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Pyrrolizidine alkaloids in honey and bee pollen

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A total of 3917 honey samples and 119 'bee pollen' samples (pollen collected by honeybees) were analysed for pyrrolizidine alkaloids (PAs). Some 0.05 M sulphuric acid was used for extraction followed by a clean-up step by means of solid-phase extraction. Separation and detection was achieved by target analysis using an LC-MS/MS system. PAs were found in 66% of the raw honeys (bulk honey not yet packaged in containers for sale in retail outlets) and in 94% of honeys available in supermarkets (retail honey). A total of 60% of the bee pollen samples were PA positive. The PA pattern was used to identify the potential origin of the PAs in honey, which was verified for the genus *Echium* by relative pollen analysis. The results give an estimate of the impact of PA-containing plants belonging to the genera *Echium*, *Senecio* and, to a certain extent, *Eupatorium* on PA levels in honey and can serve as a decision basis for beekeepers in order to find the most suitable location for the production of honey and bee pollen low in PAs.

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

Introduction

The European Community imports about 130,000 tons of honey with a market value of more than €200 million from non-European Community countries each year (Eurostat 2010). Another 90,000 tons are traded between member states of the European Community. Honey is consumed pure and is used as an ingredient in fruit spreads, breakfast cereals, sweets, bakery products, cosmetics and for medicinal purposes. It enjoys the reputation of being a natural and healthy product. Pollen collected by honeybees (hereinafter referred to as 'bee pollen') are used as a dietary supplement as they contain minerals, amino acids and proteins (Stanley and Linskens 1985).

PAs containing a double bond in the 1,2-position are potentially toxic to the liver and are under suspicion of causing cancer (World Health Organization (WHO) 1988). However, the toxicity depends strongly on the esterification of the hydroxyl groups. PAs containing a double bond in the 1,2-position are also referred to as, for example, 1,2-dehydropyrrolizidine ester alkaloids, dehydroPA (Edgar et al. 2010) or 1,2-unsaturated PA esters (Kempf et al. 2008).

Contrary to, for example, antibiotics and pesticides, PAs are of purely natural origin. PAs are produced by plants as secondary metabolites for protection against herbivores. It has been estimated that about 3% of all flowering plants (more than 6000 plant species) contain PAs (Smith and Culvenor 1981). They mostly belong to different species of the families Boraginaceae (e.g. *Heliotropium*, *Echium*, *Myosotis*, *Borago*, *Cynoglossum*), Asteraceae (e.g. *Senecio*, *Eupatorium*, *Chromolaena*, *Ageratum*) and Fabaceae (e.g. *Crotalaria*). Some of these plants (e.g. *Echium* species) are intentionally used for honey production (Lüllmann 2010), thus it is not surprising that PAs have been identified in honey and pollen collected by honeybees (Crews et al. 1997; Deinzer et al. 1977; Beales et al. 2004; Betteridge et al. 2005; Boppré et al. 2005, 2008).

In 1992 limits for PA in phytopharmaceutical products were introduced (e.g. Bundesgesundheitsamt 1992). The consumption of PAs was limited to $1\,\mu g\,PAs\,day^{-1}$, if consumed for up to 6 weeks, and $0.1\,\mu g\,PAs\,day^{-1}$ if consumed longer. For honey, bee pollen and other food stuffs no limits for PA levels exist. Kempf, Reinhard et al. (2010) gave an overview on the approaches and potential limits discussed by other countries to evaluate PA levels in food and animal feed. A comprehensive review on the potential risks of PAs in food in general is given by Edgar et al. (2010) and on the ecological context of PAs in food, feed and forage by Boppré (2011).

Edgar et al. (2002) suggested that all honeys need to be assessed for their content of 1,2-unsaturated PAs

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in order to minimise dietary PA exposure of consumers. For the scope of earlier works it was sufficient to analyse only a small number of honey samples. However, that is not enough to get an overview on the occurrence of PAs in honey (Kempf, Heil et al. 2010). In order to achieve that, more samples had to be analysed and a method for the routine analysis of PA in honey and bee pollen had to be established (Dübecke 2010). That was first done by Rijksinstituut voor Volksgezondheid en Milieu (RIVM) (2007) and Kempf et al. (2008). They performed screenings of 171 and 216 honey samples and detected PAs in 25% and 8.8% of the samples, respectively. However, very different methods were used. While RIVM (2007) used LC-MS target analysis for the detection of a small number of PAs typical for *Senecio* species, Kempf et al. (2008) developed a method to reduce those PAs, which are esters of a wide range of necic acids and retronecine as necine base, to retronecine, which was subsequently analysed using GC-MS resulting in a sum parameter. The limit of quantification was 10 $\mu g\,kg^{-1}$ for the sum-parameter method and 1 $\mu g\,kg^{-1}$ for the target analysis. Both methods give results that are likely to be an underestimate as the sum-parameter method only works for esters of retronecine or heliotridine but not for otonecine-derived PAs (e.g. senkirkine) and the target analysis ignores all PAs apart from those for which the instrument was set up.

The aim of this study was to obtain a clearer view on PA levels in honey and bee pollen from different countries. Furthermore, data were used to deduce influences of the genera *Echium*, *Eupatorium* and *Senecio* species on PA contamination of honey, as species of these genera are commonly found in many countries worldwide. Since information on single PAs is lost when using the sum-parameter method, no conclusions in terms of botanic influence on PA levels can be drawn. Thus, the method of choice was the LC-MS target analysis, which preserves information on single PAs.

A total of 3917 honey samples and 119 bee pollen samples from various countries were analysed regarding a range of 1,2-unsaturated (and thus potentially toxic) PAs. Raw honeys (bulk honey not yet packaged in containers for sale in retail outlets) and honeys available in supermarkets (usually blended honey) were considered separately. The data can serve as a decision basis for beekeepers in order to find the most suitable location for the production of honey and bee pollen low in PAs.

