## SCIENTIFIC OPINION

# Scientific Opinion on Pyrrolizidine alkaloids in food and feed[1]

**EFSA Panel on Contaminants in the Food Chain (CONTAM)**[2, 3]

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## ABSTRACT

The European Food Safety Authority (EFSA) was asked by the European Commission to deliver a scientific opinion on pyrrolizidine alkaloids (PA) in food and feed. PAs are toxins exclusively biosynthesised by plants. To date, approximately 600 different PAs are known. Results for 13,280 bulk honey and 1324 retail honey samples were provided to EFSA by one Member State and 351 feed samples were provided by a second Member State. The EFSA Panel on Contaminants in the Food Chain (CONTAM Panel) performed estimates of both acute and chronic exposure to PAs through honey for three different age groups. Although there might be other sources of PA exposure, due to lack of data the CONTAM Panel was not able to quantify dietary exposure from food other than honey. A number of PAs were identified as being of particular importance for food and feed. Based on the present knowledge of metabolism, activation, DNA adduct-formation, genotoxicity and carcinogenicity, the CONTAM Panel concluded that 1,2-unsaturated PAs may act as genotoxic carcinogens in humans. Therefore, the CONTAM Panel decided to apply the Margin of Exposure (MOE) approach. A benchmark dose lower confidence limit for a 10 % excess cancer risk ($BMDL_{10}$) of 70 µg/kg b.w. per day for induction of liver haemangiosarcomas by lasiocarpine in male rats was calculated as the reference point for comparison with the estimated dietary exposure. The CONTAM Panel concluded that there is a possible health concern for those toddlers and children who are high consumers of honey. There is generally a low risk of PA poisoning in livestock and companion animals in the EU as most PA poisonings reported recently are due to accidental exposure.



## KEY WORDS

pyrrolizidine alkaloids (PA), origin, chemistry, analysis, exposure, risk assessment, margin of exposure (MOE)

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## SUMMARY

Following a request from the European Commission, the Panel on Contaminants in the Food Chain (CONTAM Panel) was asked to deliver a scientific opinion on the risks to human and animal health related to the presence of pyrrolizidine alkaloids (PA) in food and feed. PAs are toxins exclusively biosynthesised by plants. They are typical plant secondary metabolites against herbivores. It has been estimated that approximately 6000 plant species world wide, representing 3 % of all flowering plants, may contain pyrrolizidine alkaloids. PAs are mainly found in the distantly related angiosperm families of the Boraginaceae (all genera), Asteraceae (tribes Senecioneae and Eupatorieae) and Fabaceae (genus *Crotalaria*). The PA-content of plant material depends on a large number of factors (species, plant organ, harvest, storage, extraction procedures). Reported contents vary from trace amounts up to 19 % based on dry weight. The name pyrrolizidine is the chemical description of two-fused 5-membered rings with a nitrogen atom at the bridgehead. This motif is the central structure of a variety of PAs. PAs generally consist of an amino alcohol which is referred to as necine or necine base and an acid part which is called a necic acid. Most of the known PAs are esters of hydroxylated 1-methylpyrrolizidine or otonecine-type necine bases. To date, an estimate of approximately 600 different PA structures is known. The rich diversity is derived through factors such as combination of a pool of necine bases with an even larger pool of necic acids. The variability is further increased by the possible formation of monoesters at different positions and open or cyclic diesters. In addition, many PAs frequently co-occur in two forms, their *N*-oxide (PANO) and as tertiary base PAs.

Currently, only methods with mass spectrometric (MS) detection provide the prerequisites to analyse PAs at trace levels in food and feed. Basically, two MS based approaches are applied, either in combination with gas chromatography (GC) or high performance liquid chromatography (HPLC) in tandem mass-spectrometry (MS/MS) mode.

Following a European Food Safety Authority (EFSA) call for data covering mycotoxins and phytotoxins, including pyrrolizidine alkaloids, no replies for PAs were received even by the extended deadline in January 2011. Therefore, the industry was contacted and two submissions were received from one Member State covering the presence of a range of PAs in bulk honey as well as in retail honey. Overall, results for 14,604 samples of honey were reported to EFSA of which 13,280 samples concerned bulk honey and 1324 samples covered retail honey that is mostly blended and ready for consumption. No occurrence data in food other than honey were received. A further submission of 351 results was received from the National Competent Authority in one Member State covering PAs in feed. All food and feed samples were reported to be analysed by liquid chromatography-tandem mass spectrometry (LC-MS/MS). The limit of detection (LOD) for food samples was reported as 0.5 µg/kg for most PAs and 4.5 µg/kg for all PAs in feed after correction for the dry weight matter (to 88 %). As the submissions for food and feed were each coming from one Member State, they cannot be regarded as representative for the occurrence of PAs across Europe.

The two submissions covering results of PAs found in honey included testing of different ranges of alkaloids. The submissions had eight PAs in common (echimidine, echimidine-*N*-oxide, heliotrine, lycopsamine, retrorsine, senecionine, seneciphylline and senkirkine), while one submission included a further six (heliotrine-*N*-oxide, lasiocarpine, lycopsamine-*N*-oxide, retrorsine-*N*-oxide, senecionine-*N*-oxide and seneciphylline-*N*-oxide) giving a total of fourteen, and the other a further nine (acetylechimidine, acetylechimidine-*N*-oxide, acetylechiumine-*N*-oxide, echiumine, echiumine-*N*-oxide, echiuplatine, echiuplatine-*N*-oxide, echivulgarine and echivulgarine-*N*-oxide) giving a total of seventeen PAs.

The substitution method was used for expressing left-censored results, i.e. results at or below the limit of detection (LOD) or the limit of quantification (LOQ) were assigned either a value of zero (lower bound approach) or the value of the LOD or LOQ (upper bound approach). For bulk honey, the lowest proportion of left-censored results was recorded for lycopsamine at 49 %, followed by

echimidine and echiumine at 56 % and 71 %, respectively. For retail honey, the situation was slightly different with the lowest proportion of left-censored results recorded for echimidine at 16 %, followed by lycopsamine and echiumine at 36 % and 45 %, respectively. Apart from acetylechiumine-*N*-oxide (one positive result), there were a further eleven PAs with only left-censored results reported for the retail honey of which six also reported 99 % left-censored data for the bulk honey.

The average levels of the different PAs for bulk honey varied from 0-9.7 µg/kg for the lower bound (LB) and 0.1-10 µg/kg for the upper bound (UB), and for retail honey from 0-6.5 µg/kg for the lower bound and 1-6.7 µg/kg for the upper bound. The maximum reported levels of PAs for bulk honey were for echimidine-*N*-oxide at 2031 µg/kg, echimidine at 1522 µg/kg, lycopsamine at 1448 µg/kg and seneciphylline-*N*-oxide at 1441 µg/kg. Maximum reported levels for retail honey were much lower with echimidine at 150 µg/kg, lycopsamine at 126 µg/kg and echiuplatine at 115 µg/kg.

The number of samples with any PAs above the LOD or LOQ is higher and the overall average level is lower when comparing retail honey to bulk honey. The maximum levels found in retail honey are only 10 % or less of the levels found in bulk honey. The eight PAs in common for the two submissions comprised between 75 and 90 % of the total upper bound sum of PAs in the respective alkaloid grouping. The sum of PA levels for the eight PAs in the total number of samples and for the PA sum in the groups of 14 and 17 alkaloids tested were used for the exposure calculations.

The CONTAM Panel decided that both acute and chronic exposure to PAs should be assessed. Consumption data were taken from the EFSA Comprehensive European Food Consumption Database (Comprehensive Database). Three representative age groups were selected for the exposure analysis, toddlers from 1 to 3 years of age, other children from 3 to10 years and adults from 18 to 65 years. Since there was little difference in consumption patterns between adults on the one hand and adolescents, elderly and very elderly population groups on the other, there was no need to present consumption data separately for these latter groups.

The highest acute exposure to PAs through retail honey was calculated for toddlers with a daily intake providing from 0.80 to 48.6 ng/kg body weight (b.w.) and from 3.3 to 114 ng/kg b.w. when applying the country range of minimum and maximum mean and 95$^{th}$ percentile consumption, respectively, and mean lower and upper bound PA concentrations. Consuming retail honey at the 95$^{th}$ percentile concentration level could potentially increase acute exposure two to three times compared to the mean concentration, with the highest value of 254 ng/kg b.w. calculated for toddlers consuming 40 g of retail honey in one day.

For the chronic scenario, PA exposure for the mean consumption and concentration scenario for toddlers could reach a high of 37.4 ng/kg b.w. per day in “honey consumers only”. However, it is probably closer to the result of 5.10 ng/kg b.w. per day calculated for consumption distributed over all survey participants in the respective age group, based on the uncertainty associated with interpolating a few survey days to long-term consumption and a rather low number of honey consumers in the surveys.

The theoretical exposure calculated for consumption of unblended (bulk) honey was in general about 50-100 % higher than the results of the calculations for retail honey or sometimes slightly higher than that. However, this calculation is mainly based on occurrence results for honey imported from countries outside Europe and such honey would usually be blended before retail.

Levels of PAs in 351 feed materials, sampled between 2006 and 2010 were submitted by one Member State. The compounds were merged into four groups of structurally related PAs (senecionine-, lycopsamine-, heliotrine- and monocrotaline-type PAs). In 55 % of samples, concentrations of PAs were below the LOD (4.5 µg/kg).

Livestock and domestic animals may be exposed to PAs by the consumption of forage and roughage contaminated with plant (parts) of *Senecioneae* and *Boraginaceae* spp. In particular, lucerne (alfalfa;

*Medicago sativa*) forage was occasionally found to be contaminated with substantial amounts of PAs, which is most likely due to contamination with *Senecio vulgaris*. Horses may be more exposed than other livestock due to their high consumption of lucerne. Herbal mixtures used as feed which are contaminated with PA-containing plants (or their parts) are another possible source of exposure of livestock to PAs, but these feeds generally represent only a small proportion of the diet. Overall, the data on PA occurrence in feed are too limited to undertake a reliable estimate of the animal exposure.

Based on the available literature and the occurrence data submitted on PAs in honey and feed, the CONTAM Panel identified the following PAs (including the tertiary amine as well as the corresponding *N*-oxide forms) of particular importance for food and feed:

- Senecionine-type PAs: acetylerucifoline, erucifoline, integerrimine, jacobine, jacoline, jaconine, jacozine, retrorsine, senecionine, seneciphylline. These PAs occur particularly in the *Senecioneae* (Asteraceae family), but are also found in *Crotalaria spp.* (Fabaceae family).

- Lycopsamine-type PAs: acetylechimidine and isomers, echimidine and isomers, echivulgarine, lycopsamine and isomers, vulgarine. These PAs occur in the Boraginaceae family and in the *Eupatorieae* (Asteraceae family).

- Heliotrine-type PAs: europine, heliotrine, lasiocarpine. These PAs occur in *Heliotropium spp*. (Boraginaceae family).

- Monocrotaline-type PAs: fulvine, monocrotaline, retusamine, trichodesmine. These PAs occur in *Crotalaria spp.* (Fabaceae family).

Data on several 1,2-unsaturated PAs show that they are readily absorbed from the gastrointestinal tract and undergo extensive metabolism in mammals. 1,2-unsaturated PAs undergo metabolic activation by hepatic cytochrome P450 from experimental and livestock animals and humans to reactive pyrrole metabolites.

The toxicity of the 1,2-unsaturated PAs in experimental animals is characterised by hepatotoxicity, developmental toxicity, genotoxicity and carcinogenicity. Some also exhibit pulmonary toxicity. The liver is the primary site for genotoxicity of 1,2-unsaturated PAs. The 1,2-unsaturated PAs from different structural classes (i.e., retronecine, heliotridine, and otonecine; di-esters and mono-esters) undergo metabolic activation to reactive pyrrolic intermediates and form a common set of DHP adducts at dG and dA sites in rat liver DNA. These findings suggest that a genotoxic carcinogenic mechanism is applicable for all 1,2-unsaturated-PA esters and their *N*-oxides, which can be metabolically converted into PAs.

The concomitant induction of mutations compatible with DHP adduct formation in liver cells in transgenic rats, and the formation of hemangiosarcomas and hepatomas in riddelliine (retronecine type PA)-treated male and female rats and mice, provide strong evidence for a genotoxic mechanism for hepatocarcinogenicity. In contrast to 1,2-unsaturated PAs, 1,2-saturated PAs do not undergo metabolic activation to reactive pyrrolic species responsible for hepatotoxicity and genotoxicity. Therefore the CONTAM Panel decided to base the risk characterisation on the 1,2-unsaturated PAs.

Human case reports of poisonings due to PA containing herbal medicines and teas and large outbreaks of human poisonings, including deaths associated with grain crops contaminated with PA containing weeds, have demonstrated the toxicity of 1,2-unsaturated PAs in humans, affecting predominantly liver and lung. Poisoning with 1,2-unsaturated PAs in humans is characterised by acute hepatic veno-occlusive disease (HVOD). The acute disease is associated with high mortality, and a sub-acute or chronic onset may lead to liver cirrhosis.

The lowest known doses associated with acute/short-term toxicity in humans are reported to be 3 mg PA/kg b.w. per day (exposure of a boy for a 4 day-period, lethal outcome) and 0.8 -1.7 mg PA/kg b.w.

per day (exposure of a girl for a 2 week-period, HVOD). The lowest known dose associated with long-term toxicity (HVOD) in humans is reported to be 15 µg PA/kg b.w. per day (exposure for a period of 6 months). Substantial long-term follow-up data or epidemiological studies to assess whether exposure to 1,2-unsaturated PAs results in cancer in humans are not available.

Overall, based on the present knowledge of metabolism, activation, DNA adduct-formation, genotoxicity and carcinogenicity studies, the CONTAM Panel concluded that 1,2-unsaturated PAs may act as carcinogens in humans. Therefore, the data from experimental animals are relevant to humans and the carcinogenicity data provide the most suitable basis for the risk characterisation.

Because 1,2-unsaturated PAs are genotoxic and carcinogenic, the CONTAM Panel concluded that it was not appropriate to establish a Tolerable Daily Intake (TDI), and decided to apply the Margin of Exposure (MOE) approach. A $BMDL_{10}$[4] for excess cancer risk of 70 µg/kg b.w. per day was calculated for induction of liver haemangiosarcomas by lasiocarpine in male rats and used as reference point for comparison with the estimated dietary exposure. Lasiocarpine is amongst the most toxic of the PAs that have been tested. In the data on PAs submitted to EFSA, lasiocarpine was below the LOD or LOQ in 99 % of the honey samples. Some PAs such as lycopsamine, which was one of the most frequently detected PA in honey, are more than an order of magnitude less toxic. Since the toxicity influences the carcinogenicity, the carcinogenic potency of most PAs present in honey is likely to be lower, therefore basing the risk characterisation on the $BMDL_{10}$ for lasiocarpine is a conservative approach, which is likely to allow also for concomitant exposure to co-occurring PAs.

