REPORT

Pyrrolizidine alkaloids (PAs) in honey and pollen-legal regulation of PA levels in food and animal feed required

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Pyrrolizidine alkaloids (PAs) are secondary plant constituents that comprise about 400 different structures and occur in two major forms, a tertiary form and the corresponding *N*-oxide. PAs containing a 1,2-double bond are pre-toxins and metabolically activated by the action of hepatic P-450 enzymes to toxic pyrroles. Besides the acute toxic effects, the genotoxic and tumorigenicity potential of PAs was demonstrated in some eukaryotic model systems. Recently, the potential PA contamination of food and feeding stuff attracted recurrent great deals of attention. Humans are exposed to these toxins by consumption of herbal medicine, herbal teas, dietary supplements or food containing PA plant material. In numerous studies the potential threat to human health by PAs is stated. In pharmaceuticals, the use of these plants is regulated. Considering the PA concentrations observed especially in authentic honey from PA producing plants and pollen products, the results provoke an international regulation of PAs in food.

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1 Introduction

1.1 Pyrrolizidine alkaloid occurrence

Pyrrolizidine alkaloids (PAs) are constitutively expressed toxic secondary plant defense compounds. They encompass about 400 structures isolated from more than 560 plant species [1]. Despite their rich diversity they all share a common theme; *i. e.* they are ester alkaloids composed of a necine base esterified to one or more necic acids. Considering chemosystematics and biogenetical analysis, almost all PAs can be classified into five different major structural types (Fig. 1) [1, 2].

The occurrence of PAs is restricted to the angiosperms and is mainly limited to only four plant families: the Asteraceae (Senecioneae and Eupatorieae), the Boraginaceae, the Apocynaceae and the genus *Crotalaria* within the Fabaceae [1].

The majority of known PAs can be attributed either to the senecionine-type or the lycopsamine-type. The macrocyclic diesters of the senecionine type are primary found in Senecioneae (Asteraceae). In common is the complex C_{10}-necic acid, comprising two C_5-compounds (derived from the amino acids isoleucine and, less frequently, from leucine). Furthermore, this structural type can be biosynthetically modified to the otonecine-type-PAs (Fig. 2).

Related is the group of triangularine-PAs with their open-chained C_5-acids diester structures. Triangularine-PAs appear in many species of the Boraginaceae and *Senecio*, but never exist in parallel with senecionine-type-PAs.

PAs of the monocrotaline-type consist of an 11-membered macrocyclic diester structure. Their occurrence is limited to the genus *Crotalaria* and a few species within the Boraginaceae. The lycopsamine-type-PAs represent another very diverse group of mono- and diester PAs. In this case, the necine base is esterified at C-9 with a C_7-necic acid derived from 2-isopropylbutyric acid. PAs of the lycopsamine-type are basically found in Eupatorieae (Asteraceae), the Boraginaceae and the Apocynaceae.

The major part of the known PAs belongs to these above-mentioned structural types and frequently shares two important things in common: (i) the necine base is often constituted by the 1,2-unsaturated retronecine and (ii)

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Abbreviations: COC, Committee on the Carcinogenicity of Chemicals in Food, Consumer Products and the Environment; **COT,** Committee on Toxicity; **EFSA,** European Food Safety Authority; **MOE,** margin of exposure; **PAs,** pyrrolizidine alkaloids; **PANOs,** PA-*N*-oxides

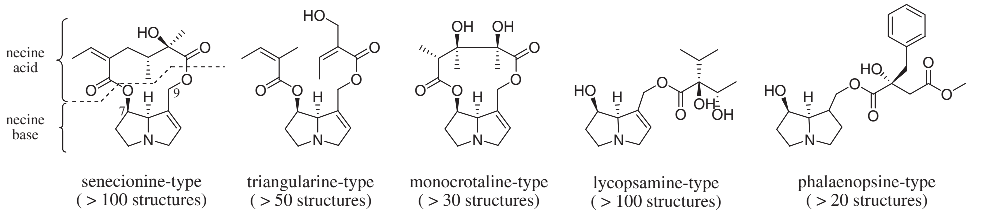

Figure 1. Major structural types of naturally occurring plant PAs.

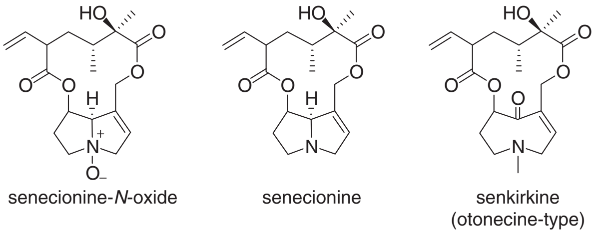

Figure 2. PANO and otonecine-type-PA using the example of senecionine.

within the plants, PAs are mainly present in the form of the corresponding *N*-oxides (Fig. 2).

Besides the already mentioned facts, PAs additionally occur sporadically in other plant families such as the structural related phalaenopsine-type-PAs, which are assembled with a saturated necine backbone structure [1].

In the Asteraceae (*Senecio* and *Eupatorium*) senecionine-type-PAs are synthesized in roots, distributed through the phloem throughout the plant and diversified by peripheral enzymatic reactions resulting in the species-specific alkaloid pattern. Within the Boraginaceae the biosynthetic origin can differ from species to species [3, 4].