Material and methods

PA references were purchased from a range of distributors. Seneciphylline was obtained from Carl Roth (Karlsruhe, Germany), echimidine and lycopsamine from Cfm Oskar Tropitzsch (Marktredwitz, Germany), heliotrine from Latoxan (Valence, France), senkirkine, senecionine-N-oxide and seneciphylline-N-oxide from Phytolab (Vestenbergsgreuth, Germany), monocrotaline, retrorsine and senecionine from Sigma-Aldrich (Steinheim, Germany). Methanol was purchased from VWR International (Darmstadt, Germany), formic acid, sulphuric acid, ammonia and ammonium acetate from Merck Chemicals (Darmstadt, Germany).

Samples of raw honey were taken directly from the export drum in which the honey was shipped and kept in plastic containers. Samples of honeys available in supermarkets (hereinafter referred to as 'retail honey') were taken after homogenisation and processing either from the container in which the honey was mixed for sale or from the jar in which it is finally offered in the supermarkets. Bee pollen samples were acquired from a variety of sources in plastic containers.

Sample preparation

Honey

To 10 g of honey 100 ng heliotrine as internal standard and 30 ml of 0.05 M sulphuric acid were added, followed by 20 min of vigorous shaking (modified after Betteridge et al. 2005; and Kempf et al. 2008). As heliotrine may occur naturally, parallel samples without internal standard were prepared. The samples were then filtered (2 µm mesh) overnight to remove particles, which would block the solid-phase extraction (SPE) cartridges during the clean-up step using HF Bond Elut LRC (500 mg/3 ml) SCX (Varian) SPE-cartridges (Kempf et al. 2008). Prior to SPE, the cartridges were washed with methanol and conditioned with 9 ml of 0.05 M sulphuric acid. The samples were applied onto the cartridges without the use of negative pressure and subsequently eluted into 8 ml glass vials using ammoniated methanol (Kempf et al. 2008) and dried at 40°C in a stream of ambient air. The dried samples were reconstituted in 1 ml deionised water, shaken vigorously and filtered into a 2 ml glass vial using 0.45 µm syringe filter.

Bee pollen

At least 10 g of bee pollen were ground and homogenised using an electric mill. To 1 g of the bee pollen homogenate 100 ng heliotrine and 20 ml of 0.05 M sulphuric acid were added, followed by 60 min of vigorous shaking. As for honey, samples were processed in parallel. Subsequently, samples were centrifuged and the supernatant kept. The solid residue was again shaken with 10 ml 0.05 M sulphuric acid and centrifuged. The supernatants were combined and filtered (2 µm mesh) overnight. The solid bee pollen

residue was discarded. SPE was performed according to the protocol for honey samples.

LC-MS/MS

The LC-system consisted of a Shimadzu degasser (DGU-20A3) and two Shimadzu LC-20AD pumps controlled by a Shimadzu CBM-20A controller unit. Injection of 10 µl of sample was done using a HTC PAL autosampler of CTC Analytics AG. Column temperature was held steady at 25°C. The column was eluted using a gradient flow (300 µl min^{-1}) of two solvents. Solvent A consisted of 99.5% water plus 0.5% formic acid; solvent B of 94.5% methanol, 5% water and 0.5% formic acid. To both solvents oxalic acid and ammonium acetate were added. Concentrations in both solvents were set to 0.1 and 2.0 mM, respectively. The mobile phase was maintained for the first 30 s at 97 : 3 (A : B) and then changed with a linear gradient to 100% solvent B over 6 min, which was kept for 1 min before a re-equilibration phase over 2.5 min restored the initial mobile phase of 97 : 3. Separation was achieved using a Thermo Hypersil Gold C18 column (50 x# 2.1 mm, 1.9 µm particle size). The short column allowed for a reduced analysis time of only 10 min per sample. An Applied Biosystems API 4000 QTRAP triple quadrupole mass spectrometer was used to detect the PAs. The instrument was set to multi-reaction monitoring (MRM) mode (source temperature 650°C, cone voltage 5500 V, collision gas on 'high', curtain gas 25 psi, ion source gas 1 and 2 at 35 and 45 psi, respectively), using one MRM transition as quantifier and another two as qualifiers for each PA. The following MRM transitions (parent ions ($[M + H]^+$) underlined, quantifier in **bold**, qualifiers plain) were used: monocrotaline (326; **94**, 120,194), echimidine (398; **120**, 83, 138), heliotrine (314; **138**, 120, 156), lycopsamine (300; **156**, 120, 138), retrorsine (352; **138**, 120, 324), senecionine (336; **94**, 120, 308), seneciphylline (334; **138**, 120, 306) and senkirkine (366; **168**, 122, 107). Quantification was done using external calibrations. The calibrations were prepared with the reference materials in water including a model honey matrix. Finally, concentrations were corrected against the recovery of the internal standard resulting in recoveries between 60% and 110% for the analysed PAs. The limit of quantitation (LOQ) was 1 µg kg^{-1} for echimidine and senkirkine, 2 µg kg^{-1} for heliotrine and 3 µg kg^{-1} for lycopsamine, retrorsine, senecionine and seneciphylline.

Results and discussion

To cover as many PAs as possible, an additional four PAs and seven PA-N-oxides were identified by comparison of their fragmentation patterns with data published by Betteridge et al. (2005), Boppré et al. (2005) and Colegate et al. (2005). As the physical properties of N-oxides and tertiary alkaloids differ to some extent, the recoveries of three N-oxides were tested using the N-oxides of senecionine, seneciphylline and echimidine. Senecionine-N-oxide and seneciphylline-N-oxide were commercially available only at the end of this study, which is why they were not analysed in the honey samples. Echimidine-N-oxide was prepared from the echimidine reference according to Cymerman and Purushothaman (1970). The results show that the recoveries are in the same range as the tertiary alkaloids and are in accordance with the findings of Betteridge et al. (2005) and Kempf et al. (2008).

The PAs were grouped according to their potential botanical origin:

- Group 1: PAs typical amongst others for *Eupatorium* species. Available reference: lycopsamine. Furthermore identified (MRM transitions in brackets): lycopsamine-N-oxide (316; **172**, 94, 226).
- Group 2: PAs typical for *Echium* species. Available reference: echimidine. Furthermore identified: echimidine-N-oxide (414; **254**, 220,120), acetyl-echimidine (440; **120**, 83, 138) (-N-oxide (456; **254**, 220,120)), echiumine (382; **120**, 83, 138) (-N-oxide (398; **254**, 220,120)), acetyl-echiumine-N-oxide (440; **254**, 220,120), echiuplatine (382; **120**, 83, 138) (-N-oxide (398; **254**, 220, 120)) and echivulgarine (480; **120**, 83, 138) (-N-oxide (496; **254**, 338, 220)).
- Group 3: PAs typical for *Senecio* species. Available references: senecionine, retrorsine, seneciphylline, senkirkine.