In relation to PAs in retail honey, the MOEs for adults are in the ranges of 57,000 - 3,500,000 and 7400 - >7,000,000, at the mean and 95$^{th}$ percentile of consumption (based on maximum UB and minimum LB across European countries). The EFSA Scientific Committee has concluded that a MOE of 10,000 or higher, based on a $BMDL_{10}$ from an animal study, is of low concern from a public health point of view. Taking into account the influence of samples with non-quantifiable levels of PAs, and the conservative nature of using the $BMDL_{10}$ for a potent PA as the reference point, these MOEs are not likely to represent a health concern.

For toddlers, the MOEs are in the ranges of 14,000 - 7,000,000 and 1200 - >7,000,000, respectively. For other children, MOEs are in the ranges of 25,000 - 1,800,000 and 3900 - >7,000,000 at the mean and 95$^{th}$ percentile population consumption of honey.

For individuals who regularly eat locally produced unblended honey, exposure to PAs could be up to twice that of people who consume retail honey.

The CONTAM Panel concluded that there is a possible health concern for those toddlers and children who are high consumers of honey.

Estimates of acute dietary exposure to PAs in honey are four orders of magnitude lower than the lowest known PA dose associated with acute/short term toxicity in humans, indicating that PAs in honey will not lead to acute toxicity.

In addition to honey, there are other possible sources of dietary exposure to PAs. Based on the few available data indicating limited carry over from animal feed, meat, milk and eggs are not likely to be major sources, but this requires confirmation. Exposure to PAs from herbal dietary supplements can potentially be very much higher than dietary exposure from honey and is known to have caused human illness. Data on PAs in herbal dietary supplements are generally not available. However, if such supplements are prepared from PA-containing plants, then they could present a risk of both acute and chronic effects in the consumer. Furthermore, borage oil and Echium oil marketed as dietary supplements, and salad crops contaminated with PA-plants such as *Senecio vulgaris* (common

[4] $BMDL_{10}$ (Benchmark dose lower confidence limit) is the 95 % lower confidence limit of the benchmark dose associated with a 10% response.

groundsel), could present a risk to the consumer, but data were not available for the CONTAM Panel to perform exposure assessments or risk characterisation for these sources.

Since the publication of the EFSA (2007) opinion on pyrrolizidine alkaloids as undesirable substance in animal feed, a number of reports have been published on the effects of PA intake by livestock and companion animals, although the clinical signs and pathological findings described in the 2007 opinion remain valid. Even though all animal species are susceptible to both acute and chronic PA intoxication, the risk of PA poisoning in the EU appears to be low. Most poisonings reported recently have been due to accidental exposure, but in the absence of integrated data on the incidence in the EU, it has not been possible to quantify this risk.

The CONTAM Panel recommended, inter alia, that ongoing efforts should be made to collect analytical data on occurrence of PAs and PANOs in relevant food and feed commodities. These should include milk, eggs and meat. The PAs monitored should include at least the compounds identified in this opinion as markers for the main PA-containing plant families. In order to improve the analytical methods, there is a need for a larger and more diverse set of certified reference standards, covering both PAs and PANOs identified as markers of the main PA containing plant families. As limited literature results on honey showed considerable differences in the PA concentrations depending on the country of origin, more data are needed to correlate the occurrence of various PAs with the geographical and botanical origin. Finally, there is a need for toxicological data relating to the PAs most commonly found in honey.

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## BACKGROUND AS PROVIDED BY THE EUROPEAN COMMISSION

Pyrrolizidine alkaloids (PAs) are toxins found naturally in a wide variety of plant species. PAs are probably the most widely distributed natural toxins and affect wildlife, livestock and humans. Over 6,000 plant species are known to contain PAs, although direct poisonings in man and animals seems to be associated with only a few species. Poisoning caused by these toxins is associated with acute and chronic liver damage and may be fatal.

Direct human cases of poisoning are occasional but are well documented; consumption of grain or grain products (flour or bread) contaminated with seeds from weed species that contain these alkaloids is commonly involved. These occurrences frequently occur as 'outbreaks' following dry season or drought conditions that favour the development of weeds in the primary crop. It has been suggested that intoxication may occur as a result of drinking milk from affected animals, but the amount of PAs excreted into the milk of animals exposed to PAs is low. The same holds true for eggs. The ingestion of honey from bees that have fed on toxic plant species has also been suggested as a possible exposure pathway, but the levels involved are low and there are no reported cases. The direct and deliberate use of toxic plant species as herbal teas or traditional medicines forms another pathway of exposure, which is well documented and has resulted in deaths.

Farm animals, particularly cattle, sheep, goats, horses, poultry and pigs are known to be susceptible to poisoning with high levels of mortality, while small animals such as rabbits appear less affected. There are reports of toxicity to fish. Outbreaks in farm animals cause severe economic losses to farmers and rural communities and there is the possibility of transfer of the toxins to humans.

## OCCURRENCE

Over 350 PAs are known and the list continues to grow. They are known to be present in more than 6000 plant species. The main sources are the families *Boraginaceae* (all genera), *Asteraceae* (tribes *Senecionae* and *Eupatoriae*), and *Fabaceae* (genus *Crotalaria*). Some plant species express several PAs or alkaloid *N*-oxides and there are some PAs that are expressed by several plant species. The toxins are commonly concentrated in the seeds and the flowering parts of the plant, with decreasing amounts in the leaves, stems and roots. Most plants produce mixtures of PAs in varying concentrations ranging from less than 0.001 % to 5 % in certain plant seeds. Some PA-bearing plants are used as ground cover, soil improvers (*Fabaceae*), ornamental plants, and for animal feed. Some, especially in the *Boraginaceae* family, are appreciated for the quality of their honey.

Excluding the use of herbal teas and medicines, the plants most commonly reported as being associated with food poisoning in humans are *Heliotropium* (in the family *Boraginaceae* ) and *Crotalaria.* These occur as weeds in cereal or legume crops and the seeds are mixed accidentally with the main crop at harvest. The toxins survive the milling, baking and subsequent processes. The situation is frequently aggravated by drought and other conditions advantageous to weed growth at the expense of the crop. Outbreaks of veno-occlusive disease and other liver disorders have been reported from parts of Central Asia, Afghanistan and India with recent outbreaks occurring in Afghanistan.

Poisoning in animals has been reported from all of the sources listed above with known outbreaks attributed to *Heliotropium*, *Trichodesma, Senecio*, and *Crotalaria* species. In general, grazing animals will avoid PA-bearing plants but may have little choice in conditions of drought or when searching for food on over-grazed or otherwise depleted pastures. If weedy crops are used for the production of hay or silage the animals can no longer exercise discrimination when feeding because the toxins survive storage processes and are completely intermixed with the fodder. Mortality is reported to be high.

## THE OPINION OF THE PANEL ON CONTAMINANTS IN THE FOOD CHAIN ON A REQUEST FROM THE COMMISSION RELATED TO PYRROLIZIDINE ALKALOIDS AS UNDESIRABLE SUBSTANCE IN ANIMAL FEED

The Panel on Contaminants in Food Chain issued on 25 January 2007 on a request from the Commission an opinion related to pyrrolizidine alkaloids as undesirable substance in animal feed.[5]

The Panel concluded that given the large variation in the pattern of toxic PAs in plant materials, analytical surveys should focus on selected alkaloids, which have been identified as major (hepato-)toxic compounds, while at the same time being representative for major PA-containing plant families. In a first analytical survey for the presence of PAs in feed materials, the following compounds should be monitored:

- the *Senecio* alkaloids senecionine, seneciophylline and erucifoline, which are found in high concentrations in the *Senecio* species occurring in Europe, and which have been associated with clinical intoxications in livestock.

- the *Crotalaria* alkaloids monocrotaline and trichodesmine. *Crotalaria* spp. represent a prominent class of tropical plants, known to produce high amounts of PAs and having been associated in the past as a source of intoxications in poultry and pigs.

- the *Heliotropium* alkaloids Heliotrine and indicine occurring predominantly in the seeds of plants of the *Heliotropium* species, which might contaminate cereals and grains intended for human and animal consumption.

- the PAs intermedine and lycopsamine, as these are representative for many species of *Anchusa, Borago, Symphytum* and *Eupatorium* species, and may serve as markers for the undesirable presence of these plant species (and/or their seed) in feed materials.

Although human exposure resulting from carry-over of PAs from feed into animal derived products seems to be low, more data should be made available on the potential carry-over into milk, considering that infants have a relatively high consumption per kg body weight. Moreover, more data are needed to quantitatively assess the contribution of honey to human exposure, as the latter is regularly found to contain residual amounts PA metabolites.

## TERMS OF REFERENCE AS PROVIDED BY THE EUROPEAN COMMISSION

In accordance with Art. 29 (1) (a) of Regulation (EC) No 178/2002 the Commission asks EFSA for a scientific opinion on the risks to human and animal health related to the presence of alkaloids in food and feed.

The scientific opinion as regards the presence of pyrrolizidine alkaloids in food should, *inter alia,* comprise the:

(a) evaluation of the toxicity of pyrrolizidine alkaloids for humans, considering all relevant toxicological endpoints and identification of the pyrrolizidine alkaloids of toxicological relevance present in food;

(b) assessment of the relevance for the monitoring of PAs in food of those which were previously identified by the CONTAM Panel as being relevant for the monitoring of the presence of PAs in feed;

[5] Opinion of the Scientific Panel on Contaminants in Food Chain on a request from the Commission related to pyrrolizidine alkaloids as undesirable substance in animal feed, adopted on 25 January 2007, The EFSA Journal (2007) 447, 1 – 51, http://www.efsa.europa.eu/en/scdocs/doc/447.pdf.

(c) exposure of the EU population to pyrrolizidine alkaloids, including the consumption patterns of specific (vulnerable) groups of the population (e.g. high consumers, children, people following a specific diet, etc) and identify the sources of exposure.

The scientific opinion as regards the presence of pyrrolizidine alkaloids in animal feed should, *inter alia,* comprise an update, if necessary, of the Opinion of the Panel on Contaminants in Food Chain on a request from the Commission related to pyrrolizidine as undesirable substance in animal feed, taking into account new data (toxicological, occurrence and other relevant information) which has become available since 2007.

**ASSESSMENT**

## 1. Introduction

Pyrrolizidine alkaloids (PAs) are toxins exclusively biosynthesised by plants. They are typical plant secondary metabolites against herbivores. It has been estimated that about 6000 plant species world wide, representing 3 % of all flowering plants, may contain PAs (Smith and Culvenor, 1981). PAs are mainly found in the distantly related angiosperm families of the Boraginaceae (all genera), Asteraceae (tribes Senecioneae and Eupatorieae) and Fabaceae (genus *Crotalaria*). The toxicological effects of acute exposure to PAs to livestock and humans are well known and documented in literature (Wiedenfeld and Edgar, 2011). In particular, human poisoning with 1,2-unsaturated PAs is known to cause liver toxicity resulting in development of hepatic veno-occlusive disease (HVOD) (Chen and Huo, 2010). The latest documented outbreak was recorded in Afghanistan in 2008 (Kakar et al., 2010) where flour contaminated with seeds of *Heliotropium* (Boraginaceae) and milk from goats appeared to be the main cause for the health damage. In laboratory experiments, 1,2-unsaturated PAs showed the potential to induce genotoxicity in different model systems, and carcinogenicity was observed following chronic oral exposure to several 1,2-unsaturated PAs (Fu et al, 2004; Chen et al., 2010). The toxicity and risk to human health associated with the ingestion of PA-containing food products have been previously summarised by the WHO-IPCS (1988).

To this day, common sources of PA-intoxication are herbal remedies and/or food supplements that contain PA-plants. Concerning phytopharmaceuticals, several European countries have regulated the use of these preparations (Kempf et al., 2010a) but through self-medication, folk- and ethnomedicine (Roeder, 2000; Yu and Li, 2005) and internet trading some of these plants are still in use. Recently, several studies demonstrated that PAs are found frequently in retail honey and food supplements containing bee pollen (Kempf et al., 2010a, 2010b). In addition, demonstrating a more complex occurrence of PAs in the human food chain, PA-containing plants were repeatedly found as plant matter contamination in retail packed salads (BfR, 2007) or contaminated fodder for livestock (Mulder et al., 2009).

There are numerous reports of PAs playing an essential role in the life cycle of specialized adapted herbivorous insects, which strongly depend on PAs in their food supply for protection against predators (Hartmann, 2009).

### 1.1. Chemistry

#### 1.1.1. Pyrrolizidine alkaloids: structures and physicochemical properties

The name pyrrolizidine is the chemical description of two-fused 5-membered rings with a nitrogen atom at the bridgehead. This motif is the central structure of a variety of alkaloids known as PAs. Appendix A contains the chemical structures of the relevant PAs discussed in this opinion. The amino alcohols are referred to as necines or necine bases and are condensed with an acid part which is called a necic acid (Mattocks, 1986; Hartmann and Witte, 1995; Roeder, 1995). Most of the known PAs are ester-PAs of hydroxylated 1-methylpyrrolizidine or otonecine-type necine bases. 1,2-unsaturated PAs can be metabolised in the liver to reactive intermediates, so called pyrrolizines. Since these carry the structural subunit of a pyrrole, they are also referred to as ‘reactive pyrroles’ in the literature.

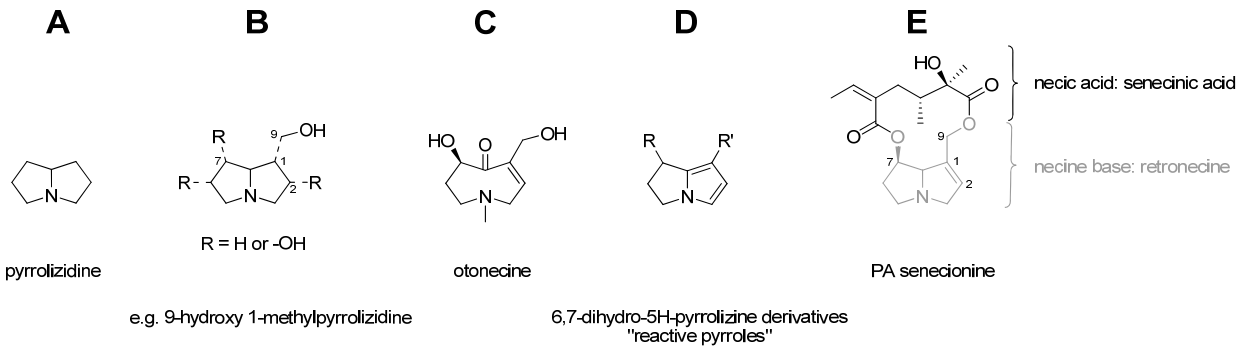


**Figure 1:** Structural features of PAs. A) core structural motif pyrrolizidine (1,2,3,6,7,8-hexahydro-5*H*-pyrrolizine); B) general description of the main necine base parts of naturally occurring PAs including the common necine base numbering; C) necine base otonecine; a core structural motif of otonecine-type PAs; D) general pyrrolizine structure motif and E) structural example of 1,2-unsaturated ester PA senecionine.

