscopy. The combined organic supernatants (cyclohexane washes) containing the waxy cover of the pollen were analyzed separately. As demonstrated in [10] for *E. vulgare*, this procedure should not extract additional PAs from other contaminating plant debris.

Pure pollen of *E. vulgare* was harvested from the inflorescence of soil-potted plants as described in Boppré *et al.* [10].

Pollinia were obtained by dissecting flowers of greenhouse-reared *Phalaenopsis* hybrids and were directly used for PA extraction.

In addition, 25–30 young flower heads of each time point of pollen collection (with exemption of *Phalaenopsis*-5 flowers) were randomly picked after the pollen collection was completed.

PA extraction of pollen, cyclohexane washings (wax cover) and flower heads was performed in a slightly modified procedure as described previously [16]. Modifications were an intense grinding with sea sand and 1 N HCl instead of 0.25 M H_2SO_4. Each sample extract was split in two halves. One half was used for the analysis of free tertiary PAs; the second half was subjected to zinc reduction to yield the total PA content of the sample (tertiary bases plus PANOs) [16].

The amounts of PAs found in flowers and flower heads were not corrected in terms of the PA content of pollen remains. Usually most of the pollen was lost during flower picking and processing so that the overall pollen content was neglected.

2.4 Sample preparation of pollen products

Pollen products ($n = 55$), available as dietary supplements, were purchased from various supermarkets in European countries (mainly Germany, France and Italy), and from Internet stores. Most commercial products were pollen loads of bees obtained through pollen traps that are regularly installed at bee hives for this purpose and are further processed in cleaning and drying procedures before they are sold. Since heliotrine, the internal standard we used, is a natural occurring PA, each pollen product analysis had to be performed *a priori* in duplicates (*cf.* Kempf *et al.* [9]). The pollen granules were mixed in a blender to break the compact pollen-bee saliva conglomerate. Duplicates (approx. 2 g each) of the blended pollen samples were ground with 5 g of sea sand (to break the exine) and 30 mL of 0.05 M sulfuric acid was added, according to Kempf *et al.* [9]. Only one sample received 100 μL of a heliotrine standard solution (40 ng/μL methanol). This suspension was shaken vigorously for 24 h in a rotary shaker. After 24 h the samples were filtered, the filtrate stored at −18°C. This procedure was repeated once for the residue in the filter paper with 50 mL 0.05 M sulfuric acid. After addition of ~500 mg zinc dust to each filtrate the mixture was stirred at room temperature for 3 h. Samples were centrifuged (4000 U/min) for 5–10 min.

HF Bond Elut LRC (500 mg 10 mL^{-1}), strong cation exchange-SPE columns (Varian, Palo Alto, CA, USA) were pre-conditioned (6 mL methanol followed by 6 mL 0.05 M sulfuric acid). The supernatants (each of the two filtrates was applied to an individual preconditioned HF Bond Elut column and the sample preparation (after combining the two eluates of each column) was further processed as described in Kempf *et al.* [9].

2.5 PA analysis of floral pollen: Method 1

Separation and identification of the individual PAs was achieved by GC-MS. The data were recorded with a Hewlett Packard 5890A gas chromatograph (Hewlett Packard, Santa Clara, CA, USA) equipped with a 30 m × 0.32 mm × 0.25 μm ft analytical column (ZB-1, Phenomenex, Aschaffenburg, Germany). The capillary column was directly coupled to a triple quadrupole mass spectrometer (TSQ 700, Finnigan MAT, Bremen, Germany). The conditions applied were: Injector and transfer line were set at 280°C; the temperature program used was: 100°C (3 min) to 300°C (3 min) at 6°C/min. Carrier gas flow was 1.6 mL/min He, and the mass spectra were recorded at 70 eV. The alkaloids were identified by their retention index, molecular ions and mass fragmentation patterns in comparison to an in-house reference database. The retention index was calculated by a set of hydrocarbons (even numbered from C_{10} to C_{28}) by linear interpolation.