Quantification of lycopsamine-N-oxide was done by using the calibration of lycopsamine and assuming the same LOQ. We are aware that the responses of the tertiary alkaloid and its N-oxide are not necessarily the same (Betteridge et al. 2005). The other additionally identified PAs were quantified using the echimidine calibration, assuming the LOQ of echimidine. It was accepted that the results have to be regarded as approximate results.

The concentrations of each PA within one group were summed up for each sample and the average of all samples of each country was calculated. The occurrence of heliotrine (used as internal standard) in honey is very rare (approximately 0.1%) and is thus not discussed. Monocrotaline, a cyclic diester typical for *Crotalaria* species, was not found in any of the samples, which is in accordance with the observation that these plants are usually visited by bumble bees and not by honeybees.

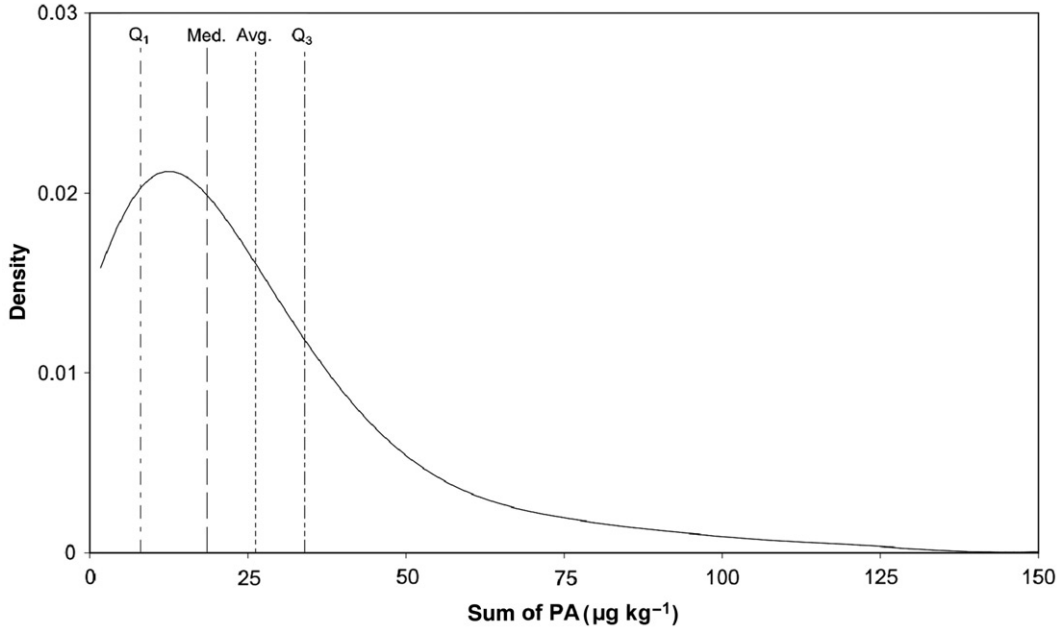

Figure 1. Kernel-density plot of the abundances of PA concentrations (kernel width = 10) of the ΣPAs (sum of PAs) in honey available in supermarkets (retail honey). The median concentration (Med.) of the PA-positive samples was 19 µg kg^{-1} and the average concentration (Avg.) was 26 µg kg^{-1}. Q_1 and Q_3 are the first and third quartiles. A total of 88% of all analysed retail honeys were below 50 µg kg^{-1}.

It has to be kept in mind that the mentioned marker PAs cannot be attributed exclusively to a single family of plants, e.g. echimidine can be found in *Symphytum officinale* (comfrey) as well. As different *Echium* species are widespread in South America and also in Spain and known to be intensively visited by honeybees, echimidine can be considered a suitable marker PA for *Echium* plants. Senecionine also occurs in plants of other genera (Hartmann and Witte 1995), but the vast majority of senecionine-containing plants belongs to the genus *Senecio*. Deinzer et al. (1977) were able to show that honeybees do produce honey from *Senecio jacobaea*, though this plant does not seem to be their preferred choice.

Eupatorieae often contain lycopsamine and/or its isomers (Hartmann and Witte 1995). As especially in Argentina and Uruguay *Eupatorium buniifolium* is very abundant (Sharma et al. 1998) and much visited by honeybees, we chose lycopsamine as marker for *Eupatorium* species. However, for other countries, e.g. Brazil, other plants could be of importance, like *Chromolaena odorata*, which is a very invasive weed in tropical regions worldwide and contains an isomer of lycopsamine.

According to Smith and Culvenor (1981) *Echium vulgare*, which is also abundant in Argentina and Uruguay, also contains lycopsamine or one of its isomers. Analysis of plant material of *E. vulgare* revealed only minor amounts of lycopsamine. Compared with the amounts of echimidine and its N-oxide found in this plant, lycopsamine originating from *Echium vulgare* can be neglected for our purpose. Nevertheless it cannot be excluded, that there are other lycopsamine-containing plants contributing lycopsamine and other PAs to honey.

Retail honey

A total of 696 samples of retail honey were analysed of which 94% were found to be PA positive showing concentrations between 1 and 267 µg kg^{-1} honey (ΣPAs, all measured PAs summed up). These values are in accordance with results found previously (RIVM 2007; Kempf et al. 2008).

The distribution of concentrations within PA-positive samples is shown as a kernel-density plot in Figure 1. The average ΣPAs' concentration in PA-positive honeys was 26 µg kg^{-1} and the median at 19 µg kg^{-1}.