To date, approximately 600 different PA structures are known (for a comprehensive overview see Mattocks, 1986; Hartmann and Witte, 1995; Roeder, 1995). The rich diversity is derived through factors such as combination of a pool of necine bases (see Figures 1 and 2) with an even larger pool of necic acids. The variability is further increased by the possible formation of monoesters at different positions (e.g. C-7 or C-9) and open or cyclic diesters. In addition, many PAs frequently co-occur in two forms, their *N*-oxide (PANO) and as tertiary base PAs (see Figure 2).

PANOs are charged molecules and are to a certain degree soluble in water, but also in polar organic solvents like methanol or acetonitrile. Tertiary PAs are soluble in polar organic solvents like methanol but also in more lipophilic solvents like dichloromethane. At low pH-values, tertiary PAs are protonised at the nitrogen atom and are to some degree water-soluble.

The UV-spectra of most 1,2-unsaturated PAs is of limited significance, showing an absorption maximum at about 214 nm. PAs are chiral molecules, hence are optical active.

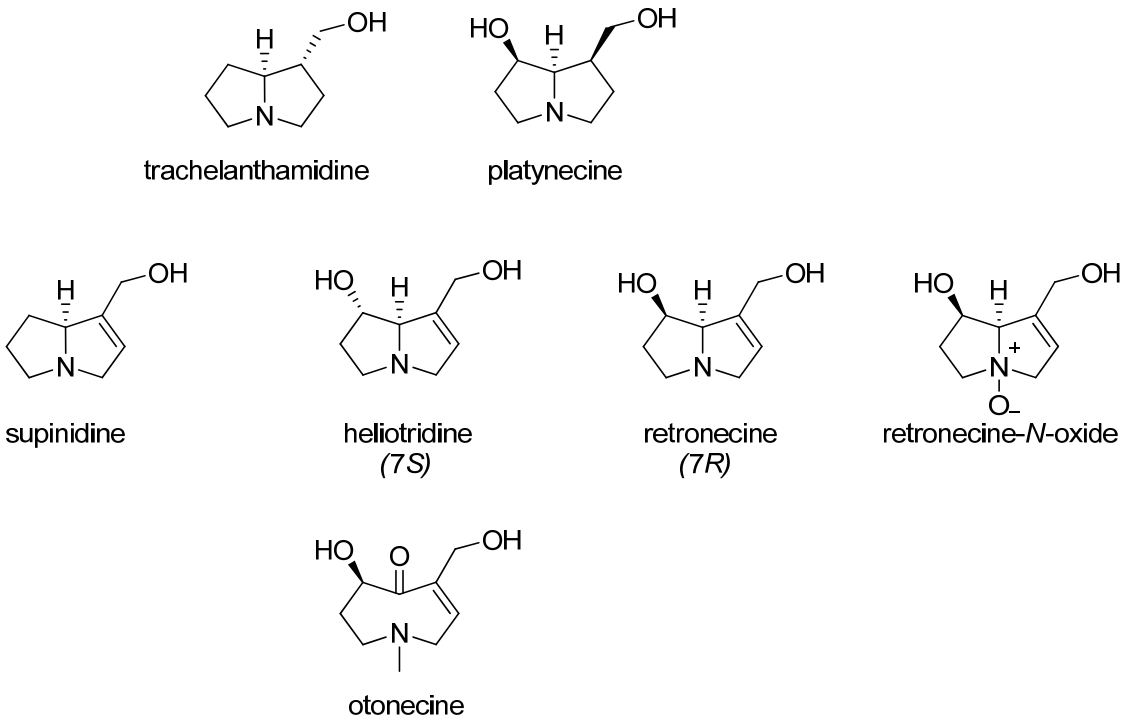


**Figure 2:** Pool of the most common necine bases of PAs and serving as an example for *N*-oxides, retronecine-*N*-oxide.

### 1.1.2. Stability

The composition of PA-patterns and quantity can change through the analytical process. Sample preparation like heat can influence the tertiary PA/PANO-ratio and the total PA-content of samples (Mattocks, 1986; Hösch et al., 1996). *N*-oxidation of PAs is an enzymatic catalysed reaction, while the reduction of PANOs occurs spontaneously in the presence of chemical or biological reducing agents (Hartmann and Toppel, 1987). However, tertiary PAs and PANOs are still present in lyophilized plant material after decades of storage at dry and dark conditions. On the other hand, complete bio-degradation of PAs under silage or composting conditions is described (Crews et al., 2009; Hough et al., 2010). Ester-PAs can be hydrolysed in aqueous solutions at pH-values above 9. To avoid hydrolysis, mainly ammonia is used to raise the pH in most of the described extraction procedures. Under mildly acidic conditions most PAs are more stable, although epoxide functionalities present in some PAs are sensitive to halogenic acids. Halogenated solvents promote quaternisation of tertiary PAs to corresponding salts with modified characteristics. In addition, photochemical reactions of amines and halogenated solvents are described (Mattocks, 1986).

### 1.1.3. Botanical origin of PAs

PAs are exclusively produced by plants. Hence, PAs found in other organisms like insects etc. are usually acquired via the forage (Hartmann, 1994, 1995). The PA-content of plant material depends on a large number of factors (species, plant organ, harvest, storage, extraction procedures) and cannot be generalized. Reported contents vary from trace amounts up to 19 % based on dry weight (Johnson et al., 1985). In most cases (exceptions mainly for PAs found in seeds of *Crotalaria* spp. or PAs in Orchidaceae) PANOs are the dominating form found in plant tissues.

The precursor in the formation of the necine base moiety of PAs is the polyamine homospermidine formed from spermidine and putrescine in a reaction catalysed by homospermidine synthase (EC

2.5.1.44) (Graser and Hartmann, 1997). The enzymatic formation of spermidine has been studied in relation to this role as an alkaloid precursor. Thus, Graser and Hartmann purified and characterised *S*-adenosylmethionine decarboxylase (SAMDC, EC 4.1.1.50) and spermidine synthase (SPDS, EC 2.5.1.16) from root cultures of *Senecio vulgaris* (Graser and Hartmann, 2000). Concerning the necic acids, complete biosynthetic labelling patterns have been obtained for the skeletons of two classes of PAs, i.e., the senecionine type and the lycopsamine type as reviewed by Hartmann and Ober (2000). Thus, experiments with labelled precursors in *S. vulgaris* and *S. pleistocephalus* showed that senecic acid in both species is formed from two units of 2-aminobutyric acid. The C-13 and C-20 positions of senecionine (Figure 3 A) came from the C-3 of 2-aminobutanoic acid, while the C-19 and C-21 positions were originally C-4 of the amino acid (Stirling et al., 1997). More about the biosynthesis of pyrrolizidine alkaloids may be found in Hartmann and Ober (2000). In *Senecio* spp. it has been shown that PAs are synthesised in the roots as PANOs, which are specifically translocated into shoots (Hartmann, 1994, 1995).

About 95 % of the known PAs are found in only five plant families, i.e. Asteraceae (Compositae), Boraginaceae, Fabaceae (Leguminosae), Orchidaceae and Apocynaceae. According to their taxonomic occurrence, PAs can be grouped into six different subclasses (Figure 3A to F) (Hartmann and Witte, 1995; Langel et al., 2011).

Senecionine-type PAs represent the largest and most diverse class. It consists of mostly macrocyclic diesters (Figure 3A) and, in lower abundance, of open chain diesters like triangularine (Figure 3B). The main necine base is retronecine, sometimes replaced by otonecine or a 1,2 saturated analogue (e.g. platynecine; see Figure 2). PAs of the senecionine-type are generic for species of the Asteraceae tribe Senecioneae and are also found in *Crotalaria* species (Fabaceae). In addition *Crotalaria* spp. frequently contain another type of PA (monocrotaline-type: Figure 3C), which is characterised by an eleven-membered macrocyclic ring instead of the twelve-membered ring found in macrocyclic senecionine-type PAs.

Another diverse group of PAs is represented by the lycopsamine-type PAs (Figure 3D). These are open chain mono- or diesters PAs containing at least one necic acid unit of 2-isopropyl-2,3-dihydroxybutyrate. The necine base can be either retronecine (C-7 *R*-configuration) or heliotridine (C-7 *S*-configuration) as shown for lasiocarpine (Figure 3D1) and is species-specific. PAs of the lycopsamine-type occur in Asteraceae (tribe Eupatorieae), the Boraginaceae and the Apocynaceae. They can be grouped together with tri-ester PAs like parsonsine (Figure 3E) that are occasionally found in Apocynaceae species. Finally, certain genera of Orchidaceae contain phalaenosine-type PAs. These are usually monoesters of simple 1,2 saturated necine bases like trachelanthamidine and aralkyl acids (Figure 3F).

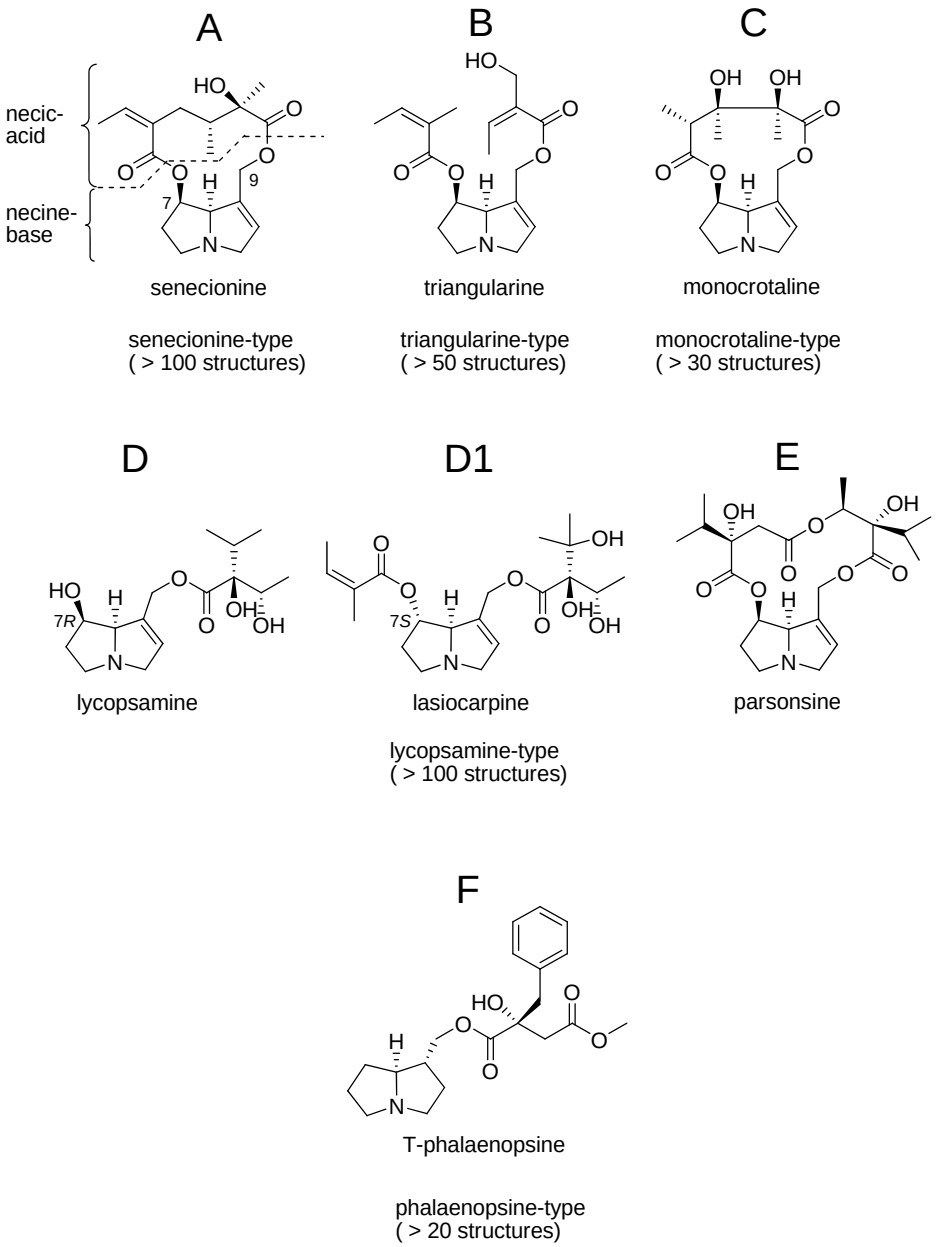


**Figure 3:** Exemplified classification of PAs following the scheme proposed by Hartmann and Witte (1995).

### 1.2. Previous assessments

Several 1,2-unsaturated PA-containing plant materials and 1,2-unsaturated PAs have been evaluated by the International Agency for Research on Cancer (IARC, 1976, 1983, 1987, 2002) (Table 1). For example, for lasiocarpine (e.g. in *Heliotropium* spp.), riddelliine (e.g. in *Senecio* spp.) and monocrotaline (e.g. in *Crotalaria* spp.) the available data gave sufficient evidence for carcinogenicity in experimental animals and they have been classified by IARC as being possibly carcinogenic to humans – group 2B (IARC, 1983, 1987, 2002). To the experimental results obtained with isatidine (syn.: retrorsine-*N*-oxide), retrorsine and senkirkine, all of them being a component of e.g. *Senecio* spp., IARC attributed the category 'limited evidence for the carcinogenicity to experimental animals' (overall evaluation: not classifiable as to its carcinogenicity to humans, group 3) (IARC, 1983, 1987).