For quantitative analysis PAs were separated using a Hewlett Packard 5890 Series II gas chromatograph (Hewlett Packard) equipped with a J&W DB-1 fused-silica capillary column (15 m × 0.25 mm), coated with a 0.25 μm film (Agilent J&W, Waldbronn, Germany). GC Conditions applied were as described for GC-MS. The eluting compounds were detected simultaneously by using a fused silica Y-splitter and an flame ionization detector and an nitrogen phosphorus detector. Quantifications were performed *via* the flame ionization detector signal and heliotrine as internal standard.

2.6 Trace analysis of PA in pollen products: Method 2

2.6.1 High-resolution GC-MS

GC-MS was carried out with a Fisons Instruments GC 8060 (Thermo Electron, Dreieich, Germany) gas chromatograph with split/splitless injection (220°C/1:20) directly coupled to a Fisons Instruments MD 800 mass spectrometer (Thermo Electron) essentially as described in Kempf *et al.* [9].

2.6.2 Quantification

Standard controlled relative quantification with heliotridine (originated from 4 μg heliotrine *per* sample) as internal standard was performed by high-resolution GC-MS under the above-mentioned conditions. Linear retention indices were 1600 and 1632 for di- trimethylsilyl-retronecine and the standard di-trimethylsilyl-heliotridine, respectively. Integration of peak area counts in electron ionization MS (EIMS) SIM mode (*m/z* 93, 183 and 299) was carried out. 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 with values obtained from authentic reference compounds; variances of <10% were tolerated. No extraction/response factors ($F = 1.0$) were considered. The data obtained was finally calculated into retronecine equivalents as previously described in Kempf *et al.* [9].

2.6.3 Detection limit of overall procedure and reliable quantification limit

A sample of homogenized pollen, not containing PAs, was spiked with five different concentrations (in the range of 1.0–20.0 μg /g) of PAs or PANOs and analyzed with the above-mentioned procedure. The results revealed a detection limit of overall procedure and reliable quantification limit with signal to noise ratios of 3:1 and 7:1 at 0.003 and 0.01 μg/g, respectively.

2.7 Microscopic pollen analysis

PA-positive pollen products were 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 pollen grains 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 PA analysis of pollen: Comparison of method 1 and method 2

Two quantitative methods were applied in this study. Method 1 was useful for floral pollen analysis. In this case the PA concentrations were in the range of milligrams *per* gram. Method 1 is a known standard procedure to analyze PAs in plant tissues at higher concentrations in EIMS scan mode. Under these circumstances full scan MS will be sensitive enough to identify minor PAs as well. In the case of commercial pollen products the PA range of interest is reduced by a factor of 1/1000 (μg/g). In this case method 1 would not generate useful full scan MS spectra in this complex matrix; therefore method 2 operating in SIM MS mode was applied for pollen products.

In order to compare the results and the reliability of both methods, we analyzed (threefold determination) one sample of floral *S. vernalis* pollen with both methods. The results were in good agreement: 4.05 mg/g ± SD 0.15 and 3.96 mg/g ± SD 0.42 (senecionine equivalents) for method 1 and method 2, respectively.

When method 1 is used, the analyzed compounds could be structurally identified. In comparison, method 2 represents a more sensitive and selective tool for the selective determination of trace amounts of 1,2-unsaturated PAs, by analyzing a sum parameter in the SIM EIMS mode. In contrast to target analysis approaches [8] method 2 does not require a large set of reference compounds or any additional information, such as botanical origin [6, 7, 10, 11] to screen and quantify the 1,2-unsaturated PA content of bee-derived samples. Each method represented a reliable tool for the determination of PAs depending on the analytical problem or application area.

3.2 PA content of floral pollen

Although recent publications have demonstrated the occurrence of PAs and PANOs in pollen of PA plants and pollen loads taken from bees visiting PA plants [10, 11, 13] the available data set is still limited.