As retail honey is usually a blend of different honeys, which originate in different countries and from a variety of plants, PAs of all three groups were expected. The most abundant PAs within each group were lycopsamine, echimidine and senecionine. PAs belonging to group 2 showed the greatest abundance and average cumulative concentration (all analysed PAs of each group summed up) of 92% and 13 µg kg^{-1}, respectively (PA-positive samples only). In 69% of the samples PAs of group 1 were found at an average concentration of 11 µg kg^{-1}. PAs from group 3 were

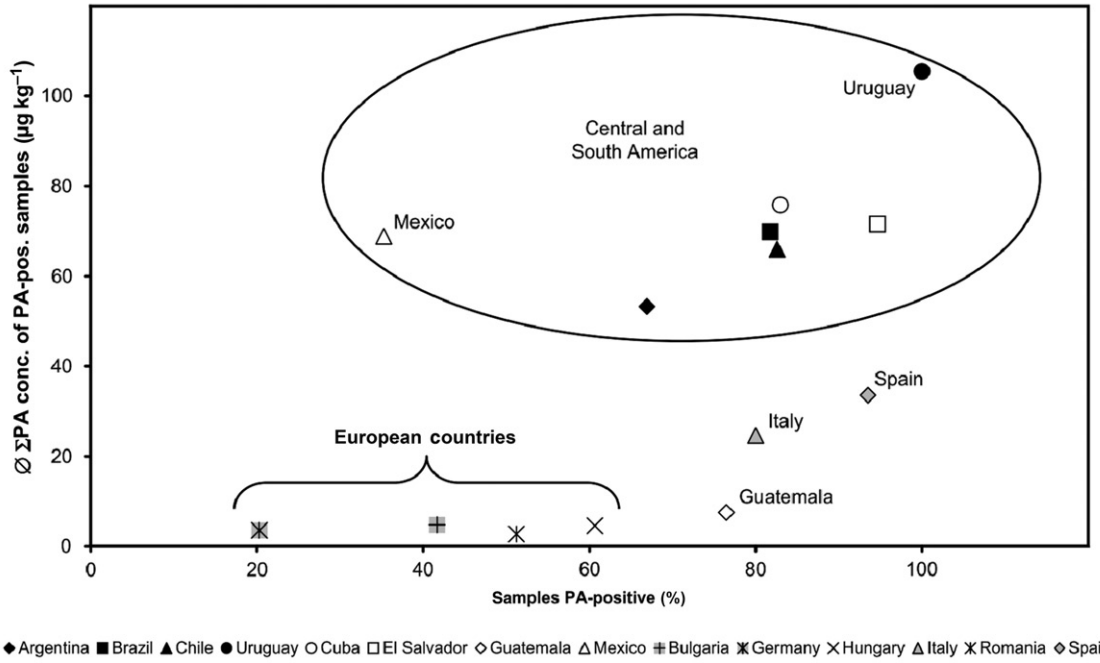

Figure 2. Relationship between the percentage of PA-positive raw honeys and their average ΣPAs concentration sorted by country of origin. Generally, raw honeys from Central and South America contain a higher amount of ΣPAs than raw honeys from European Community countries. However, ΣPAs concentrations in Guatemalan honeys are much lower than in the other Central American honeys. Mexico shows a low fraction of PA-positive honeys (35%). All honeys from Uruguay contained PAs and the average concentration was highest of all (105 µg kg^{-1}). Honeys from Italy and Spain have higher PA contents and also higher fractions of PA-positive honeys compared with the other European Community countries.

found in 40% of the samples at average concentrations of 12 µg kg^{-1}. Thus, it seems that *Echium* species have the greatest impact on PA concentrations in honey, followed by, for example, *Eupatorium* species and eventually *Senecio* species. However, it has to be remarked that the LOQ for PAs of groups 2 and 3 was 3 µg kg^{-1}, while that of group 1 was 1 µg kg^{-1}. Lowering the LOQ for PAs of groups 1 and 3 would most likely result in higher abundances of these types of PAs, but also in lower average concentrations.

Furthermore, it has to be considered that especially in honeys from tropical areas species of the genera *Chromolaena* and maybe *Ageratum* and others may also contribute substantial amounts of PAs of group 1 to the PA pool, as they contain lycopsamine or its isomers.

In Germany the PA limit for consumption of phytopharmaceuticals was limited to 1 µg day^{-1}, if taken for no more than 6 consecutive weeks, and 0.1 µg day^{-1} if consumed for more than 6 weeks (Bundesgesundheitsamt 1992). Thus, a hotel serving of honey (usually 20 g) may contain up to 50 µg kg^{-1} of PAs and would still meet the limit of 1 µg PAs day^{-1} and 5 µg kg^{-1} to meet the limit of 0.1 µg PAs day^{-1}. A total of 88% of all retail honeys were below 50 µg kg^{-1} and 22% were below 5 µg kg^{-1} ΣPAs. Assuming a limit of 1 µg day^{-1} and a consumption of 20 g of honey, only 12% of all retail honeys would violate that limit. However, the questions of how to evaluate toxicity of PAs and if it is adequate to apply the limits for phytopharmaceutical products to food stuffs are not yet answered.

Central and South America

A total of 2839 raw honey samples from Argentina, Brazil, Chile, Cuba, El Salvador, Guatemala, Mexico and Uruguay were analysed. In 68% of the samples PAs were found at ΣPAs' concentrations ranging from 1 to 1087 µg kg^{-1}. The average PA concentration of the PA-positive samples was 67 µg kg^{-1} (median = 27 µg kg^{-1}), and 46 µg kg^{-1} including the PA-negative honeys from Central and South America.

Due to blending of raw honey, the average ΣPAs' concentration of retail honey is about 2.5 times lower than ΣPAs in raw honey, but while only 68% of the raw honeys contain PAs, they were detected in 94% of the retail honeys. Blending is done to meet consumer demand for a constant level of quality in terms of, for example, taste, colour and odour of the product.

The highest average ΣPAs' concentration and abundance was found in honeys from Uruguay with 105 µg kg^{-1} and 100% of the samples being PA positive, respectively. Only 35% of the Mexican honey samples were PA positive. However, the average ΣPAs' concentration of 69 µg kg^{-1} of the PA-positive samples was within the range of the other Central and South American countries (53–76 µg kg^{-1} ΣPAs; Figure 2). Only Guatemala showed relatively low

amounts of only 8 μg kg^{-1} ΣPAs, though 76% of the samples were PA positive.