**Table 1:** Classification of pyrrolizidine alkaloids by the International Agency for Research on Cancer.

| 1,2-unsaturated Pyrrolizidine Alkaloids | Occurrence, e.g. | Evaluation IARC vol.10 (1976), vol. 31 (1983), vol. 82 (2002) | Degree of Evidence for Carcinogenicity IARC Suppl. 7 (1987), vol. 82 (2002) | | |
|---|---|---|---|---|---|
| | | | Human | Animal | Overall evaluation[(a)] |
| Isatidine (Retrorsine-*N*-oxide) | *Senecio* spp. | carcinogen in rats (oral, liver tumours) | ND | L | 3 |
| Lasiocarpine | *Heliotropium* spp. | carcinogen in rats (i.p., tumours in liver & other organs) | ND | S | 2B |
| Monocrotaline | *Crotalaria* spp. | carcinogen in rats (oral, tumours in liver) | ND | S | 2B |
| Retrorsine | *Senecio* spp. | carcinogen in rats (oral, tumours in liver & other organs) | ND | L | 3 |
| Riddelliine | *S. vulgaris* | carcinogen in rats and mice (oral, tumours in liver & other organs) | ND | S | 2B |
| Senkirkine | *Tussilago farfara* *Senecio* spp. | carcinogen in rats (i.p., liver cell adenomas; *T. farfara*, oral, hepatic haemangioendothelial sarcomas) | ND | L | 3 |
| 18-Hydroxysenkirkine | *C. laburnifolia* | insufficient data | ND | I | 3 |
| Jacobine | *S. jacobaea* | no data | ND | I | 3 |
| Seneciphylline | *Senecio* spp. | no data | ND | ND | 3 |
| Symphytine | *Symphytum* spp. | insufficient data | ND | I | 3 |

ND = no adequate data; L = limited evidence; S = sufficient evidence; I = inadequate evidence.
(a): 2B: The agent is possible carcinogenic to humans; 3: The agent is not classifiable as to its carcinogenicity to humans.

The toxicity and human risks associated with the ingestion of PA-containing products have been evaluated in detail by the World Health Organization – International Programme on Chemical Safety (WHO-IPCS) (1988, 1989a). The report summarises experimental studies and cases of human and livestock poisonings. In man, poisoning is described as acute HVOD, leading to hepatomegaly, ascites, massive pleural effusion, and in many cases progressing to cirrhosis. From human data on the use of comfrey (*Symphytum* spp.) over a period of four to six months (Ridker et al., 1985) it was concluded that ingestion rates above 15 µg/kg body weight (b.w.) per day for the mixture of echimidine and related alkaloids (equivalent to 9 µg heliotrine/kg b.w. per day, calculated using corresponding rat $LD_{50}$ data) may lead to acute or subacute liver disease. Therefore the WHO-IPCS considered it prudent to conclude that a dose equivalent to 10 µg heliotrine/kg b.w. per day may lead to disease in humans. In terms of equivalent doses of heliotrine, the total doses in the known outbreaks or cases of HVOD were estimated to range from 1000 to 167,000 µg/kg b.w., with 1000 to 120,000 µg/kg b.w. being the range for non-fatal cases and 6000 to 167,000 µg/kg for fatal cases. According to WHO-IPCS it was not possible to evaluate the human cancer risk due to PAs because of lack of information on the long-term follow-up of populations having been exposed to PAs. However, various PAs had been shown to be carcinogenic for experimental animals which implies that a potential cancer risk for humans should be seriously considered. Since the effects of PAs in humans might be cumulative at very low intake rates, it is recommended that exposure should be minimised wherever possible (WHO-IPCS, 1988, 1989a).

Furthermore, the Australian New Zealand Food Authority (ANZFA) presented a technical report on PAs in Foods (ANZFA, 2001). In this assessment, it was stated that in humans the major effects are hepatocellular injury, liver cirrhosis and HVOD, but evidence for PA-induced cancers was lacking in humans. Therefore, HVOD was considered to be the most sensitive toxicological endpoint in humans and a tentative no-observed-effect level (NOEL) for HVOD of 10 µg/kg b.w. for PAs was derived from the available human data. The ANZFA proposed a provisional tolerable daily intake (PTDI) of 1 µg/kg b.w. for PAs by applying an uncertainty factor of 10 (ANZFA, 2001).

The Dutch National Institute for Public Health and the Environment (Rijksinstituut voor Volksgezondheid en Milieu, RIVM) derived a tolerable daily intake (TDI) of 0.1 µg/kg b.w. per day for non-cancer effects. This was obtained by applying an uncertainty factor of 100 to a no-observed-adverse-effect level (NOAEL) of 10 µg/kg b.w. per day with regard to non-neoplastic changes (hepatocyte cytomegaly) observed in rats receiving riddelliine by gavage for 105 weeks (NTP, 2003; RIVM, 2005). Regarding neoplastic effects based on the same study RIVM established a virtually safe dose (VSD) for PAs of 0.00043 µg/kg b.w. per day, leading to an increased risk of at most one person in a million developing cancer. This VSD was derived from the lowest dose leading to tumour development (haemangiosarcomas), which was 1000 µg/kg b.w. per day (RIVM, 2005).

The EFSA Scientific Panel on Contaminants in the Food Chain (CONTAM Panel) issued an opinion on pyrrolizidine alkaloids as undesirable substances in animal feed in 2007 (EFSA, 2007). The CONTAM Panel identified gaps of knowledge concerning human exposure and concluded that given the large variation in the pattern of toxic PAs in plant materials, analytical surveys should focus on selected alkaloids, which have been identified as major (hepato-)toxic compounds, while at the same time being representative for major PA-containing plant families. The PAs selected for a first analytical survey for the presence in feed were senecionine, seneciophylline, erucifoline, monocrotaline, trichodesmine, heliotrine, indicine, intermedine and lycopsamine.

In 2008 the UK Committee on Toxicity of Chemicals in Food, Consumer Products and the Environment (COT) published a risk assessment on PAs in food (COT, 2008). In this statement the COT accepted the advice of the UK Committee on Carcinogenicity of Chemicals in Food, Consumer Products and the Environment (COC), that PAs should be considered as a cumulative assessment group where it is prudent to assume that PAs are genotoxic carcinogens. The COT concluded that PAs are known to cause HVOD in humans but that the available reports of human cases of poisoning did not provide sufficiently reliable exposure data to be used in establishing a health-based guidance value for PAs.

With respect to non-neoplastic effects, the COT concurred with RIVM and concluded, that, by referring to the NOAEL of 10 µg/kg b.w. per day in rats for hepatocyte cytomegaly in the above mentioned NTP study (NTP, 2003) and by applying an uncertainty factors of 100, a dose of 0.1 µg riddelliine/kg b.w. per day would not be expected to result in non-cancer effects. The ratio of $LD_{50}$ values could be used to convert other PAs to riddelliine equivalents for comparison with this dose.

Regarding neoplastic lesions, the COT recommended to use a $BMDL_{10}$[6] of 73 µg/kg b.w. per day derived from a 2 year carcinogenicity study of lasiocarpine in rats (NTP, 1978) to assess exposure for any PA. Allowing a margin of exposure (MOE) of at least 10,000 indicated that PA doses of up to 0.007 µg /kg b.w. per day are unlikely to be of concern for cancer risk. Such doses would also not be expected to result in non-cancer effects. The maximum PA concentration in honey, to maintain an MOE of 10,000 for high level infant consumers, would be 6.4 µg/kg honey (COT, 2008).

The Federal Institute of Risk Assessment (Bundesinstitut fuer Risikobewertung, BfR, Germany) assessed leaves and blossoms of *Senecio vulgaris* (common groundsel), which contain the 1,2-unsaturated PAs senecionine, seneciphylline, retrorsine and riddelliine, as contaminants of a mixed salad (degree of contamination with the plant material: 1.7 %). BfR made reference to cases of poisoning with *S. vulgaris* and other *Senecio* species in humans and animals, which resulted in hepatic injury and partly were fatal. The estimated acute exposure with 1,2-unsaturated PAs of 28 - 44 µg/kg b.w. resulting from consumption of the salad mixture was a factor of 68-152 below the dose causing death in a boy and a factor of 18-61 below the dose causing liver cirrhosis in a girl. Thus, it was concluded that acute to medium-term liver damage in consequence of the consumption of the contaminated salad could not be ruled out. Also in view of the carcinogenicity data salads contaminated with parts of *S. vulgaris* as indicated were considered to be dangerous to health. A portion size below which the 1,2-unsaturated PAs do not constitute a health risk could not be determined and therefore a tolerable intake could also not be derived. For an adult weighing 60 kilograms, the long-term intake by a contaminated salad as indicated was estimated to be 220 to 349 µg of 1,2-unsaturated PAs per day and therefore would far exceed the tolerated daily dose of 0.1 µg 1,2-unsaturated PAs for internal medicinal products without therapeutic indications or without restriction of intake to 6 weeks in Germany (Bundesgesundheitsamt, 1992). BfR advised that contamination of food with 1,2-unsaturated PAs from *S. vulgaris* should be as low as reasonable achievable (BfR, 2007; Dusemund et al., 2010).

A 'Discussion Paper on Pyrrolizidine alkaloids' summarising available evaluations, regulations, analytical methods, data on the occurrence in food and feed and data on the toxicity in animals in humans has been published recently by a working group of the Codex Alimentarius Commission (FAO/WHO, 2011). According to a first analysis of the occurrence data, it is concluded that the PAs which are the most detected in food are (acetyl)echimidine, (acetyl)lycopsamine, europine, heliotrine, jacoline, lasiocarpine, monocrotaline, retrorsine, senecionine, seneciphylline, and trichodesmine whereas in feed the most detected were echimidine, erucifoline, heliotrine, jacobine, lycopsamine, monocrotaline, retrorsine, retusamine, senecionine, and seneciphylline.

The working group recommended that the evaluation of PAs should be updated by the Joint FAO/WHO Expert Committee on Food Additives (JECFA) and that work on the development of a code of practice for the prevention and reduction of contamination of food products with pyrrolizidine alkaloids should be started.

Following an expert meeting on 4 March 2010, BfR prepared a report on the analytics and toxicity of PAs and the risk assessment of the occurrence of PAs in honey (BfR, 2011). BfR recommends to keep total exposure with 1,2-unsaturated PAs from different foods as low as possible and to not exceed an intake of 0.007 µg 1,2-unsaturated PAs/kg b.w. per day (corresponding to 0.42 µg 1,2-unsaturated PAs per day for an individual weighing 60 kg . In agreement with the COT this dose was derived from the

[6] $BMDL_{10}$ (Benchmark dose lower confidence limit) is the 95 % lower confidence limit of the benchmark dose associated with a 10% response.

$BMDL_{10}$ of 73 µg/kg b.w. per day calculated from a 2 year carcinogenicity study of lasiocarpine in rats (NTP, 1978) by applying a MOE of 10,000. BfR also supported a cumulative assessment approach of all 1,2-unsaturated PAs. With regard to the evaluation of actual available analytical data on the occurrence of 1,2-unsaturated PAs in honey, BfR recommended efforts to reduce the levels of 1,2-unsaturated PAs in certain honeys to minimise risks of high-consumers and among those especially of children, for whom MOEs fell below the tolerable MOE of 10,000 in particular when brand-loyalty was taken into account (BfR, 2011).

## 2. Legislation

In order to protect public health, Article 2 of the Council Regulation (EEC) No 315/93[7] stipulates that, where necessary, maximum tolerances for specific contaminants shall be established. Thus, a number of maximum levels (ML) for contaminants as well as natural plant toxicants are currently laid down in Commission Regulation (EC) No 1881/2006.[8] While maximum levels for nitrates and various mycotoxins were set for a number of food commodities, pyrrolizidine alkaloids are not regulated so far under this and with one exception nor under another regulation for food.

Commission Decision 2008/558/EC[9] authorises the placing on the market of refined echium oil as novel food ingredient provided inter alia that pyrrolizidine alkaloids are not detectable with a detection limit of 4 µg/kg.

Some Member States (i.e. Austria, Belgium, Germany, The Netherlands and the United Kingdom) have regulated PA-containing toxic plants or plant parts. The following is an excerpt from the CODEX ALIMENTARIUS Discussion paper on pyrrolizidine alkaloids prepared by an electronic working group in February 2011 (FAO/WHO, 2011).

In Austria only a few PA-containing plants are authorised for herbal remedies. These plants or preparations thereof can only be marketed if they are analysed by a state of the art detection method' which proves that 'the final product does not contain pyrrolizidine alkaloids'.

In Belgium, borage (*Borago officinalis*) is prohibited for use in food. Oil of borage may be used in food supplements, if it can be shown that it is free of PAs using an appropriate detection method with sufficient limit of detection (LOD) (Koninklijk besluit, 1997).

In Germany 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 PANOs) must not exceed 1 µg per day. If the application is more than 6 weeks, 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' (Bundesgesundheitsamt, 1992, cited by Kempf et al., 2010a). In Switzerland the same regulations for phytopharmaceuticals as in Germany are in force.

In The Netherlands, PAs are regulated for herbal preparations or herbal extracts. The total PA content (including PANOs) of these products must not exceed 1 µg/kg or 1 µg/L, respectively (Warenwetbesluit Kruidenpreparaten, 2001). In the Annex of the Warenwetbesluit Kruidenpreparaten, an overview is given of all plants and fungi of which it is presumed that they contain PAs.

[7] Council Regulation (EEC) No 315/93 of 8 February 1993 laying down Community procedures for contaminants in food. OJ L 37, 13.2.1993, p. 1–3.
[8] Commission Regulation (EC) No 1881/2006 of 19 December 2006 setting maximum levels for certain contaminants in foodstuffs. OJ L 364, 20.12.2006, p. 5–24.
[9] Commission Decision of 27 June 2008 authorising the placing on the market if refined echium oil as novel food ingredient under Regulation (EC) No 258/97 of the European Parliament and of the Council (2998/558/EC). OJ L 180, 9.7.2008, p. 17-19.

For grains and pulses, the Codex Alimentarius states that toxic seeds in wheat should not be present in amounts that represent a hazard to health and mentions specifically the presence of *Crotalaria* (FAO, 2010). These standards are: Maize (corn) (CODEX STAN 153-1985[10]), Certain pulses (CODEX STAN 171-1989[11]), Sorghum grains (CODEX STAN 172-1989[12]), Wheat and durum wheat (CODEX STAN 199-1995[13]), Oats (CODEX STAN 201-1995[14]).