For PAs occurring in Asteraceae, Boraginaceae and the Fabaceae it is proven that the primary synthesized tertiary PAs are mostly converted to their corresponding *N*-oxides. Once synthesized the PAs only undergo biosynthetic diversification, but no turnover was observed [5]. Storage and accumulation in the specific plant organs was ascertained [6]. In *Senecio*, PAs were preferentially accumulated in the inflorescence [7] and are stored within cellular vacuoles [8].

1.2 Toxicity

In correlation to structural features 1,2-unsaturated PAs show a more or less pronounced toxicity. One generally needs to distinguish between acute and chronic toxicity and genotoxicity.

A prerequisite for PA toxicity is the existence of a carbon–carbon double bond in 1,2-position of the necine base. Furthermore, an ester functionality in position C-7 or C-9 or both positions is required. If these requirements are met, these PA structures can undergo biotransformation, mainly by liver cytochrome P-450 monooxygenases, into the corresponding dehydropyrrolizidinalkaloides (pyrroles). These pyrroles are highly electrophilic compounds that readily and irreversibly react with nucleophilic cell components such as proteins, DNA or amino acids and might cause liver damage; but transport and consequently damage of other body tissues is possible as well [9].

The extent of the observed toxicity depends fundamentally on the structure and the resulting metabolic pathways and detoxification rates as well as can vary greatly from organism to organism [9]. Thus, differences in acute toxicity could be correlated with the esterification of both hydroxyl functionalities of the necine base and the additional presence of an α,β-double bond in the acid substructure of the PA ester [10]. PA expositions over longer periods of time are mainly known to damage liver, lung or blood vessels or to a lesser extend kidney, gastrointestinal tract, pancreas and bone marrow. This may become manifest in venous occlusions in liver and lung, megalocytosis, inhibition of cell division (mitosis) and liver cirrhosis [10–12].

In addition, following metabolic activation, PAs show a number of genotoxic effects. These include DNA binding and DNA cross linking, mutagenicity, teratogenicity and carcinogenicity [12].

More detailed information on toxicological aspects of PAs is available and it is referred to already published review articles (references [9, 11]). The classic work on acute toxicity and structure-activity relationships of PAs in the mammal model (rat) was published by Culvenor *et al.* [13]; PA genotoxicity studies are represented by the work of Fu *et al.* [12] and Frei *et al.* [14].

2 PAs in honey and pollen loads

2.1 Risk exposure for humans

A well-known major threat is the direct use of PA containing remedies in traditional medicine [15].

In the past, secondary contamination of food with PAs was repeatedly observed. In foods of animal origin, the PAs are passed on through the feed and the host organism into food for human consumption, such as eggs [16] or milk [17]. Severe intoxications were reported on several occasions for grain contaminated with PA plants seeds or parts of PA plants [9]. Recently, PAs were repeatedly detected in packed lettuce boxes (*Eruca sativa*, arugula). The contamination could be traced back to the presence of accompanying *Senecio vulgaris* plant parts. In August 2009, the discovery led to short-term ban of arugula by a German supermarket chain (http://www.bfr.bund.de/cm/208/salatmischung_mit_pyrrolizidinalkaloid_haltigem_geiskraut_verunreinigt.pdf; last accessed 07.08.2009; http://www.zeit.de/newsticker/2009/8/12/HAUPTSTORY-RUCOLA-MI22069532xml; last accessed 23.08.2009) [18, 19].

2.2 Honey

Reports of honeys containing PAs were first published in the late 70s and early 80s; affected was honey from *Senecio jacobaea* (Asteraceae) in the United States and *Echium* honey in Australia (*Echium plantagineum*, Boraginaceae) [20, 21]. According to a review article that deals with PA occurrence in honey, there are a number of PA plants, well documented by pollen analysis, that serve as nectar supply for honey bees, although a direct evidence of PAs by analytical means was not confirmed at this point [22]. According to ref. [22], the most common representative plants are *Echium* spp., *Senecio* spp., *Eupatorium* spp. and *Borago* spp. So far, there is no direct evidence whether the nectar of PA plants contains PAs in general or only in some cases, but it can be assumed due to many observed PA positive honeys that nectar of PA plants will contain PAs [20, 23]. In the course of the first evidence on PAs in honey, several isolated cases and small studies, mostly related to specialty honeys or based on locations with increased PA plant occurrence, were reported. Since 2002, an increased concern on the presence of PAs in the food chain is observed, and studies with larger sample numbers and a less stringent sample selection were conducted to gain insight into the general market situation. The results of all reports and studies are summarized in Table 1 and categorized into number of samples, the plant of origin, ratio of PA positive samples and average and maximum PA levels.

The majority of the data summarized in Table 1 refers to analyses for honeys that were suspicious because of available background information or that were derived from test hives placed in locations with known PA plant occurrence and it is therefore ineligible to assess an average PA level for retail honey. In terms of an overall market situation only two similar designed studies have been published so far: on the one hand from the food control authority of The Netherlands [30] and on the other hand a recently published study by Kempf *et al.* [32]. Both studies have in common that no specifically suspicious honeys from regions with a high proportion of PA plants or honeys with high PA pollen levels were analyzed, but a large number of randomly selected retail samples. Both studies may therefore reflect the market situation of Western European countries with a high honey import ratio.