The concentration span of the ΣPAs' concentration covers in most countries three orders of magnitude. Still, apart from Cuba and Uruguay, more than 75% of the samples of each country were below 100 μg kg^{-1}. Guatemalan samples in turn were in the range from 3 to only 28 μg kg^{-1} ΣPAs with 75% of the PA-positive samples containing less than 10 μg kg^{-1} ΣPAs (Figure 3A).

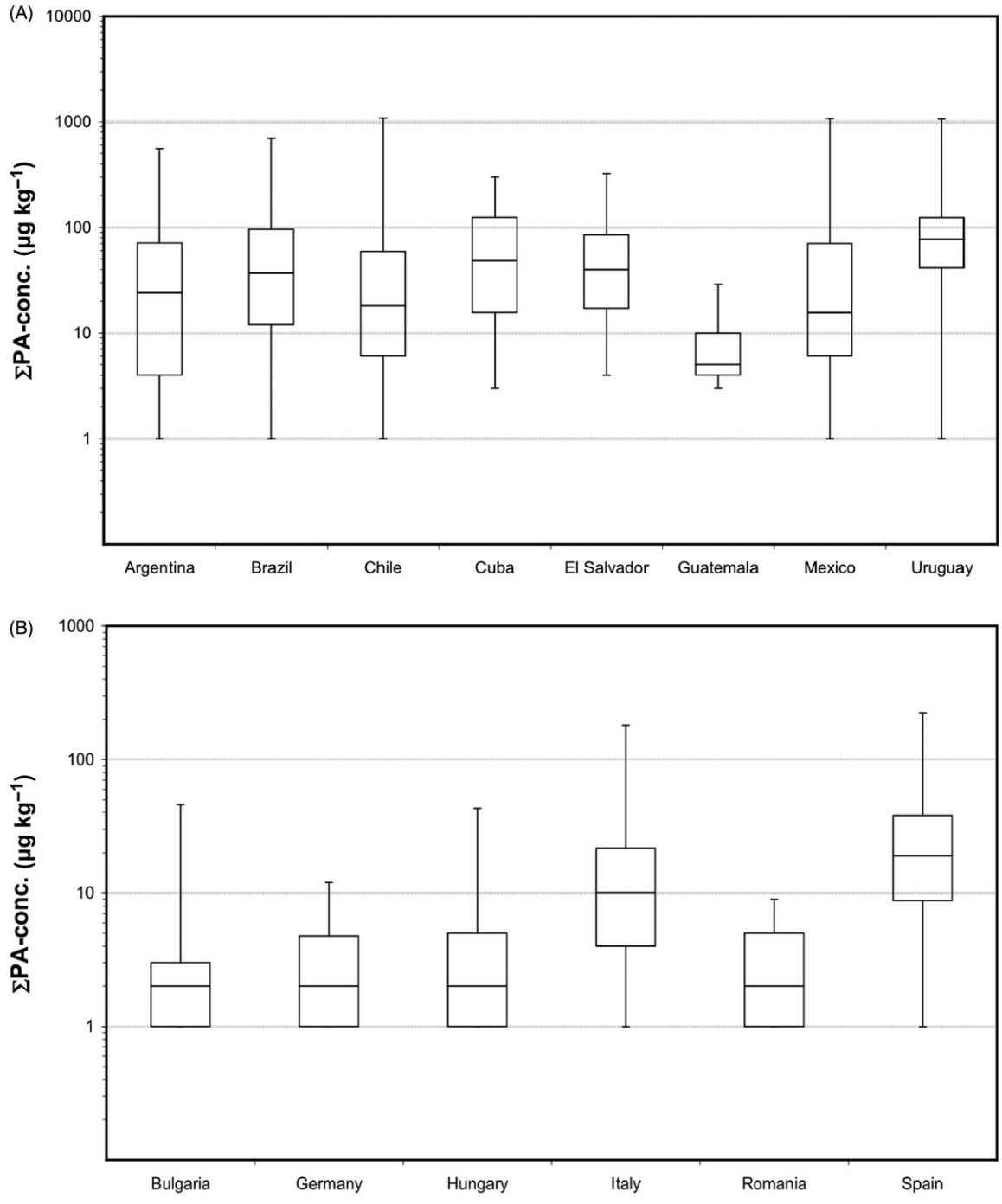

Figure 3. (A) Distribution of ΣPAs concentrations in Central and South American raw honeys. Whiskers represent the maximum and minimum values found in PA-positive samples. The range of ΣPAs' concentration covers three orders of magnitude except for Guatemalan samples. Apart from samples from Cuban and Uruguayan, 75% of the PA-positive samples of each country were below 100 μg kg^{-1} ΣPAs. (B) ΣPAs' concentrations in raw honeys from European countries. Italian and Spanish honeys contain considerably more ΣPAs than the samples from the other European countries. Concentrations are generally much lower compared with honeys from Central and South America.

In order to gain information about the contribution of each group of PAs to the ΣPAs in honey, the results were plotted as a ternary plot (Figure 4). Each point shows the relative fractions of PAs of the different groups in a sample. Honeys from Argentina show a great variation in composition. Most samples were mostly comprised of groups 2 and 3 PAs and, to a much lesser extent, group 1 PAs. A number of samples lack group 1 PAs totally, while others lack group 3 PAs (Figure 4A). Thus, for Argentina *Senecio* and *Echium* species show the greatest influence on PA pattern and *Eupatorium* species mostly played a minor role. Samples from Uruguay are usually a mixture of all three groups of PAs, though some samples lack group 1 PAs while others in turn contain mostly PAs of group 1, with only a low fraction of group 2 PAs and no group 3 PAs contributing to the ΣPAs (data not shown). Compared with Argentina, *Senecio* species seem to contribute less to the ΣPAs, while *Echium* and *Eupatorium* species show a greater impact.