We have tried to obtain pollen of all major PA-containing plant families. The identified PAs were in accordance with published data (see Table 1). PAs were detected in pollen and flower samples of all but one plant family (*Crotalaria juncea and C. sagittalis* (Fabaceae); data not shown). In general, the PA content determined for floral pollen (method 1) was in the same range as previously found with LC-MS [11]. The PA concentration of *Echium* pollen was somewhat lower (factor of 10) than those reported by Boppré *et al.* [10] but close to concentrations published later for pollen loads of foraging bees [11]. Pollen sacs and flowers of *C. sagitallis* were devoid of PAs. Whether Crotalaria pollen in general is devoid of PAs remains to be established. We were not able to rear plants of *C. juncea* in the green house with male flower parts and there are numerous examples especially for *Crotalaria spp.*, where PAs are not equally distributed in all parts of the plant [17, 18]. Perhaps the

Table 1. Total amounts of PAs and ratios of PANOs in pollen and flower heads of known PA-plants

Plant species	Pollen		Flowers or flower heads		PAs detected
	Total PAs (mg/g)	PANO proportion of total PAs (%)	Total PAs (mg/g)	PANO proportion of total PAs (%)	
Senecio vernalis	4.1	71	2.3	84	*cf.* Figs. 1 and 2
Senecio jacobaea	3.3	95	3.4	74	[24]
Eupatorium cannabinum	0.6	83	4.2	69	[a]
Echium vulgare	0.9	81	2.0	98	[25]
Phalaenopsis hybrids[b]	0.6	0	4.4	3	[26]

a) Rinderine (46%), intermedine (31%), echinatine (23%).
b) Pollinia.

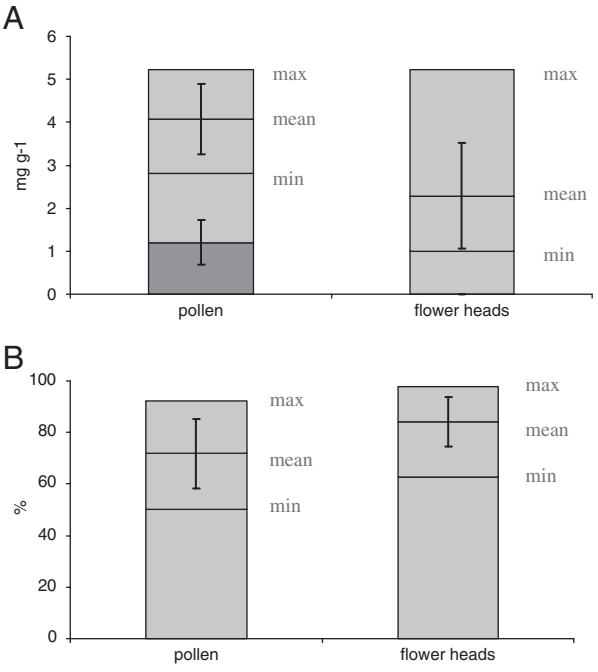

Figure 1. Total amounts of PAs (A) and ratios of PANOs (B) in pollen and flower heads of *S. vernalis*. The bars show means ± SD, minimum and maximum values of the dataset ($n = 12$). The dark color represents the amount of PAs found in the wax cover of the pollen grains.

species we were able obtain for analysis was, by chance, devoid of PAs in its floral parts, although we detected trace amounts of monocrotaline in seed pods of our *C. sagittalis* plants.

In pollen and flower heads of Asteraceae PAs are dominantly found as *N*-oxides. Only in the pollinia of *Phalaenopsis* PAs are present almost exclusively in the tertiary form (Table 1). Usually the qualitative PA composition was identical in pollen or flower heads of each species but the PA pattern varied considerably in their relative abundance.

To investigate this in more detail we used *S. vernalis* as model system. The data set obtained for pollen or flower heads of *S. vernalis* showed no significant differences in total PA content or individual PA ratios in terms of location, time point or rooted *versus* clipped plants. But significant differences were observed between the two sample sets: pollen and flower heads (Figs. 1 and 2).