Both studies, however, differ fundamentally in the methods used. While in Kempf *et al.* [32] the toxicologically relevant 1,2-unsaturated PAs and their *N*-oxides were converted to a single sum parameter and showed an LOQ of 0.01 ppm, the Dutch applied LC-MS and target analysis. The LC-MS approach was more sensitive (LOQ: 0.001 ppm) but, due to the lack of available reference PAs, the study was limited on the detection and quantification of eight PAs and three PA-*N*-oxides (PANOs) (mainly PAs from *Senecio* spp.). Essentially, this results in some differences in the assessment of the corresponding data sets. As to Kempf *et al.* [32] 9% of the honey samples were PA positive ($n = 216$; GC-MS) compared with 28% ($n = 170$, LC-MS) in the (Dutch study (http://www.vwa.nl/cdlpub/servlet/CDLServlet?p_file_id = 22703; last accessed 07.10.2009) [30]. The PA content ranged from 0.041 to 0.259 μg/g and the average content was approximately 0.1 μg/g (calculated as senecionine equivalents). In contrast, the Dutch study showed 0.002–0.365 μg PAs *per* gram of honey and an average content of 0.007 μg/g can be calculated.

Taking into account only those honey samples that are within the range of the higher LOQ of the GC-MS study conducted by Kempf *et al.* [32], it follows a more unified picture. Consequently, 11.7% of the honey samples of the Dutch study would be tested positive and the average PA content would be 0.05 μg/g. Considering that only 11 PA structures could be quantified, it can be assumed that the resulting average PA content should be calculated as too low. From this point of view, both studies show very similar tendencies: approximately 10% of the honeys on the German, respectively the Dutch market contain PAs with an average of 0.05–0.1 μg/g. In contrast to other studies, Kempf *et al.* [32] correlated some PA levels with corresponding mellisopalynological data. In all PA positive honey samples studied so far pollen from PA plants were detected as well. In the latter study, these pollen were mainly originating from *Echium* spp. and, to a much lesser extent, from *Eupatorium* spp. So far, the relative pollen frequency (according to DIN 10760) cannot be correlated to the PA levels found in honey samples. Whether the determination of an absolute pollen frequency is a promising alternative remains to be seen.

Furthermore, among the 216 samples investigated by Kempf *et al.* [32] the origin of the honeys is covered as well using the label and/or pollen analysis. In this case, the largest proportion (94 honeys, including 46 from Germany) was of European origin, followed by honeys from "European countries and non-European countries" (see Fig. 3). While in the first sample set no PA positive samples were detected, the second set showed a disproportionate frequency of PA positive samples (20%, compared with about 10% on average for all samples).

One possible reason behind this is most likely to be found in the German/European agricultural landscape and the fact that PA plants are only rarely used economically; thus the probability of PA transfer into honey is minimized. However, in Southern Europe (*e.g.* Spain) the occurrence of *Echium* spp. is documented and in certain regions *Echium* spp. is probably strongly represented [29, 33]. However,

Table 1. Summary of literature on PAs in honey

Number of samples	PA plants	PA positive samples (%)	Average/ maximum levels of PAs (μg/g)	Characteristics	Method	Literature
4	*S. jacobaea*	100	3.4/3.9	-	GC-MS	[20]
5	*E. plantagineum*	100	0.58/0.95	-	GC-MS	[21]
13	*S. jacobaea*[a]	39	0.02/0.06	Honey of a test hives next to S. *jacobaea*	LC-MS	http://archive.food.gov.uk/maff/archive/food/infsheet/1995/no52/52honey.htm; last accessed 12.10.2009 [24, 25]
8	*S. jacobaea*	100	< 0.002	Honey with unknown origin; from local beekeepers		
2	*S. jacobaea*	100	0.95/1.5	Honey with suspicion of *S. jacobaea* content, assigned due to organoleptic properties		
13	*E. plantagineum*	100	0.91/2.63	-	LC-MS	[26, 27]
9	*E. plantagineum*-Mix	100	0.27/1.03	-		
4	*Heliotropium amplexicaule*	100	0.81/1.65	-		
2	*Heliotropium europaeum*	100	0.19/0.25	-		
35	Unknown	46	0.13/0.81	Assigned to non-PA producing plants		
5	*E. vulgare, Echium lucida*, unknown	80	0.39/1.3	Retail honeys		
9	*E. vulgare*	78	0.79/2.85	Five honeys assigned to *E. vulgare*	LC-MS	[28]
127	*Echium* spp.	17	n.d.	-	Derivatization; spectro-photometric	[29]
171	*S. jacobaea*	25	0.007/0.36	Retail honeys (mainly analyzed for *S.jacobaea* PAs)	LC-MS	[30]
16	*S. jacobaea,*	75–88[b]	n.d.	Honeys from six test hives next to *S. jacobaea* and *B. officinalis*	LC-MS	[31]
16	*B. officinalis*	56[c]				
216	Unknown	9	0.056/0.12	Retail honeys	GC-MS	[32]

n.d. not determined.

a) Only one sample out of three locations was PA positive.

b) Only trace amounts of seneciphylline and senecionine were detected.

c) Only lycopsamine or intermedine was detected.

this seems not to be reflected in the 94 analyzed European honey samples, but it can most likely be explained by import statistics. For the Germany market, only a small amount of honey is imported from southern Europe, while the dominating import regions are South America and Eastern Europe (http://www.warenverein.de/pdf/wv_jb_2008_web.pdf; last accessed 20.10.2009) [34].