Undesirable substances in feed are regulated by Directive 2002/32/EC.[15] Pyrrolizidine alkaloids are not listed as such in the Annex of this Directive. However, several plant species or specific parts of these plant species containing pyrrolizidine alkaloids, such as *Crotalaria* spp. as well as weed seeds and unground and uncrushed fruits containing alkaloids, glucosides or other toxic substances separately or in combination are explicitly listed as undesirable substances. The maximum content (relative to a feedingstuff with a moisture content of 12 %) for *Crotalaria* spp. is 100 mg/kg and for weed seeds and uncrushed fruits 3000 mg/kg for all feedingstuffs. It should be noted that this Directive does not provide limits for individual or groups of pyrrolizidine alkaloids but refers to weed seeds and uncrushed fruits that can be detected by microscopic examination.

## 3. Methods of analysis

Many different plants or plant organs, food and feedingstuffs, and biological fluids have been analysed for PAs in the past and most of the common analytical techniques were applied in the detection of these compounds (Mattocks, 1986; Crews et al., 2010). Hence, the next paragraphs will only focus on the most recent and most common techniques used for the trace analysis of PAs in complex matrices. A discussion of a larger variety of methods used for the determination of PAs can be found in a recent review by Crews et al. (2010).

### 3.1. Extraction and sample preparation

#### 3.1.1. Extraction

Considering the large structural variety of PAs, especially the co-occurrence of PAs and corresponding PANOs, the extraction method has to ensure the efficient simultaneous extraction of both types.

In general, PAs are alkaloids of which the reduced (tertiary) form contains a characteristic basic nitrogen and therefore classical alkaloid extraction with semi-polar to polar organic solvents or acidified aqueous conditions are prevalent. In the oxidised form (PANOs) they are polar molecules that can be readily extracted by polar solvents such as methanol, or diluted aqueous acids (Crews et al., 2010). Since both PA-forms are rather polar, the extraction material can be treated with non polar solvents first (like pentane or petroleum ether) to remove non polar compounds like fats, waxes, terpenes etc. which results in cleaner extracts that show less interference with subsequent analytical procedures. Alternatively, this clean-up step can be introduced in a later stage and the acidic aqueous extracts can be processed by liquid-liquid extraction with a non polar solvent such as dichloromethane to reduce the amount of interfering non polar compounds. It is well known that extraction conditions can affect PA-stability, ratio of PANO to PA and yield (Mattocks, 1986; Crews et al., 2010). Therefore, appropriate extraction conditions need to be tested and evaluated for different matrices.

[10] www.codexalimentarius.net/download/standards/51/CXS_153e.pdf
[11] www.codexalimentarius.net/download/standards/56/CXS_171e.pdf
[12] www.codexalimentarius.net/download/standards/57/CXS_172e.pdf
[13] www.codexalimentarius.net/download/standards/62/CXS_199e.pdf
[14] www.codexalimentarius.net/download/standards/64/CXS_201e.pdf
[15] Directive 2002/32/EC of the European Parliament and of the Council of 7 May 2002 on undesirable substances in animal feed. OJ L 140, 30.5.2002, p. 10.

Depending on the methods for analysing PAs/PANOs, different sample preparations can be chosen. Techniques where PAs have to be volatilized for chromatographic separation (e.g. gas chromatographic techniques) are not suitable to detect PANOs. To enable detection PANOs are converted into the corresponding PAs. Under these conditions the result would reflect the total PA (tertiary form + PANOs) in form of tertiary PAs. Several reduction techniques are reported (Crews et al., 2010), the most common one being the reduction with nascent hydrogen for several hours using elemental zinc powder in acidic aqueous solution.

After reduction, the aqueous samples can be further purified by raising the pH and liquid-liquid extraction of the PAs, which are no longer protonated under these conditions, into medium-polar organic solvents like dichloromethane. Alternatively, the PAs can be partitioned into an organic solvent by using columns packed with inert diatomaceous earth (Hartmann and Toppel, 1987). The PAs are recovered by passing organic solvents through the columns, while the aqueous part is captured by the column matrix (liquid-liquid extraction with a solid matrix support). To gain information on the ratio of PANOs in such samples, a second extract (or splitting of the initial extract in two equal portions) has to be analysed under identical conditions, but omitting the reduction step, resulting in the amount of the present tertiary PAs only. The difference of total PA and the tertiary PA amount reflects the amount originally present as PANOs.

Techniques where direct detection of PANOs is desired require a different sample preparation. The fact that no reduction step is needed, may lead to simplified and less elaborate protocols. A recent method for the simultaneous detection of PAs and PANOs in *Jacobaea vulgaris* with subsequent liquid chromatography-tandem mass spectrometry (LC-MS/MS) (Joosten et al., 2010) or ultra high performance liquid chromatography-tandem mass spectrometry (UHPLC-MS/MS) detection uses a single step extraction of lyophilized ground plant material with 2 % aqueous formic acid solution for 30 min, microfiltration and dilution prior to analysis (Joosten et al., 2011).

### 3.1.2. Solid phase extraction (SPE)

Trace analysis of PAs usually requires a clean-up step to increase PA-concentration and/or to remove interfering compounds. PA-extracts can be further processed by SPE procedures. Several SPE materials like Ergosil, C18-material and strong cation exchange (SCX) have been successfully applied (Crews et al., 2010). At present, the latter one (SCX) has become a frequently used tool especially in trace analysis of PAs in complex matrices such as honey (Mattocks, 1961; Betteridge, et al., 2005; Kempf et al., 2008, Zhou et al., 2010, Dübecke et al., 2011). A benefit of SCX-SPE is that simultaneously both PAs and PANOs can be trapped and specifically eluted. A general procedure would comprise an acidic aqueous PA extraction, then applying the extracts on top of a SCX-SPE cartridge, removal of interfering compounds by rinsing with water and/or methanol and eluting of all PA-forms by applying basic conditions such as ammonia in methanol. In practice, it seems necessary that SCX-SPE cartridges should be tested and adapted to the analytical problem. For instance, analysis of PAs in honey showed blocking problems or different recovery rates for different brands or materials (Betteridge, et al., 2005; Kempf et al., 2008).

## 3.2. Instrumental analysis

Many analytical techniques have been successfully adapted to detect PAs (Mattocks, 1986; Rizk, 1991; Roeder, 1999; Crews et al., 2010). The methods that have become the most established ones will be briefly summarized below.

### 3.2.1. Spectrophotometry and Thin Layer Chromatography (TLC)

The spectrophotometric detection for 1,2-unsaturated PAs and PANOs (with the exclusion of otonecine-type PAs) is based on a colour reaction and was improved by Mattocks (1968a; Mattocks and Jukes,

1987). The tertiary PAs are oxidized to the corresponding PANOs, dehydrogenated to the pyrroles and subsequently coupled with 4-dimethylaminobenzaldehyde (Ehrlich's reagent) to form a magenta coloured dye which can be measured spectrophotometrically at 563 nm and shows detection limits in the mg/kg range (or 1 µg total 1,2-unsaturated PA). A similar sensitive method for determination of PAs is based on a stoichiometric reaction of protonated alkaloids with methyl orange. Methyl orange is then released from the complex and measured photometrically at 525 nm (Birecka et al., 1981).

TLC-methods were introduced in the past to separate and isolate individual PAs and several stationary and mobile phases have been published (for a general overview see Mattocks, 1986). In general, visualization on TLC can be achieved by similar methods as described above for the spectrophotometric PA-detection (Mattocks, 1986). 1,2-saturated PAs or otonecine-type PAs can be detected by spraying with Dragendorff's reagent (Mattocks and Pigott, 1990) or sodium nitrite solution (Hovermale and Craig, 2002). The latter two reagents are generally used to identify nitrogen-containing compounds, hence they are not specific for PAs alone.

TLC-based methods were applied in the past to detect PAs in PA-plant derived seed oils. Mierendorff (1995) determined the PA-content of *Borago officinalis* seed oils by TLC. A *Symphytum* extract and monocrotaline were used as references. The method comprises liquid-liquid extraction, reduction with zinc, two-fold TLC separation and visual detection by a method of Mattocks (1967). Under favourable conditions a LOD of 0.1 µg can be achieved. A similar approach was reported by Parvais et al. (1994). Calibration was performed using narceine as an external reference standard alkaloid and a detection limit of 0.1 mg/kg oil was achieved.

### 3.2.2. Nuclear magnetic resonance (NMR)

Besides X-ray analysis, NMR methods are essential and prevalently used in the structural elucidation of purified PAs and data collections of $^{1}H$ and $^{13}C$-NMR data are available (Roeder, 1990; Logie et al., 1994). Quantitative determinations of 1,2-unsaturated PAs can be achieved by $^{1}H$ NMR spectroscopy. Signals originating from the vinylic C-2 hydrogen resonate at 6.2 ppm and 5.8 ppm for macrocyclic diesters and non-cyclic mono or diesters, respectively. Using appropriate internal standards (like *para*-dinitrobenzene) allows the determination of the total 1,2-unsaturated PA-level of an extract (Molyneux et al., 1979; Pieters and Vlietinck, 1985). $^{1}H$ NMR techniques have recently been used to identify jacobine and jacobine-*N*-oxide in plant extracts using a metabolomics approach (Leiss et al., 2009). Whether this approach together with an improved availability of high-field NMR instrumentation can be used to detect and quantify trace levels of PAs in complex matrices remains to be seen.

### 3.2.3. Immunological Methods

Immunological based tests are widespread and important laboratory methods for a rapid, selective and sensitive detection of analytes in complex matrices. Large sample sets can be processed in parallel and often no concentration or work-up procedure is required. Even though (HP)LC-MS must be regarded as the method of choice when a complex mixture of different PAs and PANOs are to be analysed quantitatively, still immunoassays must be mentioned as a possibility (Crews et al., 2010).

Using a common structural feature of many toxic PAs, the necine base retronecine, it was demonstrated that retronecine-protein (bovine serum albumin - BSA) conjugate raised antibodies could detect retronecine but also monocrotaline in a competitive inhibition enzyme-linked immunosorbent assay (ELISA) system (Bober et al., 1989). Later the group reported on a similar production of class-specific antibodies to the retronecine moiety using a retronamine-BSA conjugate; an antigen with a higher stability (Bober et al., 1991). Furthermore, they developed the first compound specific antibodies, i.e. to the pyrrolizidine alkaloid monocrotaline. This latter was demonstrated to detect quaternised monocrotaline ($I_{50}$ = 0.25 ppm at pH 7.6), *N*-methylated monocrotaline ($I_{50}$ = 5.3 ppm at pH 7.6), and protonated monocrotaline ($I_{50}$ = 6.0 ppm at pH 6.0). According to the authors, antibodies to

monocrotaline did not cross-react with retrorsine, retrorsine-*N*-oxide, riddelliine or retronecine (Bober et al., 1991).

Roeder and Pflueger (1995) reported on a successful analysis of the 'total alkaloids' (representing the concentrations of sencionine and integerrimine together with their *N*-oxides) as present in zinc dust reduced methanolic extracts of *Senecio rupestris* using antibodies to a retrorsine hemisuccinate-BSA conjugate. Using immunogens based on 'quaternary' (*N*-alkylated) pyrrolizidinium salts linked to either BSA or chicken egg ovalbumin (OVA) by a six carbon bifunctional linking arm, Roseman et al. (1996) developed polyclonal antibodies with a very high specificity towards retrorsine, monocrotaline and retronecine, respectively. ELISA analysis based on these antibodies showed a sensitivity in the µg/kg range using extracts of *Senecio vulgaris* and *Crotalaria retusa* (Roseman et al., 1996).

Lee and co-workers (2001) developed ELISA methods to detect and (semi)quantify PAs in bovine blood and plants. They developed two different systems to detect riddelliine and riddelliine plus riddelliine-*N*-oxide, respectively and investigated their cross reactivities to a number of other PAs such as senecionine, seneciphylline and retrorsine. Even though such cross reactivities were seen, the second system proved to be well suited for the quantification of riddelliine plus riddelliine-*N*-oxide in a mixture of *Senecio riddellii* and alfalfa plant materials. Finally, it should be mentioned that producing monoclonal antibodies against retrorsine conjugated to bovine-thyroglobine, Zündorf et al. (1998) obtained antibodies with a certain affinity to 8 different pyrrolizidine alkaloids and no cross reaction to 12 other such compounds. The authors concluded that beside the necic structure itself the dominant and discriminative epitope consists of the exocyclic ethylidene group of the various diesters.

Until now, four immunoassays have been developed using retrorsine-protein conjugates, three using retronecine-protein conjugates, one using monocrotaline-protein conjugate and one using riddelliine-protein conjugates as reviewed by Lee et al. (2001). Most were based on polyclonal antibodies. Only one of the retrorsine-protein conjugate assays was established with monoclonal antibodies (Zündorf et al., 1998). A major disadvantage is that most established antibodies so far only detect closely related PAs (e.g. 12-membered macrocyclic ester(s) but no otonecines, open chain esters, or 11- or 13 membered macrocyclic PAs) (Zündorf et al., 1998). Concerning the detection and quantification limits, Lee et al. (2001) analysed riddelliine-type PAs in blood with an LOD of 25 µg/l, corresponding to 48 pg riddelliine.

In conclusion, a number of ELISA systems have been developed with different uses, however, nonesuch are available commercially and all suffer from limited cross reactivities preventing more general use.

### 3.2.4. Gas chromatography (GC) and gas chromatography-mass spectrometry (GC-MS)

GC techniques have been widely used in the determination of PAs. The main limitation is that PANOs cannot be analysed directly by GC, since they cannot be volatilised without destruction. Hence, the PANOs need to be converted to the corresponding tertiary PAs by an appropriate upstream reduction step (see 3.2 Extraction and sample preparation). The typical GC-columns used for PA-analysis range from unpolar (100 % dimethyl polysiloxane) to polar stationary phases (14 %-cyanopropyl-methylpolysiloxane) (Witte et al., 1993).

Because of the high resolving power of modern high resolution capillary gas chromatography (HRGC), satisfactory separations of PA-isomers can be achieved and existing retention index data facilitate the identification of individual PAs (Witte et al., 1993). However, one must consider that many PAs exhibit one or more polar groups, mainly hydroxylations, which make high demands on the inertness of the total GC-system to achieve satisfactory peak shapes.

Available retention index data are especially valuable in combination with specific GC-detectors. Frequently, the GC-column is split onto two parallel detectors, flame ionization detector (FID) and nitrogen-phosphorus detector (NPD), which is operated in the nitrogen mode and selectively and

sensitively records nitrogen containing molecules. This combination helps to identify the alkaloids and distinguish from non-nitrogen containing compounds (no NPD signal) and simultaneously the FID signal can be used for quantification.