The maximum PA level in both organs reached up to 5 mg/g, but in general the PA level was higher in pollen than it was in flower heads (factor of 2). Furthermore, the PA composition differed between pollen and flowers. The PA pattern of pollen is dominated by seneciphylline while retrorsine and usaramine only occurred in flower heads (Fig. 2). It is known that senecionine is synthesized in the roots of *Senecio* spp. [16]; so it can not be excluded that the differences in PA patterns of pollen compared with flowers is a consequence of differences in transport/storage, but it seems as if the increase of seneciphylline in pollen is due to a pollen-specific conversion of senecionine into seneciphylline (Fig. 2). Only flower heads seem to be capable of senecionine hydroxylation. The diversity of the observed PA bouquet in *Senecio* is the result of simple enzymatic modifications of the core structure (hydroxylation, dehydrogenation). Since these modifications vary between organs and are at the same time specific to individuals, a large plasticity of PA pattern throughout the plant can be observed [15, 19]. Remarkably, the PA pattern of the wax cover of pollen resembles the pattern found in flowers heads

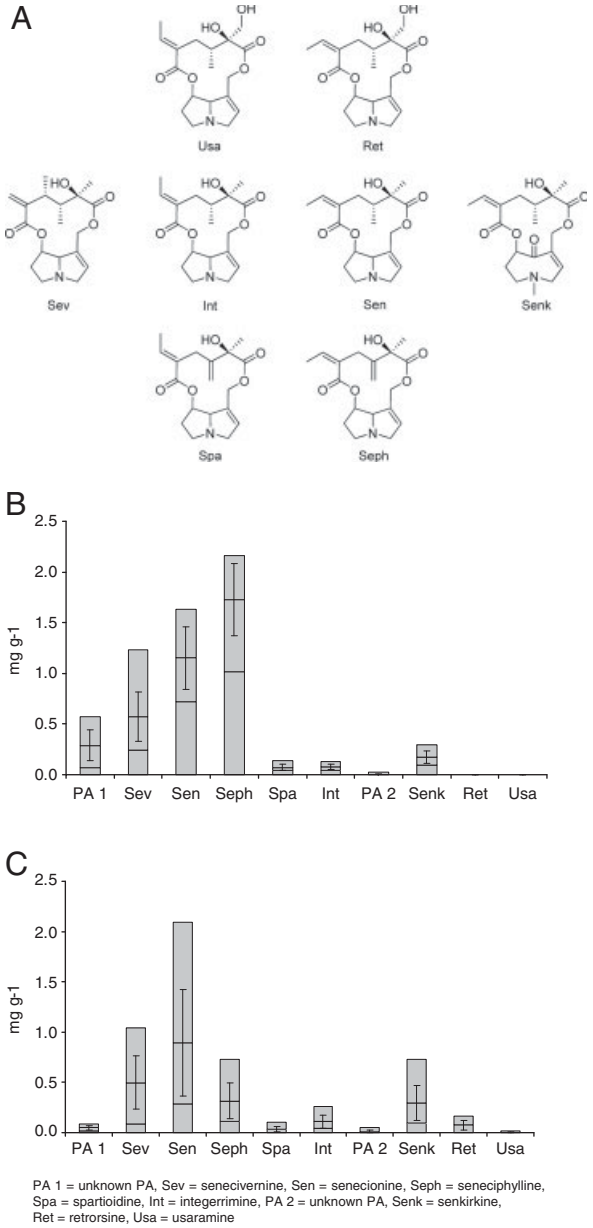

Figure 2. PA structures (A), distribution of individual PAs in pollen (B) and flower heads (C) of *S. vernalis*. The bars show means ± SD, minimum and maximum values of each PA in the data set ($n = 12$).

instead the one found for pollen (data not shown) and can be explained by the role of the tapetum cells, which apply the wax to the pollen and are part of the anther but not of the pollen. The wax cover accounts on average for 25% of the total pollen PA content (Fig. 1) but is composed largely of tertiary PAs rather PANOs, which, given the much greater lipophilicity of the former, is not surprising.