Interestingly, PAs of group 2 only contributed a minor fraction to the ΣPAs of samples from Brazil and

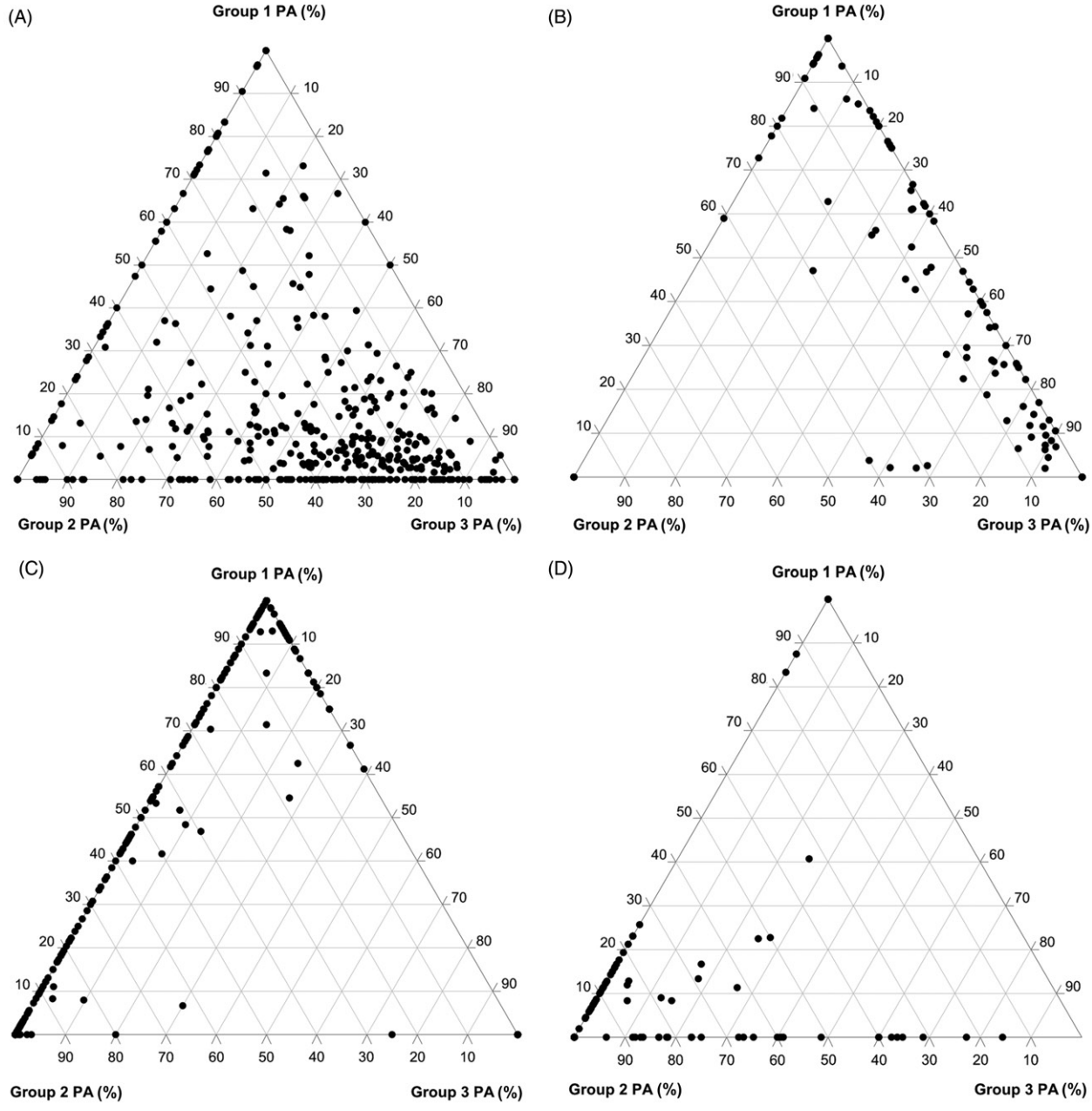

Figure 4. Contribution of the different groups of PAs to the ΣPAs in raw honeys. (A) Argentina: PAs of group 3 (typical for *Senecio* sp.), followed by PAs of group 2 (typical for *Echium* sp.) are dominant. PAs of group 1 (typical for *Eupatorium* sp.) play only a minor role. (B) Brazil: PAs of group 2 were generally low or absent. PAs of group 1 and 3 are dominant. (C) Chile: PAs of group 3 were generally low or absent. PAs of group 1 and 2 are dominant. (D) Spain: PAs of group 2 are dominant.

some totally lack group 2 PAs (Figure 4B). Thus, *Echium* species do not influence the PA pattern in Brazilian honeys strongly. This could be verified by pollen analysis, as only a low amount of Echium pollen could be found in Brazilian samples. The PA pool comprised mostly groups 1 and 3 PAs, which points to a strong influence of *Senecio* and *Eupatorium* species. As the climate in Brazil is mostly tropical, other plants, e.g. *Chromolaena odorata*, might have contributed lycopsamine or an isomer and thus influence the PA pattern. Thus, it is likely that plants belonging to other genera than *Eupatorium* play a role in terms of PA contribution to Brazilian honey.

Contrary to Brazil, group 3 PAs typical for *Senecio* species play only a minor role in honeys from Chile, while the PA pool consists of groups 1 and 2 PAs typical for *Eupatorium* and *Echium* species (Figure 4C).

In samples from Argentina, Chile and Uruguay, *Echium* pollen were found, which is consistent with the detection of PAs of group 2.

European Community honeys

Altogether 381 raw honey samples from Bulgaria, Germany, Hungary, Italy, Romania and Spain were analysed, of which 65% were tested PA positive. The ΣPAs' concentrations ranged from 1 to 225 $\mu g\,kg^{-1}$. The average PA concentration of the PA-positive samples was 26 $\mu g\,kg^{-1}$ (median = 12 $\mu g\,kg^{-1}$) and 17 $\mu g\,kg^{-1}$ including the PA-negative honeys.

Compared with honeys from Central and South America, honeys from European Community countries mostly contained a lower amount of ΣPAs as well as a lower percentage of PA-positive samples. Only in samples from Italy and Spain were PAs equally frequent as in Central and South American honeys, but the average ΣPAs' concentration (only PA-positive samples) was only about half as high (Italy = 25 $\mu g\,kg^{-1}$, Spain = 34 $\mu g\,kg^{-1}$; Figure 2).