2.3 PAs in pollen

Recent results on PA content of floral pollen, on pollen loads from bees and the potential changes of PA content during pollen drying and storage are available [35]. Drying of pollen loads, as it is carried out for commercial products, had little influence on the PA content. The PA content of pollen loads are significantly lower than PA levels detected in floral

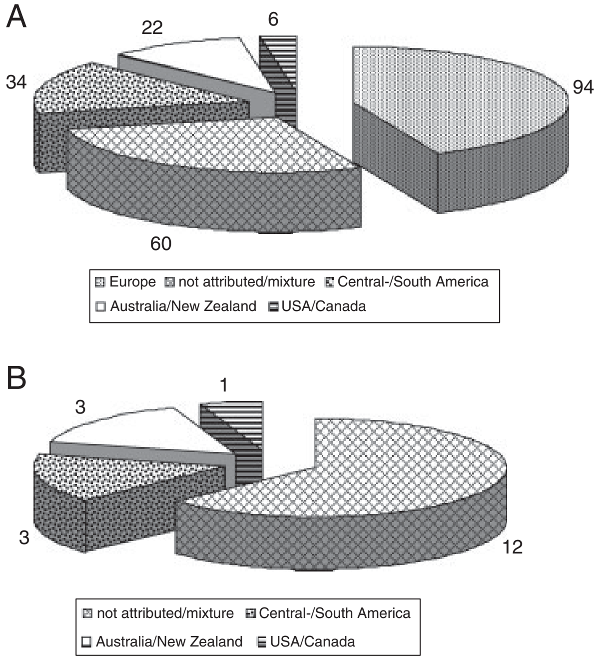

Figure 3. Geographical distribution of the analyzed honey samples (A) and geographical distribution of the PA positive honeys (B) according to Kempf *et al.* (reproduced with permission) [32].

Table 2. PA content of floral pollen and pollen loads

PA plant	PA amount (mg/g)	Literature/ method
S. jacobaea[a]	0.175[b]	[38]/GC-MS
E. vulgare[a]	8.2/14.0[c]	[36]/LC-MS
E. vulgare[d]	0.35±0.05[c]	[35]/LC-MS
Eupatorium cannabinum[d]	0.120±0.02[c]	
S. jacobaea[a]	0.8[c]	
S. jacobaea[d]	0.1[c]	
Senecio ovatus[a]	0.155±0.02[c]	
S. ovatus[d]	0.07±0.02[c]	
E. plantagineum[d]	0.028±0.015[c] 0.006±0.001[c]	
S. vernalis[a]	4.1	[37]/GC-MS
S. jacobaea[a]	3.3	
E. cannabinum[a]	0.6	
E. vulgare[a]	0.9	
Phalaenopsis hybrid[a) e)]	0.6	

a) Floral pollen.
b) Senecionine-equivalents (estimated).
c) Lasiocarpine-equivalents.
d) Pollen loads.
e) Only 1,2-saturated PAs.

pollen (8–14 mg/g for *Echium vulgare* pollen; [36]). Our own studies have demonstrated that, with exception of *Crotalaria* spp., the pollen of all relevant PA plant families contained PAs.

The currently available data on PAs in floral pollen and pollen loads are summarized in Table 2. In addition, pollen from *Borago officinalis* did not show significant amounts of PAs (own data, not published). With the exception of *Phalaenopsis*, 70–95% of the PAs are present in form of their corresponding PANOs [37]. All data concerning PAs in pollen showed that, in comparison to honey, pollen exhibited much higher PA concentrations (see Tables 1 and 2; µg/g for honey *versus* mg/g for pollen).

Due to the high PA content of pollen the origin of PAs in honey is still not yet resolved definitely. Considering a theoretical PA content of 0.05–0.1 µg *per* gram honey, which was found as the average of the PA positive honeys in the two larger studies [30, 32], this would correspond to 10–100 µg PA plant pollen *per* gram of honey. "But to what extent PAs from pollen add to the total PA content or how much PAs of pollen migrates into the honey matrix during storage or processing still remains to be seen." In addition, there are no data available on natural PA concentrations of nectar. It is likely that each of these possible PA sources, depending on the corresponding nectar/pollen plant, contributes to the final PA content of honey. Based on previously found PA concentrations in pollen it can be expected that pollen products intended for human consumption will show higher PA levels than honey. Recently, this was demonstrated by a survey of 55 commercial pollen products. Seventeen samples were PA positive (31%) and the average PA content was 5.2 µg/g (retronecine equivalents, [37]).

It is noteworthy that, in direct comparison to the analysis of 216 honey samples, the PA positive pollen products showed both, a higher frequency of occurrence (31 *versus* 9%), and a much higher average PA content (5.2 µg/g compared with 0.056 µg/g retronecine equivalents) [32, 37]. On the other hand, the pollen analysis of PA positive samples of both, honey and pollen, matched in most cases. According to that the dominating presence of *Echium* spp. pollen is an indicator for the most common responsible PA plant [32, 37].