3.3 Method development for trace analysis of 1,2-unsaturated PAs in pollen products

Initiated by our experiences with the sample preparation of honey, we orientated our isolation strategy to our recently published method of Kempf *et al.* [9]. In comparison to honey, grinding with sea sand is necessary to break the compact and hard exine. Preliminary tests were conducted, and demonstrated, that a twofold sea sand treatment followed by extraction is required to extract the PAs exhaustively. A third treatment did not increase PA recovery from pollen products (data not shown). The method comprised a strong cation exchange-SPE enrichment combined with the conversion of all PA monoesters and diesters including their corresponding *N*-oxides into the corresponding necine bases, leading to the formation of just a single sum parameter for quantification. Since all 1,2-unsaturated PAs of one sample are converted into one single compound, this strategy was also helpful to achieve a reasonable sensitivity.

In our test series, the Varian HF Bond Elut LRC SPE column (Varian) exhibited a mean recovery efficiency of 80% $\pm$ SD 3.8, using tertiary PAs or their *N*-oxides, respectively. This is comparable to our previous results obtained for honey, a completely different matrix. The somewhat lower mean recovery for pollen (80% *versus* 83%) might be due to the additional filtration steps. Plugging of the column, as occasionally observed with honey samples, did not occur.

3.4 Quantification of 1,2-unsaturated PA content in pollen products

Our method was successfully adapted to analyze 1,2-unsaturated PAs in pollen products. Fortunately, during the course of analysis of all 55 pollen products (and previously with 216 honey samples [9]) heliotridine-type PAs were never found, as they may have interfered with quantification.

With the above-mentioned sample preparation the achieved detection limit of overall procedure and reliable quantification limit were 0.003 and 0.01 μg/g with signal to noise ratios of 3:1 and 7:1, respectively (data not shown).

The result is a single sum parameter indicating the toxic principle (1,2-unsaturation of the necine base) and is calculated as retronecine equivalent (mean of four independent work-ups). Retronecine (molecular weight = 155 g/mol) represents only a small part of the originally occurring mono- or diester PAs (*e.g.* senecionine molecular weight = 335 g/mol). Hence, the retronecine equivalents listed here can be multiplied by a factor of approx. 2 to yield the original total 1,2-unsaturated PA amount in micrograms *per* gram found in these products. Other 1,2-unsaturated PAs such as otonecine and supinidine-like PAs, which are relatively rare [1], were not detected by our method. As a consequence, all of the present results are an ''underestimate'' of the actual toxic PA content and represent the lowest level of 1,2-unsaturated PAs in a specific sample.

3.5 Pollen products

Up to now the reported levels of PAs in pollen are much higher than the PA levels found in honey [6, 7, 9, 10]. However, in some cases, despite low levels of PA pollen being found in honeys, these honeys contained significant amounts of 1,2-unsaturated PAs [9]. Therefore, the determination of PA plant pollen may not always be the right choice to identify honeys or pollen products containing high PA levels and analytical methods are needed to surely recognize these products.

Stimulated by the preliminary data on high PA levels found in floral pollen [10] and pollen loads of foraging bees [11] we decided to analyze pollen products that are available as food supplements.

All the samples were purchased from various market places and drug stores in Germany and other European countries (*e.g.* France, Italy), as well as from Internet stores without any selection criteria. No additional information, such as apiarist statements about the habitats of the bee colonies or pollen analyses, were available beforehand. Since the method presented here is not based on specific target PA analysis of a specific plant or group of plants, the information of the PA plant source is not required beforehand to set up an appropriate analytical method.

In total, 55 pollen products including products labeled ''pollen granules'' were tested. From these 33 were from Europe, four from USA and Mexico, one from New Zealand, one from Asia and 16 samples were of non-specified origin, (Fig. 3A). Within these products 17 samples (31%) revealed a PA content in the range from 1.08 to 16.35 μg/g (*cf.* Table 2). The average PA content was 5.17 μg/g. The detected amounts surpass the recently published average PA content in honey [9] by almost two orders of magnitude. Furthermore, these results are in good agreement with the data available for pollen sacs of bees foraging on PA plants [11]. On the other hand, the levels are much lower than levels found in floral pollen, which is the consequence of natural ''dilution'' of PA pollen by non-PA pollen and nectar.