For PA analysis, GC is most commonly used in combination with mass spectrometric detection in electron impact (EI) mode. The mass spectrum of PAs is dominated by signals unique for the necine base part of the molecule. Hence, PAs show rather characteristic, group specific fragments (diesters, 7- or 9-monoester, otonecine- or saturated-PAs) that assist to elucidate the structure or the mode of ester connection in the molecule (Bredenkamp, 1991). The distinct fragmentation pattern of 1,2-unsaturated PAs can be used to set up dual MS detection methods (alternate switching of single ion monitoring (SIM) and scan modus: SIM/Scan) which allow fast and reliable detection of PAs in complex matrices. The combination of known electron ionization (EI)-MS and retention index data is a powerful tool to unequivocally identify PAs in complex mixtures (Witte et al., 1993).

A method in use requires the conversion of PANOs to the corresponding PAs (zinc in acidic aqueous solution) and uses the characteristic EI-PA-fragments of *m/z* 93, 120, 136 for retronecine type PAs and *m/z* 110, 151, 168 for otonecine type-PAs in SIM/Scan mode, where the scan mode can be used to confirm and identify the corresponding PA via its complete EI-MS. It is an untargeted screening approach and does not require any advance information of expected PAs. A coarse quantification is achieved with two standards senecionine for retronecine type PAs and senkirkine for otonecine type PAs achieving a limit of quantification (LOQ) of 60 µg/kg (Schulzki, 2010).

Some of the structural more complex PAs show rather low intensity or no stable molecular ion peak (Hendriks et al., 1991). In these cases positive or negative chemical ionization was successfully applied to increase the abundance of ions with molecular weight information (Hendriks, et al., 1991) which is especially useful for structure elucidation. However, these techniques are of declining importance with increasing availability of soft ionizations techniques from liquid phases like electrospray ionization (ESI).

To overcome volatilization, separation and peak shape difficulties of multi hydroxylated PAs, several derivatisation reagents were applied in the past. The most common ones are the boronate derivatives for vicinal diols or trimethylsilyl ethers or the combination of both (Wretensjö and Karlberg, 2003; Crews et al., 2010). However, because of general drawbacks of derivatisation in gas chromatography (extra steps, reproducibility, stability, side reactions and incompleteness of derivatisation) these techniques were not widely adopted.

A new GC-MS approach for the detection and quantitation of most of the 1,2-unsaturated PAs as a single sum parameter was introduced in 2008 (Kempf et al., 2008). The sample preparation comprises SCX-SPE, followed by two reduction steps using zinc and $LiAlH_4$ and by a final derivatisation step to yield the corresponding trimethyl silyl ethers of the core necine base structures, which showed desirable chromatographic and spectroscopic properties. During the procedure all individual PAs and PANOs were converted into their respective necine base backbone, retaining the well described structural feature of PA-toxicity, the 1,2-double bond. The resulting derivatives were analysed by GC-MS in the SIM mode and quantification was achieved by adding the internal standard heliotrine and a double work-up strategy. It is an untargeted screening approach and does not require any advance information on expected PAs. The results are expressed as a single sum parameter (retronecine equivalents). The method was applied to honey, pollen and several honey containing foods like mead, sweets, etc and showed a LOD of 10 µg/kg retronecine equivalents (S/N ratio of 7:1) which approximates to 20 µg original PA per kg foodstuff (Kempf et al. 2008, 2010b, 2011a). However, otonecine type PAs are not covered through this approach.

Seed oil products of *Borago officinalis* were analysed by GC-MS following standard PA-extraction and PANO-reduction procedures. The detection limit was reported as 3 ng/5 ml (0.6 µg/l) seed oil (Langer and Franz, 1997). Later, an alternative method applying GC-EI-MS in SIM mode was reported to

analyse the PA-content of crude and refined borage oil. The method comprises liquid-liquid extraction, zinc reduction and double derivatisation including transformation into alkyl boronate derivatives and silyl ethers to improve sensitivity especially for lycopsamine-type PAs to obtain an LOD of 20 µg/kg (Wretensjö and Karlberg, 2003).

The transfer of PAs into eggs has been studied using GC-MS methods. Edgar and Smith (2000) used standard extraction and *N*-oxide reduction procedures. PAs were identified by fast-atom bombardment mass-spectrometry (FAB-MS) and GC-EI-MS. To improve GC-MS separation, the extracts were derivatised by acetylation and combined acetyl-methylboronation. The content of individual PAs in the total alkaloid extracts were estimated by comparing peak areas to an external standard curve generated from authentic lasiocarpine. LODs or LOQs for the overall method were not reported.

In 2008, studies of feeding Japanese quail with aerial parts of *Heliotropium dolosum*, *Heliotropium circinatum* and *Senecio vernalis* also considered the PA transfer to eggs. Sample preparation included defatting with petroleum, methanol extraction, zinc reduction and inert diatomaceous earth work-up. Analysis was performed with GC-MS in full scan mode. Senecionine was used as the external standard for quantitation and a LOD of 0.137 µg/ml was specified (Eröksüz et al., 2008).

In conclusion, HRGC-EI-MS in combination with EI-MS databases and retention index data is a powerful tool to detect and identify PAs in complex matrices, but there are drawbacks in terms of detection limits and analysis of polar (e.g. polyhydroxylated) PAs and especially the incompatibility to directly detect PANOs.

### 3.2.5. Liquid chromatography (LC) and liquid chromatography-(tandem) mass spectrometry (LC-MS/(MS))

High performance liquid chromatography (HPLC) and liquid chromatography (LC) separation of PAs has gained more and more interest, especially through the increasing availability of LC-MS instruments. LC offers the advantage of a simultaneous detection of PAs and PANOs that means fewer steps of sample preparation and a reduced risk of alteration of the analytes (Brown et al., 1994). Crews et al. (2010) have reviewed the recent literature on LC and LC-MS detection of PAs/PANOs. In summary, $C_{18}$-based stationary phases and either acids (mainly formic or acetic acid) or bases (ammonium hydroxide or diethylamine) were used as modifiers for the mobile phases (organic part of the system was mainly methanol or acetonitrile). Because PAs and PANOs do not show characteristic ultraviolet (UV) spectra (exhibiting only a non-specific UV-maximum of 214 nm), simple UV detection is of limited value. However, LC techniques are frequently and successfully used to purify PAs for structural identification including the enantioseparation of isomers like intermedine/lycopsamine (Hartmann et al., 2004; Pawar et al., 2010). Recently, a HPLC approach was published where retronecine-type PAs are converted by pre-column derivatisation to a common core structure derivative (7-methoxy-1-methoxymethyl-6,7-dihydro-5H-pyrrolizine) and analysed by reverse phase HPLC with photodiode array detection (HPLC-diode array detection (DAD) (absorption maximum at 223 nm)). The LOQ reported for standard compounds was around 1 ng/mL. This sum parameter method was applied to determine the total retronecine-type PA content of plant extracts from *Ligularia* and *Senecio* spp. in the range of 0.01 to 0.15 % (Xiong et al., 2009c).

Coupling of HPLC with MS-instruments for the analysis of PAs and PANOs has become the method of choice in recent years. Besides occasional reports of early or less frequently used ionization techniques, electrospray ionization (ESI) is currently dominating (Crews et al., 2010). It is particularly appropriate because of the polarity of the PAs and especially the PANOs (Crews et al., 2010). The commonly used atmospheric-pressure chemical ionization (APCI) technique showed good stability for PA-analysis but tends to have lower sensitivity for polar PANOs (Beales et al., 2004). Most frequently, ion traps or triple quadrupole instruments are used for detection. Just as in electron impact (EI), collision-induced dissociation (CID) results in PA-type dependent key fragments which are useful for the development of compound/class specific MS-methods (like single reaction monitoring (SRM), multiple reaction

monitoring (MRM) or precursor ion scan (PIS)) for the specific and sensitive detection of PAs in complex matrices, lowering the need of tedious sample preparation.

Many of the latest published methods are taking advantage of the characteristic CID fragment ions of *m/z* 94, 120/118 and 138/136 for 1,2-unsaturated PAs/PANOs respectively or *m/z* 122, 150 and 168 for 1,2-unsaturated otonecine-type PAs and pursue MRM approaches on triple quadrupole analysers for a selective and sensitive detection (Xiong et al., 2009b; Zhou et al., 2010; Kempf et al., 2011b; Dübecke et al., 2011; Hoogenboom et al., 2011). Furthermore, UHPLC was successfully applied to increase chromatographic resolving power and sensitivity, and at the same time decreasing the analytical run-time (Xiong et al., 2009a; Zhou et al., 2010; Hoogenboom et al., 2011). In addition, on the mass spectrometer side, the increased availability of high resolving mass analysers that provide accurate mass information were successfully introduced to the field (Crews et al., 2009; Xiong et al., 2009b). The main advantages of these instruments are applications where PAs or derivatives with unknown structure need to be analysed, like in metabolism or toxicology studies of PAs. Whether the high resolution capabilities can improve selectivity and sensitivity of existing methods or reduces sample preparation time remains to be seen.

Another recent LC-MS approach is to perform simultaneous screening and quantification. PIS utilising the characteristic fragment ions of 1,2-unsaturated PAs is combined with MRM experiments. This approach allows the simultaneous detection and quantification of known/expected PAs (MRM) and detection of unexpected/unknown 1,2-unsaturated PAs in a single run (Zhou et al., 2010). The specificity of this detection method was evaluated with 7 standard compounds and the analytes could be detected below concentrations of 0.05 µg/mL. PANOs were only tentatively covered. The LOQs of the simultaneous MRM approach for known PA targets were indicated between 0.8-36 pg/ml for diluted stock solutions.

A complete UHPLC-MS/MS based method comprising sample workup, detection and quantification of PA-contamination of fodder/hay/silage by plant parts of the genus *Senecio* was presented recently and showed a LOD of 3-10 µg/kg and a LOQ of 10-30 µg/kg (Mulder et al., 2009). It can be presumed that similar approaches and detection limits can be utilized for PA-contamination in vegetables, salads, herbs or plant based remedies for human consumption.

Several HPLC-MS based methods have been published using targeted MRM approaches to detect 1,2-unsaturated PAs and PANOs in honey or pollen. Sample preparation includes either SPE on SCX material or online pre-concentration on reversed phase-material (Betteridge et al., 2005; VWA, 2007; Dübecke et al., 2011; Kempf et al., 2011b).

Betteridge et al. (2005) utilized pre-concentration by SCX-SPE and MS/MS quantification as lasiocarpine/lasiocarpine-*N*-oxide equivalents with an ion-trap mass spectrometer. No LOD/ LOQs were given but the lowest detected amount for an individual PA was 5 µg/kg.

A similar approach was reported from Dübecke et al. (2011). The method comprises SCX-SPE and MRM detection with a triple quadrupole mass spectrometer. The LOQ was 1 µg/kg for echimidine and senkirkine, 2 µg/kg for heliotrine and 3 µg/kg for lycopsamine, retrorsine, senecionine and seneciphylline. Quantification for these reference PAs was obtained by external calibration and non-reference PAs were included into the quantitation using ESI-MS data from literature and calibration curves of structural related PAs.

A modified sample preparation was reported by Kempf et al. (2011b). A QuEChERS [16] workup is combined with an automated online concentration prior to HPLC separation and triple quadrupole mass spectrometric detection. A LOQ (S/N 10:1) of 1 µg/kg for 14 out of 16 target PAs and PANOs (except monocrotaline and monocrotaline-*N*-oxide: LOD 50 µg/kg) was achieved. Recoveries ranged from

[16] QuEChERS: Quick, Easy, Cheap, Effective, Rugged and Safe; acronym coined for an analytical approach to determine pesticide residues.

97.5 % (echimidine-*N*-oxide) to 104.6 % (lycopsamine-*N*-oxide) and the relative standard deviation from 4.9 % (heliotrine-*N*-oxide) to 38.8 % (seneciphylline). The LC-MS methods reported by Kempf et al. (2011b) and Dübecke et al. (2011) were applied to analyse the PA content of several thousands bulk honey samples.

In the framework of a carry-over study of PAs of *Jacobaea vulgaris* (ragwort) into milk, an LC-MS/MS method was developed to measure PA and PANO residues in milk (Hoogenboom et al., 2011). Milk samples were mixed with acidic methanol and the fat fraction was removed by a freezing out step. Subsequently, the samples were concentrated by evaporating of the solvent. Heliotrine was added as internal standard to correct for daily recovery and a LOQ for individual compounds between 0.05 and 0.2 µg/l was achieved

Methods for the detection of PAs in animal tissues are rare. Fletcher et al. (2011) used LC-MS/MS for the analysis of PAs in tissues of cows fed with *Crotalaria novae-hollandiae*, *Heliotropium amplexicaule* or *Senecio brigalowensis*. Details of the method were not disclosed, but the LOD was stated as being 1 µg/kg for individual PAs.

### 3.3. Availability of reference standards

The large structural variety of naturally occurring PAs is a challenge for a comprehensive trace analysis and quantification of PAs in feed and food and the availability of standards represent a bottleneck. Only a fraction of the known naturally occurring PAs is commercially available as certified reference standards (see Table 2). Concerning corresponding *N*-oxides the situation is even worse. In addition, isotopically labelled standards for PAs/PANOs, which would be useful as internal standards in LC-MS/MS approaches, are not available.

**Table 2:** Available reference standards as reported in the latest publications on PA-analysis in food and feed. The list does not claim to be exhaustive.

| Compound | CAS Number | Literature references |
|---|---|---|
| Echimidine | 520-68-3 | 1, 2 |
| Heliotrine | 303-33-3 | 1, 2, 3, 4 |
| Lasiocarpine | 303-34-3 | 1 |
| Lycopsamine | 10285-07-1 | 1, 2 |
| Monocrotaline | 315-22-0 | 1, 2, 3 |
| Otosenine | 16958-29-5 | 4 |
| Retrorsine | 480-54-6 | 1, 2, 3, 4 |
| Retrorsine-*N*-oxide | 15503-86-3 | 4 |
| Senecionine | 130-01-8 | 1, 2, 3, 4 |
| Senecionine-*N*-oxide | 13268-67-2 | 2, 4 |
| Seneciphylline | 480-81-9 | 1, 2, 3, 4 |
| Seneciphylline-*N*-oxide | 38710-26-8 | 2, 4 |
| Senkirkine | 2318-18-5 | 1, 2, 3, 4 |

[1] Kempf et al., 2011b
[2] Dübecke et al., 2011
[3] Kempf et al., 2010b
[4] Hoogenboom et al., 2011

So far, individual marker PAs that allow screening for most of the relevant plant species, involved in known PA-contaminations of food and feed e.g. *Senecio, Echium, Crotalaria and Eupatorium* spp. are available. On the other hand, much more standard-PAs would be needed for accurate quantification approaches (e.g. the main *Jacobaea vulgaris*-PAs such as jacobine, jaconine, jacoline and jacozine, erucifoline and acetylerucifoline are not yet available). In those cases, in-house standards and/or correlations to known PA-standards are applied to estimate the total PA-content. Less frequent PAs like amabiline/supinine or platyphylline-type PAs are not available either.