2.4 PAs and honey bees

The knowledge about PA occurrence in honey raises the question whether PAs may affect the development of the honey bee and/or their nectar collecting behavior. The elucidation of these relationships could be included in beekeeping practice, thus being a preventive measure to reduce PA contamination of honey. The results of the first experiments were published recently [39].

In laboratory feeding experiments a mix of tertiary 1,2-unsaturated PAs isolated from *Senecio vernalis* was compared with results obtained for the corresponding PANOs. In a second test series, pure monocrotaline was matched against the data obtained for the corresponding 1,2-saturated PA (1,2-dihydromonocrotaline).

The tests were evaluated with respect to food consumption and bee mortality in relation to the PA concentration of the food (0, 0.02, 0.2 and 2% PAs in 50% sucrose solution). A deterrent effect of PANOs was demonstrated for concentrations $>0.2\%$, but increasing PANO concentrations were not accompanied with an increase of mortality. However, in the test series with tertiary PA mix from *S. vernalis* and monocrotaline no deterrent effect was detected, but a significant increase of mortality induced by the ingestion of tertiary PAs was observed. As expected, the synthesized 1,2-saturated PA (1,2-dihydromonocrotaline) did not show toxic effects on the honey bee.

Further approaches have shown that bees are not able to detoxify tertiary PAs *via N*-oxidation, as it is was demonstrated in the past for some specialized PA adapted insects [40]. On the other hand, up to 70% of the ingested PANOs were metabolized by bees into the corresponding tertiary PAs (representing a toxification of non-toxic PANOs).

Overall, adult honey bees have the ability to recognize PANOs and avoid PANO containing food (the dominant PA form in PA plant flowers). This deterrent effect, however, occurs only at concentrations above 0.2%. Consequently, it is easy to imagine that given an attractive nectar or pollen supply by a PA plant, bees will most likely not discriminate this nectar/pollen based on the PA content. In addition, under laboratory conditions, adult worker bees were relatively tolerant towards 1,2-unsaturated tertiary PAs (toxic PA form) in their diet; PA amounts of up to 50 μg *per* individual bee showed no negative effects.

Trophallaxis experiments were conducted to verify the hypothesis whether ingested PAs are passed on from bee to bee. These model experiments showed that up to 15% of the ingested PAs can be exchanged and transferred from bee to bee [39].

These experiments confirm the causal chain: starting with PA uptake, transport and transfer into the final product honey or pollen. Therefore, the naturally assumed PA concentrations in floral pollen or nectar are most likely no selection criterion for honey bees. Thus, a prosperous strategy to prevent the PAs intake into honey/pollen products would be a careful selection of the locations by bee keepers to place the bee hives, together with the knowledge of PA plants that are attractive to honey bees.

3 Analytical methods

There is a multitude of existing methods to analyze PAs. Due to the mandatory sensitivity of less than 1 ppm, only MS-based methods come to consideration. So far, two different strategies are established. One is the detection of individual PAs using LC-MS [28], while the other method is based on the conversion of the toxicologically relevant 1,2-unsaturated PAs into a common sum parameter and subsequent GC-MS detection [32]. For honey samples, in any case, it is necessary to concentrate the PAs by SPE on strong cation exchange materials [26, 32] prior to the analysis.

In general, the LC-MS method is more sensitive (detection limit approx. 1 ppb) and allows the simultaneous detection of tertiary PAs and PANOs. However, a serious problem of this approach is the limited availability of commercial reference PAs and especially PANOs, as well as the subsequent drawbacks in method development (especially MS-MS methods) and quantification. Therefore, all PA analytes, where no reference is available, have to be addressed with surrogates [28]. Furthermore, unknown PAs and samples with unknown PA background, respectively, can only be processed incompletely, since these PAs usually remain undiscovered in a target analysis approach. In addition, and in particular, true for LC-MS, matrix ionization effects varying in an unpredictable manner on a day-to-day and sample-to-sample basis can vastly increase the uncertainty in the results.

A very recent review has focused on current methods for PA extraction in plants and foodstuff, sample cleanup and subsequent determination mainly by means of LC-MS [41]. There, the major drawback that is associated with the LC-MS approach of PA analysis, *i. e.* the lack of an adequate supply of PA standards, is also discussed in detail.

The analysis *via* sum parameter and GC-MS detection facilitates the quantification of the toxicologically most relevant PA type to a great extent. All 1,2-unsaturated PAs and 1,2-unsaturated PANOs are converted into a common basic structure that can be easily quantified with means of an appropriate internal standard [32]. Besides, this method covers unknown 1,2-unsaturated PAs as well and can be generally applied even if there is no background information on the botanical origin or target PAs of the samples. However, in comparison to the LC-MS approach, this method shows a lower sensitivity (10 ppb) and extra expenditure for sample preparation. In addition, only the 1,2-unsaturated PAs are measured and can be quantified, but other toxicological relevant PA types (otonecine- or supinidine-type-PAs) are not covered and, since only the common backbone structure is analyzed as a sum, there is no possibility to deduce on the structure or distribution of the original PAs.

4 Recommendations and regulations concerning PAs

To our knowledge there are currently no legal regulations concerning maximum PA levels in foodstuff or animal feed worldwide. In section 4 we summarize the legal situation or discussions on regulations concerning PAs in different countries or regions. Our list contains all information that we were able to access but does not claim to be complete.