Nine out of the 17 PA-positive pollen products were of European origin (Spain/5, Rumania/2, Italy/1 and France/1); the remaining eight were not labeled (Table 2 and Fig. 3B). As shown by subsequent pollen analysis, all ''PA-positive''

pollen products exhibited a significant amount of PA plant pollen (mostly *Echium* spp.) (*cf.* Table 2). All PA-positive samples were without conspicuous declaration towards plant origin. In general, a good correlation between high PA contents and the relative amount of PA plant pollen could be observed. For example, the three samples (no. 12, 40 and 45) with the highest PA content, *i.e.* 16.35, 11.65 and 13.36 μg/g, showed 83, 75 and 77% *Echium* spp., respectively, the highest degree of PA plant pollen. But the reverse is not necessarily true; a high PA pollen content did not necessarily correlate with high PA content (*cf.* sample no. 33, showed 67% pollen from *Echium* spp. but only a PA content of 2.38 μg/g). So far the results of the palynological analysis, which in our case was especially focused on pollen of PA plants, indicates that the relative pollen analysis is only of limited value for predicting PA content of honey. On the other hand, in all PA-positive samples (pollen or honey) the PA occurrence is accompanied by PA plant pollen (most frequently *Echium* spp.). So far the occurrence of PA pollen was always an indicator for a PA-positive honey or pollen sample.

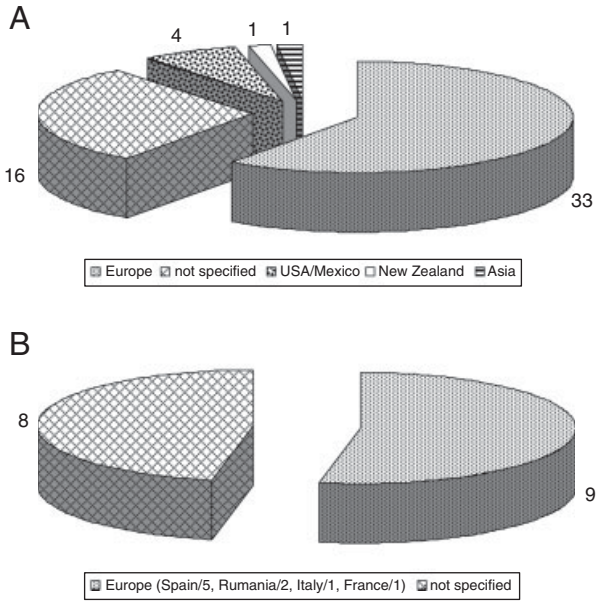

Figure 3. (A) Regional arrangement of the analyzed pollen products (according to label). (B) Regional arrangement of the PA-positive tested samples (according to label).

4 Concluding remarks

The suggested serving size of pollen products recommended by suppliers for mixing pollen in drinks, yogurt or to sprinkle on top of breakfast cereals is about 10 g (1–2 tablespoons) *per* day.

Table 2. Comparison of PA concentrations and results of microscopic pollen analysis of commercial pollen products ($n = 55$); only PA-positive products are listed

Total pyrrolizidine alkaloids[a] (μg/g)			Pollen analysis	Geographic origin	
Sample no.	Mean[b] (μg/g)	SD (μg/g)		By pollen analysis	As labeled
2	5.28	0.11	66% *Echium* spp.	Spain	Not specified
3	1.79	0.18	23% *Echium* spp.	n.p.[c]	Not specified
8	4.64	0.52	66% *Echium* spp.	Spain	Spain
9	2.42	0.67	27% *Echium* spp.	Spain	Not specified
12	16.35	1.98	83% *Echium* spp.	Spain	Rumania
13	1.81	0.24	12% *Echium* spp.	Spain (particular East-European regions)	Not specified
19	2.44	0.23	22% *Eupatorium* spp.,	n.p.[c]	Rumania
24	1.08	0.13	25% *Echium* spp., <1% *Senecio* spp.	Spain (particular East-European regions)	Spain
28	4.46	0.63	58% *Echium* spp.	Spain	Not specified
33	2.38	0.22	67% *Echium* spp.	n.p.[c]	Italy
34	2.46	0.15	44% *Echium* spp.,6% *Eupatorium* spp.	Mediterranean regions	France
37	2.83	0.28	47% *Echium* spp.	n.p.[c]	Spain
38	1.74	0.26	21% *Echium* spp.	n.p.[c]	Not specified
40	11.65	1.78	75% *Echium* spp.	n.p.[c]	Not specified
45	13.36	0.49	77% *Echium* spp.	Spain	Spain
50	6.97	0.46	60% *Echium* spp.	n.p.[c]	Not specified
52	6.20	0.03	43% *Echium* spp.	Spain	Spain