### 3.4. Summary of analytical methods

In general, colorimetric detection or TLC separation in combination with PA-detection via Ehrlich's reagent might be still suitable methods for an untargeted PA-screening, but they lack sensitivity and selectivity for the trace analysis of PAs in complex matrices. Immunological approaches on the other hand, are only sensitive for a narrow range of structurally closely related PAs. Currently, only MS provides the prerequisites to analyse PAs at trace levels. Basically, two MS-based approaches are applied. There are multiple variants of LC-MS/MS methods and in addition a sum parameter method based on GC-MS has been developed. In all cases, pre-concentration and sample clean-up prior to analysis are required.

LC-MS/MS methods offer low detection limits of approximately 1 µg/kg or less and the ability to analyse PAs and PANOs simultaneously in one run as the main advantages. In general, LC-MS offers the possibility for a reduced or automated sample workup, which will result in faster turn-around times and a higher efficiency. It was demonstrated that the PA-analysis could be integrated into a QuEChERS workflow (Kempf et al., 2011b). The major disadvantage of this approach is that most methods use a targeted MS/MS setup and relevant PAs may be missed, and for accurate quantitation (certified) reference standards are essential. To date not more than approx. 10 different PAs are commercially available, while the number of available PANOs is even lower. Isotopically labelled compounds are not commercially available at all. In addition, this is unlikely to change since there is no simple chemical synthesis or *in vitro* biosynthesis and isolation possible. PIS in LC-MS-based methods provide the ability to detect unknown/unexpected PAs (Zhou et al., 2010) but still lack accurate quantitation.

On the other hand, the sum parameter method based on GC-MS, uses one (true) internal standard and quantitation is simplified, since only two peaks need to be evaluated. It offers the general possibility to introduce a stable isotope labelled precursor since most 1,2-unsaturated PAs (except otonecine type PAs) are converted into one single substance, the chance to miss unknown/unexpected PAs is low and no information concerning the PA-plant source is required prior to analysis. It was demonstrated, that this principle could be easily adjusted to analyse PAs in different matrices (Kempf et al., 2011a). The main drawbacks of this approach are a higher detection limit of 10 µg/kg retronecine equivalents (corresponding to approximately 20 µg PA/kg), elaborate sample preparation, and information on the PA/PANO ratio is lost during the transformation into the single sum parameter.

Currently, both approaches supplement each other. The methods in use are approximations to quantify PAs in complex mixtures. Especially the reliable availability of PA-reference compounds needs to be improved in the future.

So far, no inter-laboratory validated methods have been published, nor have any proficiency tests been performed or organized, for the matrices of interest.

# 4. Occurrence of pyrrolizidine alkaloids in food and feed

## 4.1. Previously reported occurrence results

### 4.1.1. Food

PAs are of exclusive plant origin. This, in turn, means that any food or feed, that contains (parts of) PA-plants, will contain PAs. As a general rule, the closer the relation of the food to the PA-plant(s), the higher will be the PA-level in the final product.

Therefore, the highest PA-levels occur in PA-plants and herbal mixtures containing PA-plants, which might be consumed as herbal remedies, supplements, salads contaminated accidentally with PA-plants or grains contaminated with PA-plant seeds. For example, the latest documented PA-outbreak (Kakar et al., 2010) could be tracked to wheat flour contaminated with *Heliotropium* seeds and qurut, a cheese common throughout Central Asia (here derived from goats milk) which also showed *Heliotropium* PAs and trichodesmine, a PA typical for *Crotalaria* spp. Recently, PAs were repeatedly detected in packed salads and the contamination could be traced back to the presence of accompanying *Senecio vulgaris* plant parts (BfR, 2007).

The focus of recent years was directed towards the PA-content of bee-derived products (Kempf et al., 2010a). Below, the results of studies that cover a larger number of PAs in the analytical method and/or samples will be summarized.

Pollen of PA plants may contain similar PA-concentrations as observed for the originating plant tissue. 70–95 % of the PAs were present in the *N*-oxide form and levels up to 4.1 mg/g pollen were reported for *Senecio* spp. pollen (Kempf et al., 2010b).

A first study on pollen products, containing largely unaltered bee pollen baskets, which are sold/used as food supplements, was published in 2010 (Kempf et al., 2010b). In total, 55 commercially available pollen products were analysed. Seventeen samples (31 %) contained 1,2-unsaturated PAs in the range from 1080 to 16,350 µg/kg (average 5179 µg/kg), calculated as retronecine equivalents.

A second study analysed 119 pollen samples by LC-MS/MS (Dübecke et al., 2011). Sixty percent of the samples were found to be PA-positive, and the total sum of detected PAs ranged from 11 to 37,855 µg/kg (average 1846 µg/kg). In both studies *Echium* spp. were identified by a large margin as the responsible plant source, followed by *Eupatorium* and occasionally *Senecio* spp.

Because of the dilution with bee pollen baskets not containing PA-plant pollen, the PA levels in the retail pollen products are lower as compared to pure PA-plant pollen.

Three studies have been published containing data on the PA and PANO content of bulk and retail honey samples (VWA, 2007; Kempf et al., 2008; Dübecke et al., 2011).

In 2006, a set of 171 honey samples was collected from the Dutch market and analysed for the presence of PAs (VWA, 2007). The set comprised retail honeys (imported blended honeys) as well as honeys obtained from local producers. Samples were analysed by LC-MS/MS for the presence of 11 PAs representing mainly *Senecio* spp. as plant source. The LOQ for the different PAs varied between 0.5 and 2 µg/kg. In 43 honey samples (28 %) PAs were detected in amounts varying between 1 and 365 µg/kg. The average content in the samples was 6.9 µg/kg. In two samples a total PA concentration exceeding 100 µg/kg was detected: a flower honey containing 365 µg/kg and an erica honey containing 212 µg/kg. Senecionine was the PA found with the highest incidence (34 samples). It should be noted that in this study lycopsamine/echimidine type PAs were not analysed. Furthermore, the origin of the samples in most cases was not indicated.

Kempf et al. (2008) analysed 216 retail honeys mainly of the German market with the GC-MS sum parameter method. Among them 19 samples (8.7 %) contained PAs, in the range of 19 to 120 µg/kg (average 56 µg/kg), calculated as retronecine equivalents (Kempf et al., 2008).

The third study analysed a total of 3917 honey samples (mainly bulk honey) including 696 samples of retail honey (mainly from the German market) of which 94 % were found to be PA positive showing concentrations between 1 and 267 µg/kg. The average PA-concentration in PA-positive retail honeys was 26 µg/kg and the median was at 19 µg/kg. A total of 2839 bulk honey samples (traded as bulk, before blending, portioning, filling and packaging) were analysed. In 68 % of the samples, PAs were found at concentrations ranging from 1 to 1087 µg/kg. The average PA concentration of the PA-positive samples was 67 µg/kg (median 27 µg/kg), and 46 µg/kg including the PA-negative honeys. Due to blending of bulk honey, the average PA-concentration of retail honey was about 2.5 times lower than PAs in bulk honey. On the other hand, the rate of PA-positive samples increased from 68 % of the bulk to 94 % of retail honeys. Dübecke et al. (2011) evaluated the PA-results in correlation to the geographic origin of the tested bulk honey samples. Especially honey from South and Central America showed the highest rates of PA-positive samples and average PA-content (40 to 100 % and 50 to 100 µg/kg, respectively), followed by countries of mainly southern Europe with high positive rates but lower average PA-content (80-95 % and 25-35 µg/kg), respectively) and countries of central and eastern Europe with lower PA-positive rates and low average PA-concentrations (20-60 % and 5 µg/kg, respectively). In accordance to the analytical results from pollen products, *Echium* spp. were identified by a large margin as the main responsible plant source, followed by *Eupatorium* and occasionally *Senecio* spp. in the PA-positive honey samples.

In comparison to pollen, it can be concluded that retail honey contained 30 to 100 times less PAs than pollen products (Kempf et al., 2008, 2010b; Dübecke et al., 2011).

Commission Decision 2011/163/EU[17] establishes a list of third countries from which Member States are authorised to import products of animal origin. Currently, 38 third countries are listed with respect to honey. In 2010 more than 148,000 tonnes natural honey were imported into the 27 EU Member States.[18] This is slightly higher than the imported quantities in 2009 and 2008 that amounted to 137,000 and 142,000 tonnes, respectively. Figure 4 shows the contributions from those countries of origin with a share of more than 1 % of total imported honey in 2010.

[17]Commission Decision 2011/163/EU of 16 March 2011 on the approval of plans submitted by third countries in accordance with Article 29 of Council Directive 96/23/EC; OJ L 70, 17.3.2011, p. 40-46.
[18]Source: Eurostat (http://epp.eurostat.ec.europa.eu/portal/page/portal/eurostat/home/).

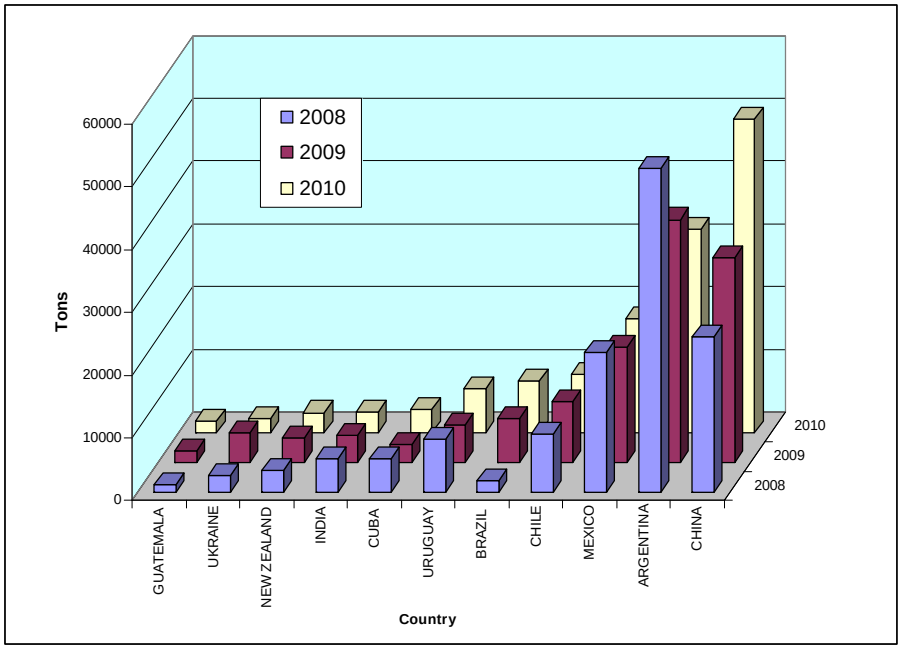


**Figure 4:** Countries of origin contributing >1 % of honey imported into the EU 27 (in tonnes).

Geographic background information on bee-derived products is of great importance since some European countries import substantial amounts to meet the domestic market demands (Statistics Eurostat). For example, countries like Belgium, Germany, UK, Ireland or The Netherlands import honey in the range of 78 to 93 % while other countries like Bulgaria, Greece, Hungary or Portugal meet the demands almost exclusively by domestic production (see Figure 5).

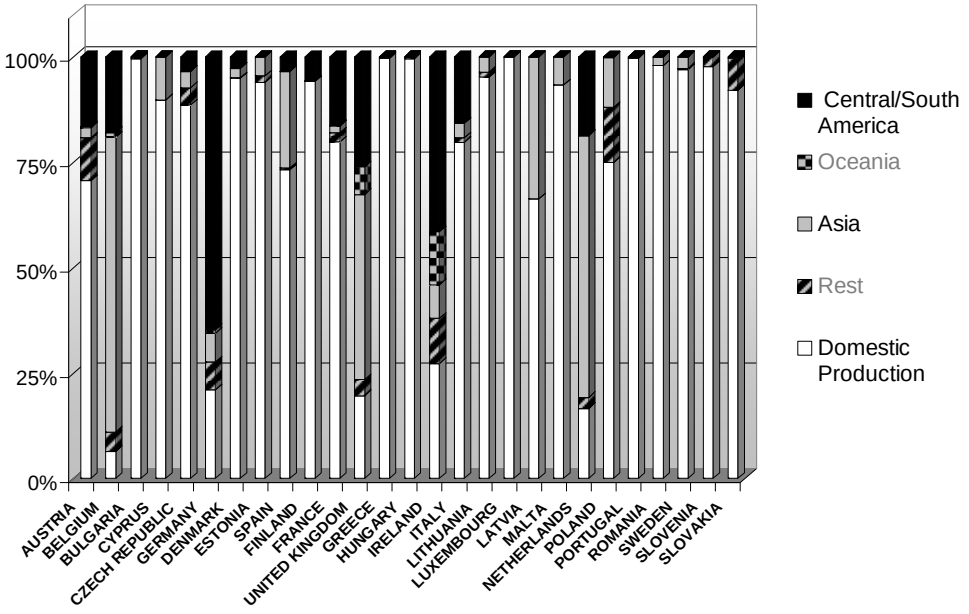


**Figure 5:** Geographic origin of honey for the different national markets in the EU (2010).

Recent studies on the PA-content mainly on bulk honey imported to Germany have demonstrated that at least for the time period of investigation, honey of Central and South America showed high rates of PA-positive samples and at the same time high individual PA-levels (Dübecke et al., 2011). As shown

in Figure 6, the German, Irish and to some extent the UK honey markets are influenced by substantial amounts of honey imported from Central or South America and may therefore experience higher PA-levels in retail honey than countries with different honey markets.