4.1 WHO

Already in 1988 the WHO published a program on ''Environmental health criteria for pyrrolizidine alkaloids'' and pointed out the potential health risk by PA contaminations in the food chain (http://www.inchem.org/documents/ehc/ehc/ehc080.htm; last accessed 12.10.2009) [42]. Thus, the main hazard of serious PA intoxications is caused by grain contaminated with PA seeds and the use of PA plants in traditional folk remedies. Based on the observed genotoxic potential of PAs, the commission recommended reducing the contamination in the human food chain to the lowest possible degree. Therefore, the WHO advice of monitoring the PA levels of honey and dairy products from regions with known high PA frequency could be an approach to achieve this goal [42].

4.2 Germany

Since 1992, PA containing phytopharmaceuticals are regulated in a Federal Pharmaceutical Ordinance. According to these regulations, only a few proven active PA plants and preparations thereof, which are listed by name, can be marketed. With regard to the PA content the following limits were established: at customary oral intake the total amount of 1,2 unsaturated PAs (including the PANOs) must not exceed 1 μg *per* day. If the application is more than 6 wks, the limit is further reduced to 0.1 μg *per* day. In addition, the package insert for orally used products needs to contain the warning notice "*do not use during pregnancy or lactation*" [43].

After an intensive risk assessment and expert hearings the authorities justified the regulation that these actions would substantially reduce the risk associated with the ingestion of PAs. The decision was based on the toxicological interpretation of results of several test systems, where 1,2-unsaturated PAs showed markedly mutagenic and genotoxic potential after metabolic activation. Furthermore, it was assumed that the mechanisms for acute, chronic and carcinogenic effects observed in the rodent models can be transferred onto humans [43].

In 2007, a multidisciplinary committee of the Federal Institute of Risk Assessment (Bundesinstitut für Risikobewertung) started to reevaluate and if applicable to complement the zero tolerance principle of the European Union for certain compound and compound classes in food and feeding stuff (http://www.bfr.bund.de/cm/208/nulltoleranzen_in_lebens_und_futtermitteln.pdf; last accessed 07.10.2009) [44]. In this context, the zero tolerance principle should be applied if the risk cannot be calculated or if limits of exposure cannot be derived because of the lack of valid scientific data. In many cases zero tolerance should be applied if the compound possesses carcinogenic or mutagenic potential.

The results were summarized in a position paper. There, it is requested that genotoxically relevant contaminations of plant origin (*e.g.* the explicit named PAs) should be generally treated in the category of zero tolerance [44].

Furthermore, the Bundesinstitut für Risikobewertung comments on the European Food Safety Authority (EFSA) demand to utilize the concept of "margin of exposure" (MOE); that it will not often be applicable due to incomplete data sets. Another discussed concept for potentially genotoxic compounds would be the "threshold of toxicological concern" by Kroes *et al.* [45]. This approach proposes a maximum daily amount of exposure of 0.15 μg *per* person *per* day. However, the implementation of this concept is still seen as critical by the authorities. First of all, it is a rarely implemented novelty and therefore it is still too early for a final evaluation and secondly, the current practical implementation often fails due to the lack of the required analytical sensitivity [44].

4.3 UK

In the past, similar to the German regulation for herbal remedies, comfrey and preparations thereof were banned in the UK. In 2007, the UK Committee on Toxicity (COT) was asked for a statement on PAs in food, in particular on honey and milk. The statement comprehensively summarizes the literature data on all aspects concerning PA toxicology like hepatotoxicity, pulmonary toxicity, carcinogenicity or genotoxicity (http://cot.food.gov.uk/pdfs/cotstatementpa200806.pdf; last accessed 12.10.2009) [46]. Considering UK honey in particular, the COT refers to the above-mentioned MAFF study [24] and a project "Collection and Analysis of Honey Samples Potentially Contaminated with Pyrrolizidine Alkaloids from Ragwort and Borage" funded by the UK Food Standards Agency [31] (http://www.food.gov.uk/science/research/researchinfo/foodcomponentsresearch/riskassessment/t01projlist/t01037/; last accessed 26.11. 2009). Six locations with either *S. jacobaea* (ragwort) or *B. officinalis* (blue borage, borage) occurrence were sampled several times throughout the 2005 season. Concerning ragwort, it was concluded that even under unfavorable conditions this plant will be of no concern, since neither pollen analysis nor analytical results showed significant amounts of PAs.

Honeys originating from *Borago* sides showed significant pollen counts and could be correlated with the detection of a PA (lycopsamine or intermedine). But in neither case a reliable quantification of the corresponding PAs were achieved and the final results and conclusions remained rather vague.

Therefore, in order to establish a risk assessment, the Committee on the Carcinogenicity of Chemicals in Food, Consumer Products and the Environment (COC) was asked by the COT for an advice on the carcinogenicity of PAs.

Seven PAs, riddelliine, lasiocarpine, clivorine, petastitenine, senkirkine, symphytine and monocrotaline and two PA metabolites, dehydroheliotridine and dehydroretronecine, were assessed based on available toxicological data, such as the US National Toxicology Program (NTP; http://

ntp.niehs.nih.gov). Based on the available NTP data for riddelliine and lasiocarpine, the COC suggests a $BMDL_{10}$ of 0.073 mg/kg body weight *per* day to be used for any MOE approach to the risk assessment (http://cot.food.gov.uk/pdfs/cocsection2008.pdf; last accessed 13.10.2009) [47].