Usually more than 75% of the PA-positive samples of each country contained less than 10 $\mu g\,kg^{-1}$ ΣPAs. Only in samples from Italy and Spain were higher concentrations of up to 225 $\mu g\,kg^{-1}$ found (Figure 3B). The PA pattern reveals a strong contribution of PAs of group 2 (*Echium* species) to the PA pool and a much lesser contribution of PAs of groups 1 and 3 (Figure 4D). The situation in honeys from Italy is very similar to that in Spanish honeys, which points to a comparable botanical setting in terms of PA plants in both countries. The presence of *Echium* pollen in Italian and Spanish samples was verified by pollen analysis.

Honeys from Bulgaria, Germany and Romania only contained minor amounts of PAs of groups 2 and 3 (1–43 $\mu g\,kg^{-1}$). Interestingly, the only PAs of group 3 found was senkirkine (always below 10 $\mu g\,kg^{-1}$). All other PAs typical for *Senecio* species were absent. In Hungarian samples, PAs of group 1 were also found, but also only in minor amounts (average = 6 $\mu g\,kg^{-1}$).

Pollen

Bee pollen is traded to a much lesser extend compared with honey and only 119 bee pollen samples were available for analysis. The origin of the samples was taken either from the label or determined by pollen analysis as was done for the honey samples. For most of the samples PAs of group 1 were not quantified, as at the time of analysis no reference material was available. However, 60% of the bee pollen samples were PA positive with ΣPAs' concentrations ranging from 11 to 37,855 $\mu g\,kg^{-1}$. The concentrations are in agreement with those found by Kempf, Reinhard et al. (2010). Average concentrations of the PA-positive samples were 1846 $\mu g\,kg^{-1}$ and the median was at 192 $\mu g\,kg^{-1}$.

The distribution of concentrations within PA-positive samples irrespective of their origin is shown as a kernel-density plot in Figure 5. The average ΣPAs' concentration of the PA-positive bee pollen of 1846 $\mu g\,kg^{-1}$ exceeds the average ΣPAs' concentration in raw honey about 30-fold.

As PAs of group 1, which are common in honey, were mostly not analysed, this value is likely to be higher. In Vietnamese honey, PAs of group 1 were often found (QSI unpublished), thus it can be assumed to be present in bee pollen from that country as well. Indeed, for the only two samples from Vietnam that were also analysed for PAs of group 1, concentrations of up to 37,855 $\mu g\,kg^{-1}$ were found.

Pollen analysis of the sample with the highest concentration of PAs of group 1 revealed that 67% comprised of *Eupatorium*-type pollen, which is not unlikely as *Eupatorium* is known to contain PAs of group 1 (Hartmann and Witte 1995). However, pollen of *Chromolaena odorata* look very similar and are difficult to distinguish from pollen of *Eupatorium* species. As *C. odorata* is also well known to occur in Vietnam, it could contribute PAs to bee pollen as well.

For most of the analysed samples, group 2 PAs were found, which are typical for *Echium* species. As with honey, the PA concentrations in samples from South-East Europe show usually low ΣPAs' concentrations and those from Spain much higher concentrations.

Due to the high ΣPAs' concentrations in bee pollen, which are about one to two orders of magnitude higher than in honey, it is likely that bee pollen contributes a substantial amount of PAs to honey (Boppré et al. 2005; Kempf, Wittig et al. 2010).

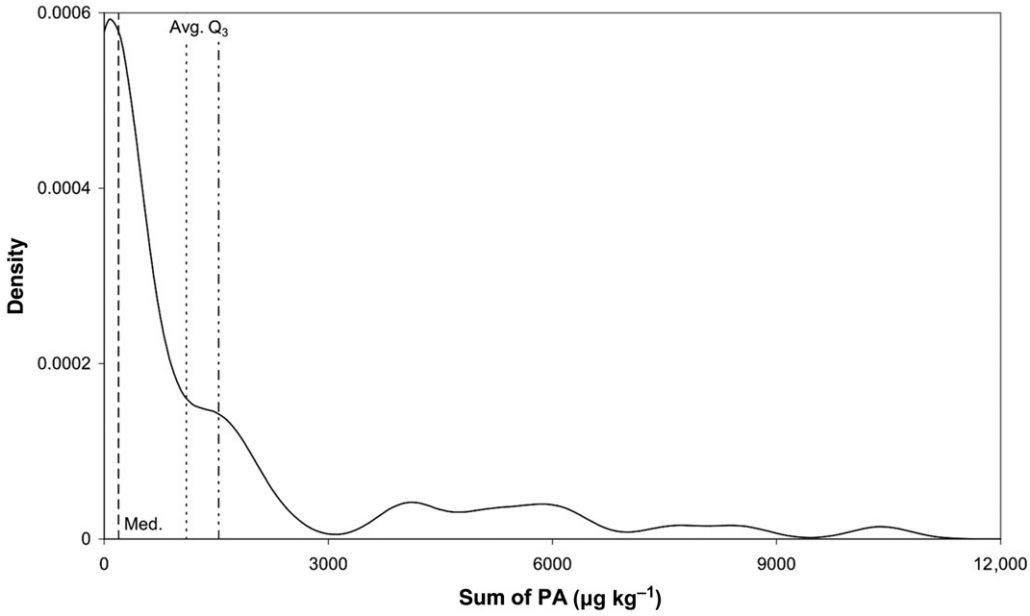

Figure 5. Kernel-density plot of the abundances of PA concentrations (kernel width = 400) of the ΣPAs (sum of PAs) in pollen collected by honeybees. The median concentration (Med.) of the PA-positive samples was 192 µg kg^{-1} and the average concentration (Avg.) was 1846 µg kg^{-1}. Q_3 is the third quartile.

PA-N-oxides (PANOs)

PAs are usually produced in the roots and/or shoots of plants. Thus, the PAs must be transported through the plant to other plant parts. As the solubility of PANOs in water is higher than that of the tertiary PAs, PAs in plants are usually synthesised as or converted to PANOs. For other plant parts, e.g. seeds, the ratio of PA/PANO can be higher than in other plant parts, as seeds are lipophilic and the tertiary PAs are better suited for storage in such environments (Hartmann and Witte 1995).