a) Data given as retronecine equivalents.
b) mean ($n = 4$).
c) n.p., not performed.

We were unable to find reliable data on the *per*-capita consumption of pollen granules in Europe or other countries. There are also no regulations we are aware of on tolerable PA levels in food in general.

In terms of phytopharmaceuticals the German authorities have prohibited herbal products containing 1,2-unsaturated PAs [20]. Exemptions are made only for a few specific plants where the authorities limit the daily PA uptake to a maximum of 1.0 μg 1,2-unsaturated PA/day. This is further restricted to no more than 6 wk of continuous use *per* year [20].

Assuming an average PA content of 5.17 μg/g, a 30% probability of PA occurrence and a daily dose of 10 g this would result in an average PA intake of 15 μg (retronecine equivalents) *per* day for pollen products consumers. This calculation is based on retronecine equivalents and does not include otonecine type PAs. Therefore, it represents the lowest calculable value. The determination of a single sum parameter, which is based on the 1,2-unsaturation of the originally occurring PA(s), can be directly linked to the current effective regulations for herbal pharmaceuticals in Germany [20]. In respect to these regulations one can assume at least a factor of 2 to convert the retronecine equivalents into the concentrations of the original mono- and diester PAs in the sample (the calculations of the German authorities are based on the amount of the original mono and diester PAs). In doing so, the average daily PA dose would be 30 μg. For individuals who regularly consume pollen products (more than 6 wk *per* year) the limit of 0.1 μg *per* day would be exceeded by a factor of more than 300.

The commission of the European Union has recently authorized refined *Echium* oil as novel food ingredient [21]. The use of the product is limited by its level of stearidonic acid but PAs are mentioned in the specifications of this product as well: […] *Pyrrolizidine alkaloids - Not detectable with a detection limit of* $4\,\mu g\,kg^{-1}$ [21].

Although there are no regulations for food so far, the Dutch authorities for food safety have discussed the complex toxicological problem in detail [8]. In their view, the virtual safe dose for carcinogenicity (based on riddelliine) and a tolerable daily intake for acute liver toxicity was 0.43 and 100 ng *per* kg body weight *per* day, respectively. Based on these calculations pollen consumers are well above the calculated limits. A 70 kg person exceeds the tolerable daily intake on average by a factor of 4 or calculated for a 20 kg child it would be a factor of 15. In 2001 the US Food and Drug Administration advised that PAs should not be used as an ingredient in dietary supplements (http://www.cfsan.fda.gov/~dms/dspltr06.html; accessed on May 14th) [22]. According to a recent position paper proposed by the German Bundesinstitut für Risikoforschung (http://www.bfr.bund.de/cm/208/nulltoleranzen_in_lebens_und_futtermitteln.pdf; accessed on May 14th) [23] there should be a zero tolerance for 1,2-unsaturated PAs in foodstuff and animal feed.

PA content of pollen is alarming in regard to two aspects. First, PA-positive pollen products occur more frequently (31%) compared with honey (9%) and in addition the average PA content of pollen is higher than those found in honey (on average 5.17 μg/g *versus* 0.056 μg/g [9], calculated as retronecine equivalents).

The PA concentration found in pollen products raises the question whether these products should be sold as food supplements without monitoring the PA content and provoke the discussion of a more general regulation of PAs in foodstuff.

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