Taking into account the opinion of WHO [41], the ANZFA (Australia New Zealand Food Authority) (http://www.foodstandards.gov.au/_srcfiles/TR2.pdf; last accessed 23.10. 2009) [48], COC [47] and a 2 year rat model study of riddelliine, COT considered this as an appropriate basis for assessing the risk of non-cancer effects of PAs and concludes "*that non-cancer effects would not be expected at doses of riddelliine up to 0.1 μg/kg b.w./day.*" COT also accepted the advice of COC that "*it is prudent to assume that PAs are genotoxic carcinogens*" and considered that "*MOEs of 10,000 and above, corresponding to doses up to 0.007 μg/kg b.w./day, would be unlikely to be of concern.*" Assuming a worst case scenario (high level infant consumer) this would result in "*a maximum PA-concentration in honey, which would still maintain an MOE of 10,000 compared to the BMDL10…would be 6.4 μg/kg honey.*" [46].

Assuming an average PA content of 50–100 μg/kg in 10% of all retail honeys, as it can be concluded by the two studies available for European honeys [30, 32], this would result in an average incidence of 5–10 μg/kg honey, which corresponds, at least for the group of high level infant consumers, with the COT suggested upper limit of 6.4 μg/kg [46].

4.4 Austria

The legal situation in Austria affecting PAs in herbal remedies is similar to the German regulations. Only a few PA plants are authorized. These plants or preparations thereof can only be marketed if they are analyzed by a "… *state of the art detection method*" which proves that "… *the final product does not contain pyrrolizidine alkaloids.*" [49].

4.5 The Netherlands

A similar regulation exists in The Netherlands for herbal preparations or extracts [50]. This decision defines that the total PA content (including PANOs) of these products must not exceed 1 μg/kg or 1 μg/L, respectively. Considering a "normal" dose for herbal remedies of a few grams or milliliters *per* day, it shows clearly that the regulations of the neighboring countries Austria and The Netherlands are many times more stringent than the German regulation.

Recently, the Dutch National Institute for Public Health and the Environment (RIVM) suggested a virtually safe dose for PAs of 0.00043 μg/kg/day leading to an increased risk of at most one person in a million developing cancer. For non-cancer effects, a tolerable daily intake of 0.1 μg/kg b.w./day is mentioned [30, 51]. The report summarizes that the consumption of honey with elevated PA levels should be avoided; therefore, studies should be intensified to be able to further minimize the risk. It is concluded that at this point, no warning notice for the consumers is necessary.

4.6 Europe

In an EFSA statement, made in 2007, concerning PAs in feedstuff it is stated that from a human-epidemiology perspective, the sinusoidal obstruction syndrome (hepatic veno-occlusive disease) is verifiable linked to PA uptake, whereas the carcinogenic potential of the PAs does not seem to be well documented until now. The carcinogenic potential of PAs, according to EFSA, so far has only been convincingly demonstrated in *in vitro* models and in rodents [52]. In contrast to former recommendations, this directive also demands the principle of non-dilution. That is, contaminated feedstuff (and as a consequence also secondary products derived thereof) must not be mixed with uncontaminated material, to meet tolerable limits. Instead, this material should be rather decontaminated or destroyed, to minimize the entry of harmful compounds into the food chain. Regarding the PA problem in feedstuff, EFSA recommends to gain an initial overview on which PA plants and/or marker PA need to be considered to be of relevance in animal feed (*e.g.* marker PA for *Senecio*: senecionine, seneciphylline and erucifoline or the markers intermedine and lycopsamine for *Anchusa* spp., *Borago* spp., *Symphytum* spp. and *Eupatorium* spp.). In addition, EFSA is calling to generate more quantitative data sets on PA levels in milk (because of the high proportion of the diet of infants and young children) and honey [52].

Recently, the European Commission has approved refined *E. plantagineum* oil as a novel food ingredient. The specifications for the product include that PAs must not be detectable (zero tolerance; detection limit of 4 μg/kg oil) [53]. To our knowledge, this is the first time that a limit for PAs is listed in combination with foodstuff.

4.7 Australia/New Zealand

For Australia and New Zealand a common opinion announced by ANZFA exists [48]. According to this statement, there is evidence that PAs show carcinogenic effects in rodents, but contrawise there is no scientific evidence that these results can be extrapolated to the human organism. Hence, PAs are only considered a human risk factor in a chronic exposure scenario eventually causing sinusoidal obstruction syndrome (VOD). Based on this assumption a provisional tolerable daily intake of 1 μg *per* kg body weight was calculated [48].

On the other hand, recent studies based on experimental results for the PA riddelliine showed clearly tumor-inducing effects in rodents. In similar experiments on human liver microsomes, a similar PA metabolism was observed and therefore it was concluded that, given the mechanistic similarities, the results of rodent experiments is highly relevant and can be transferred to some extent to humans [54].