The average concentrations of the tertiary PAs in bee pollen are one- to two-fold higher than those of their N-oxides (data not shown). The abundances of PAs and PANOs in bee pollen are roughly the same. Boppré et al. (2008) found 3.5–4.4 times more PANOs than tertiary PAs in *Senecio ovatus* and approximately four to ten times more PANOs than tertiary PAs in bee pollen from *Echium plantagineum*. They were also able to show that slight heating can reduce the amount of PANOs, thus changing the ratio of PA/PANO. For their analyses they used bee pollen taken directly from the honeybees, while in this study the bee pollen samples were usually several weeks or months old and probably stored under varying conditions in terms of temperature. Those factors might have reduced the amount of PANOs in the samples used in this study.

In honey the difference is even greater. The abundance of tertiary alkaloids is usually four- to 17-fold higher than those of the corresponding PANOs. The average concentrations of PAs and PANOs in positive samples are roughly the same for echimidine/-N-oxide, acetylechimidine/-N-oxide and echiumine/-N-oxide, but for echiuplatine and echivulgarine concentrations of the tertiary alkaloids are about three-fold higher than those of their N-oxides, which is comparable with the results of Betteridge et al. (2005). However, the abundance of lycopsamine is only 1.6-fold higher compared with its N-oxide and the average concentration of the N-oxide is about three-fold higher than that of the tertiary alkaloid (Figure 6).

Generally, the ratio of PAs/PANOs changes on the way from plant to honey in the following order: plant < bee pollen < honey. However, we encountered a small number of samples (mostly *Echium*-derived honeys) with higher concentrations of PANO than of the corresponding tertiary PAs.

Conclusions

A total of 94% of the retail honeys contain PAs, but in 88% of the samples the concentrations were below 50 µg kg^{-1} (including samples below the LOQ). Thus, consumption of one hotel serving of honey (20 g) would still meet the limit for phytopharmaceuticals of 1 µg PAs day^{-1} (if not consumed for more than 6 weeks). The effect of blending is apparent when comparing PA patterns, concentrations and abundances in raw and retail honey.

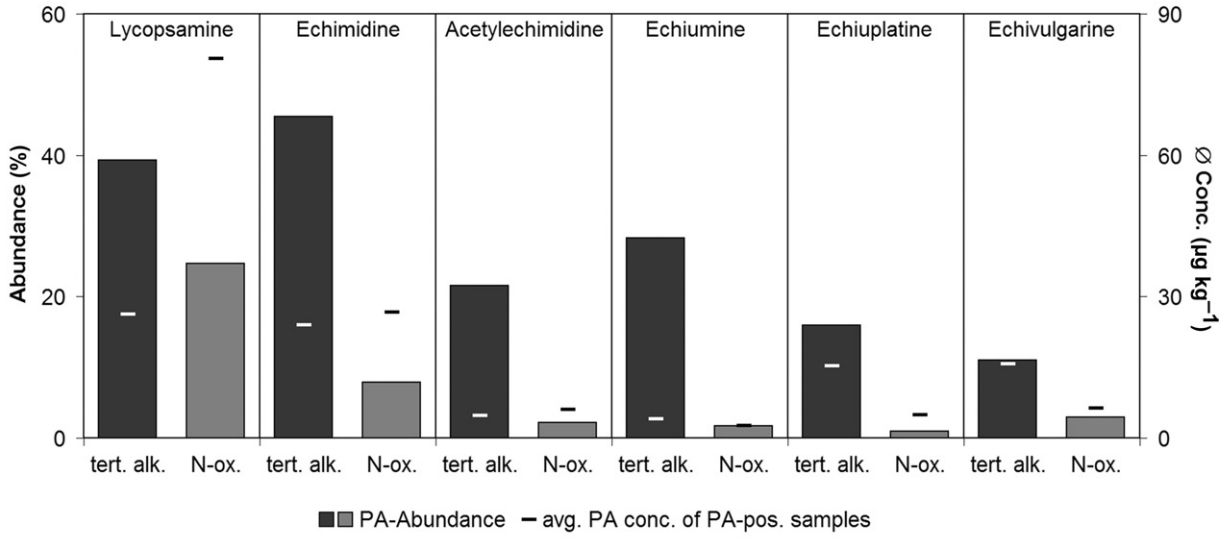

Figure 6. Abundance of a range of PAs and their corresponding N-oxides and the average concentration of PA/-N-oxide-positive honey samples. The tertiary alkaloids are more abundant in honey than their N-oxides, but the average concentrations of the PAs are roughly at the same level or slightly higher than the concentrations of the corresponding N-oxides. Only for lycopsamine is the situation is different: the abundance of the tertiary alkaloid is just slightly higher than that of its N-oxide, but the average concentration is only about one-third compared with its N-oxide.

Raw honeys (bulk honey not yet packaged in containers for sale in retail outlets) may differ substantially in PA pattern, concentrations and abundances. While the PA pattern of raw honeys from Central American countries is very similar, PA patterns in South American raw honeys show great differences due to available plants in the vicinity of the beehives, as could be indirectly verified for *Echium* species by relative pollen analysis (Louveaux et al. 1970).

European raw honeys generally contain much lower amounts of PAs and mostly PAs of group 2. Only Italian and Spanish honeys show higher amounts of PAs mostly of group 2, which are typical for *Echium* species. The presence of *Echium* pollen in samples with high concentrations of PAs of group 2 was verified by pollen analysis.

The amounts of PAs found in some bee pollen samples could lead to negative health effects when these bee pollens are consumed, as consumption of only one teaspoon of bee pollen (about 5 g) may contain up to 189 µg of PAs, which is far beyond the existing German limit of 1 µg day^{-1} for the consumption of phytopharmaceuticals for not more than 6 weeks. Nevertheless, 40% of the bee pollen samples were PA negative.

Still, it needs to be kept in mind that only a limited number of PAs were analysed. Thus, there is the possibility that not all PAs present in honey and bee pollen were detected by using this target analysis. The results thus can be regarded as preliminary values, which are likely to increase with the number of PAs included in the target analysis.

If beekeepers avoid placing beehives in areas abundant in the above-mentioned plant genera as much as possible, a substantial reduction of the PA concentrations could be achieved. However, there are other PA-containing plants that may also contribute to the PA pool in honey and which were not considered in this study.

The more that PAs are included in the analysis, the better the potential source plant can be identified. This study is just a first step in identifying plants contributing PAs to honey and bee pollen and it can serve as a basis for further studies including a wider range of PAs.

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