The FSANZ (Food Standards Australia New Zealand) has asked honey producers in Australia/New Zealand to mix honeys that are mainly derived from *E. plantagineum* (Paterson's Curse/Salvation Jane) to blend it with other honeys to achieve the required limits (http://www.foodstandards.gov.au/newsroom/factsheets/factsheets2004/consumersadvisedtoli2347.cfm; last accessed 07.10.2009) [55]. This recommendation seems counterproductive to the general desire to reduce the overall level of PAs in the human food chain. Instead it would be more reasonable to take highly contaminated honeys off the market rather than to contaminate additionally PA free honeys. This measure is also in contrast to the EFSA directive of non-dilution of contaminated food and feedstuff [52].

4.8 USA

In 2001, FDA (US Food and Drug Administration) has advised manufacturers of oral preparations derived from *Symphytum* spp. or products containing PAs from other sources to withdraw these from the market. Further, FDA refers to its authority to confiscate these products if necessary. According to FDA the decision is based on the clear relationship between PAs and literature reports on serious adverse health effects. Due to insufficient data, however, the authority finds itself unable to set a safe level for oral exposure (http://www.cfsan.fda.gov/~dms/dspltr 06.html; last accessed 14.10.2009) [56].

5 Concluding remarks

As elaborated in the previous section, there is currently no national or international uniform toxicological and/or legal assessment of PAs in foods. At least a European consensus seems to crystallize, where PAs are an undesired class of compounds in food and feedstuff.

At this point, it should be considered that the presence of PAs in food does not only affect honey and pollen, but involves, as demonstrated in the past, more plant and animal foods (like salad, eggs, milk). Hence, additive effects can be assumed. In contrast to these foods, there are several independent studies of the recent past available that provide quantitative data for PA levels in honey and pollen and could serve as basis for discussion and risk assessment.

Due to the already long lasting and controversially discussed toxicological classification and the complex analysis of certainly more than 200 relevant PAs and PANOs (currently about ten reference PAs are commercially available, but no PANO standards so far), a complete toxicological evaluation of PAs in foodstuff seems rather unrealistic for the near future.

That prospect encouraged us to implement a new methodical approach: instead of an individual PA determination we established the conversion of the toxicologically most relevant 1,2-unsaturated PAs/PANOs (with the exception of otonecine-type-PAs) into a single PA sum parameter [32, 37]. This approach offers the advantage of a highly simplified analysis without the need of additional reference PAs. In addition, unknown 1,2-unsaturated PAs are likewise detected and the sum parameter is directly proportional to the amount of the originally present single PAs. Although it is to some extent a simplification of the complex PA matter, the results obtained in such sum parameter methods are probably best suited to establish a PA limit. Quantifications based on single PA determination would always dependent on the commercial availability of all PAs/PANOs present in the sample as references. In addition, the implementation of an analysis using a stable isotope-labeled reference PA would be much easily achieved and optimized utilizing a sum parameter method. At this point, it should be mentioned that the currently valid PA limit in Germany for herbal remedies is also established as sum parameter (but the calculation there is based on the sum of all individual 1,2-unsaturated PAs, hence considering the molecular weight of each individual PA).

For honey in particular (and to some extent for milk) it would be desirable to identify most of the responsible PA plants that serve as nectar/pollen sources for bees. In this specific case, this can be achieved by pollen analysis of PA contaminated honeys. Subsequently, marker PA could be established, which analytically cover the most common PA nectar plants (for example, *Echium* honey, *Eupatorium* and *Senecio*-specific PAs). With the help of such marker (reference) PAs sensitive LC-MS-MS methods can be established. This would be helpful for an analytical approach and it would enable a reliable quantification of those target-PAs in bee products with only little efforts.

With the establishment of a PA limit for bee products it should be taken into consideration that it would be desirable in terms of a general reduction of PAs in the food chain to strictly enforce the principle of non-dilution and withdraw honey/pollen with high PA levels from the market. However, this appears rather unrealistic due to the usual practice of blending different batches of honey (or as explicitly recommended for *Echium* honeys in Australia/New Zealand).

Given the present results, adult bees seem relatively tolerant towards a PA incorporation into the hive [39]. Consequently, it is necessary that local producers are aware of the responsible PA plants and counteract the potential PA incorporation by an appropriate choice of placing their bee hives.

Hence, in terms of a preventive consumer protection and minimized PA entry into the food chain, it would be most effective to directly educate the producers/beekeepers and train them to avoid PA accumulation in honey/pollen in the first place. Besides, and especially regarding Western European honey markets like Germany or The Netherlands, which are largely supplied by imports from Non-EU countries [34], this would also indicate an expanded political and economic cooperation to provide on-site information and education. Further effective precautionary measure should be that no honey is extracted from brood combs, since this would result in additional PA pollen and increase the PA load of the resulting honey.

In our view, the possibility of PA reduction in honey/pollen and thus also in the general food chain seems already possible at the current time.

First, various analytical methods are available to identify and reject highly contaminated samples. Second, there is already knowledge of certain PA plants preferred by bees and therefore preventive measures to minimize the PA entry into bee products for human consumption are possible by carefully selecting the locations for bee hives.

Both actions together would be highly effective and significantly reduce the PA level and help to maintain the popular image of the natural product honey.

Regarding the PA levels in pollen products, the available data necessarily leads to the question, whether these products should be sold without prior PA analysis.

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