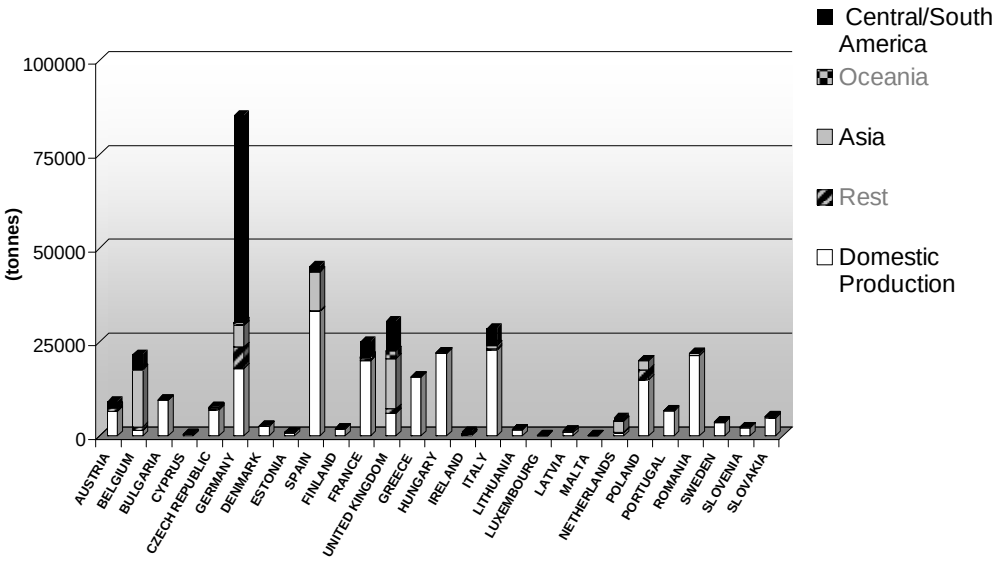


**Figure 6:** Amount (tonnes) and geographic origin of honey on the national markets in the EU in 2010.

In addition, since most of the data published or submitted to EFSA reflected only the German market or bulk honey imported to Germany, only little evidence can be derived for other main honey producing regions (e.g. Asia, especially China, see Figure 4) and EU-countries that mainly import from Asia (like Belgium, UK or The Netherlands).

Similar is true for countries where local demands are met by domestic production but for which no PA-analysis results were available. There is first evidence that honey in Spain contains *Echium* spp. pollen, which in turn could result in PA-contamination of the respective honeys (Kempf et al., 2010a), but no data on local honeys of these areas is available at present.

A recent limited study with small sample sets demonstrated the downstream PA-contamination in the food chain by honey containing PAs (Kempf et al., 2011a). Several food types such as mead (n = 19), candy (n = 10), fennel honey (n = 9), soft drinks (n = 5), power bars and cereals (n = 7), jelly babies (n = 3), baby food (n = 3), supplements (n = 3) and fruit sauce (n = 1) containing honey as an ingredient in the range of 5 % to 37 % were analysed by a GC-MS sum parameter method. Eight out of 60 retail samples (13 %) were tested PA-positive. PAs were found in mead, candy and fennel honey. The PA-concentration ranged from 10 µg/kg to 484 µg/kg (average 100 µg/kg).

Bee derived products are the only group of foodstuffs for which wide-ranging studies are available. Although carry-over of plant PAs into food of animal origin could be demonstrated for milk/milk products, eggs or meat, no comprehensive studies for these products are available that would allow further risk evaluation. The same applies for salads, flour, vegetables/spices or herbal mixtures and teas, where accidental PA-plant contamination occurred in the past.

### 4.1.2. Feed

A survey was conducted in The Netherlands on the presence of PAs in animal forage (Mulder et al., 2009). In the framework of the Dutch National Monitoring Plan on animal feedingstuffs in total

147 samples of grass silage, hay, dried grass and alfalfa were collected during 2006-2008 and analysed by LC-MS/MS. The method comprised 40 macrocyclic PAs and PANOs of the senecionine-type and the LOD reported was 10 µg/kg for individual PAs. In 31 out of 147 samples (21 %) PAs were detected in amounts varying between 10 and 5401 µg/kg, with an average content of 121 µg/kg. Large differences in PA content were found between the various forage categories. In grass silage PAs were only rarely detected; in only three out of 56 samples were measurable amounts of PAs found (maximum content 28 µg/kg). The 37 hay samples (mostly obtained from nature reserves) did not contain PAs except for one sample containing 549 µg/kg. Of the 23 (artificially) dried grass samples, only four contained PAs, with the highest concentration being 288 µg/kg. A high PA incidence was found in the alfalfa (lucerne) category. Of 31 samples analysed, 23 (74 %) contained at least traces of one or more PAs. In 16 samples relatively low amounts were found (between 10 and 100 µg/kg), while in four samples the amount was between 100 and 1000 µg/kg. In three samples (10 %) high amounts of PAs were detected, respectively 3524, 3765 and 5401 µg/kg. The average PA concentration found in alfalfa samples was 455 µg/kg, compared to 15 µg/kg or less for the other categories. This study focussed particularly on the PAs present in *Jacobaea vulgaris* (ragwort), *Senecio vulgaris* (common groundsel) and *S. inaequidens* (narrow leafed ragwort). Comparison with reference plants indicated that in most cases the forage samples, and in particular the alfalfa samples, were contaminated with *S. vulgaris*, with other ragwort species present in only two samples.

## 4.2. Current occurrence results

### 4.2.1. Data collection summary

On 20 October 2010, EFSA published a call for data covering mycotoxins and phytotoxins, including PAs. No replies for PAs had been received by the deadline of 15 December 2010 and the call was extended to 17 January 2011, with the same result. The industry was contacted with the assistance of the Confederation of the Food and Drink Industries of the European Union (now FoodDrinkEurope) and two submissions were received covering the presence of a range of PAs in bulk honey as well as in retail honey. Both submissions were received from the same Member State (Germany) and thus the results cannot be regarded as being representative for the occurrence of PAs in honey across the European Union. A submission was also received from the National Competent Authority in a second Member State covering PAs in feed.

### 4.2.2. Distribution of samples across food categories and feed

Overall, results for 14,604 samples of honey were reported to EFSA of which 13,280 samples concerned bulk honey, which could be comparable to honey purchased locally from a single source, and 1324 samples covered retail honey that is mostly blended and ready for consumption. In addition, 351 results were reported for a variety of different feed samples. Results for a total of 64 different PAs (with another 10 identified only as an iso-form since the specific structure was not identified) were reported, but not all samples were analysed for the whole range of substances. The range of substances covered for the two food categories and the overall feed category is shown in Table 3.

### 4.2.3. Analytical methods used

The method used for the analyses of PAs was given as LC-MS/MS for all samples. The LOD for food samples was reported as 0.5 µg/kg and the LOQ as 1.0 µg/kg for most PAs except for heliotrine (0.5-1.0; 1.0-2.0), lycopsamine (0.5-2.0; 1.0-3.0), retrorsine (0.5-2.0; 1.0-3.0), seneciphylline (0.5-2.0; 1.0-3.0) and senecionine (0.5-2.0; 1.0-3.0). The LOD for all feed samples was reported as 4.5 µg/kg for all PAs after correction for the dry weight matter (to 88 %). The LOQ was not given for feed samples.

**Table 3:** The number of samples analysed for the range of PAs for the two food categories (BH = bulk honey and RH=retail honey) and the overall feed category.

| Substance | BH | RH | Feed | Substance | BH | RH | Feed | Substance | BH | RH | Feed |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Acetylechimidine | 4897 | 1116 | 351 | Florosenine | | | 351 | Lasiocarpine-*N*-oxide | | | 351 |
| Acetylechimidine-*N*-oxide | 4897 | 1116 | 351 | Heleurine-*N*-oxide | | | 351 | Lycopsamine | 13,280 | 1324 | 351 |
| Acetylechiumine-*N*-oxide | 4897 | 1116 | | Heliotrine | 13,280 | 1324 | 351 | Lycopsamine-*N*-oxide | 8383 | 208 | 351 |
| Acetylerucifoline | | | 351 | Heliotrine-*N*-oxide | 8383 | 208 | 351 | Monocrotaline | | | 351 |
| Acetylerucifoline-*N*-oxide | | | 351 | Integerrimine | | | 351 | Monocrotaline-*N*-oxide | | | 351 |
| Acetyllycopsamine | | | 351 | Integerrimine-*N*-oxide | | | 351 | Onetine | | | 351 |
| Acetyllycopsamine-*N*-oxide | | | 351 | Iso-acetylechimidine[(a)] | | | 351 | Otosenine | | | 351 |
| Acetylseneciphylline | | | 351 | Iso-acetylechimidine-N-oxide | | | 351 | Retrorsine | 13,280 | 1324 | 351 |
| Acetylseneciphylline-*N*-oxide | | | 351 | Iso-acetyllycopsamine | | | 351 | Retrorsine-*N*-oxide | 8383 | 208 | 351 |
| Dehydrojaconine | | | 351 | Iso-acetyllycopsamine-N-oxide | | | 351 | Riddelliine | | | 351 |
| Desacetyldoronine | | | 351 | Iso-echimidine | | | 351 | Riddelliine-*N*-oxide | | | 351 |
| Doronine | | | 351 | Iso-echimidine-*N*-oxide | | | 351 | Senecionine | 13,280 | 1324 | 351 |
| Echimidine | 13,280 | 1324 | 351 | Iso-lycopsamine-1 | | | 351 | Senecionine-*N*-oxide | 8383 | 208 | 351 |
| Echimidine-*N*-oxide | 13,280 | 1324 | 351 | Iso-lycopsamine-2 | | | 351 | Seneciphylline | 13,280 | 1324 | 351 |
| Echiumine | 4897 | 1116 | 351 | Iso-lycopsamine-*N*-oxide-1 | | | 351 | Seneciphylline-*N*-oxide | 8383 | 208 | 351 |
| Echiumine-*N*-oxide | 4897 | 1116 | 351 | Iso-lycopsamine-*N*-oxide-2 | | | 351 | Senecivernine | | | 351 |
| Echiuplatine | 4897 | 1116 | | Jacobine | | | 351 | Senecivernine-*N*-oxide | | | 351 |
| Echiuplatine-*N*-oxide | 4897 | 1116 | | Jacobine-*N*-oxide | | | 351 | Senkirkine | 13,280 | 1324 | 351 |
| Echiovulgarine | 4897 | 1114 | | Jacoline | | | 351 | Spartioidine | | | 351 |
| Echiovulgarine-*N*-oxide | 4897 | 1114 | | Jacoline-*N*-oxide | | | 351 | Spartioidine-*N*-oxide | | | 351 |
| Erucifoline | | | 351 | Jaconine | | | 351 | Trichodesmine | | | 351 |
| Erucifoline-*N*-oxide | | | 351 | Jaconine-*N*-oxide | | | 351 | Trichodesmine-*N*-oxide | | | 351 |
| Europine | | | 351 | Jacozine | | | 351 | Usaramine | | | 351 |
| Europine-*N*-oxide | | | 351 | Jacozine-*N*-oxide | | | 351 | Usaramine-*N*-oxide | | | 351 |
| Floridanine | | | 351 | Lasiocarpine | 8383 | 208 | 351 | | | | |

(a): The prefix iso denotes that an additional isomer with unknown stereochemistry of the indicated compound has been reported. In the case of lycopsamine and lycopsamine-*N*-oxide two additional isomers with unknown stereochemistry have been reported. Possible isomers of lycopsamine are intermedine, echinatine, rinderine, indicine. A possible isomer of echimidine is heliosupine.

### 4.2.4. Occurrence data by food and feed category

#### 4.2.4.1. Food

The substitution method was used for expressing left-censored results, i.e. results at or below the LOD or the LOQ. The lower bound (LB) was calculated by setting left-censored results to zero while the upper bound (UB) was calculated by setting left-censored data to the respective detection or quantification limits according to the reporting. The statistical descriptions for the distribution of the submitted analytical results for PAs in bulk and retail honey are shown in Table 4. It should be noted that only left-censored results were reported for acetylechiumine-*N*-oxide except for one result on bulk honey. For bulk honey, the lowest proportion of left-censored results was recorded for lycopsamine at 49 %, followed by echimidine and echiumine at 56 % and 71 %, respectively. For retail honey, the situation was slightly different with the lowest proportion of left-censored results recorded for echimidine at 16 %, followed by lycopsamine and echiumine at 36 % and 45 %, respectively. Apart from acetylechiumine-*N*-oxide, there were a further eleven PAs with only left-censored results reported for the retail honey of which six also reported 99 % left-censored data for the bulk honey. As an exception for these six compounds and for acetylechiumine-*N*-oxide, where it was considered likely that most results would approach zero, both the lower and upper bound was set to zero for all left-censored results.

The average levels of the different PAs for bulk honey varied between 0-9.7 µg/kg for the lower bound and 0.1-10 µg/kg for the upper bound, and for retail honey between 0-6.5 µg/kg for the lower bound and 1-6.7 µg/kg for the upper bound. The maximum levels of PAs for bulk honey were reported for echimidine-*N*-oxide at 2031 µg/kg, echimidine at 1522 µg/kg, lycopsamine at 1448 µg/kg and seneciphylline-*N*-oxide at 1441 µg/kg. Maximum levels for retail honey were much lower with echimidine at 150 µg/kg, lycopsamine at 126 µg/kg and echiuplatine at 115 µg/kg.

The two submissions covering results of PAs found in honey included testing of different ranges of alkaloids. The submissions had eight PAs in common marked 8 in Table 4 (echimidine, echimidine-*N*-oxide, heliotrine, lycopsamine, retrorsine, senecionine, seneciphylline and senkirkine), while one submission included a further six marked 14 in Table 4 (heliotrine-*N*-oxide, lasiocarpine, lycopsamine-*N*-oxide, retrorsine-*N*-oxide, senecionine-*N*-oxide and seneciphylline-N-oxide) giving a total of fourteen and the other a further nine, marked 17 in Table 4 (acetylechimidine, acetylechimidine-*N*-oxide, acetylechiumine-*N*-oxide, echiumine, echiumine-*N*-oxide, echiuplatine, echiuplatine-*N*-oxide, echivulgarine and echivulgarine-*N*-oxide) giving a total of seventeen.

**Table 4:** Lower (LB) and upper (UB) bound mean, maximum and respective percentile (P) levels of PAs in bulk and retail honey in µg/kg and the proportion of left-censored samples (LC %). The number in brackets after the substance name indicates if analysed for all samples (8) or only for some samples (included in groupings of 14 and 17 only).

| Substance | | Bulk honey | | | | | | Retail honey | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | LC % | Mean | P50 | P95 | P99 | Max | LC % | Mean | P50 | P95 | P99 | Max |
| Acetylechimidine (17) | LB | 79 | 1.0 | 0 | 4.0 | 18 | 176 | 69 | 0.4 | 0 | 2.0 | 3.0 | 7.0 |
| | UB | | 1.8 | 1.0 | 4.0 | 18 | 176 | | 1.1 | 1.0 | 2.0 | 3.0 | 7.0 |
| Acetylechimidine-*N*-oxide (17) | LB | 98 | 0.1 | 0 | 0 | 2.0 | 67 | 100 | 0 | 0 | 0 | 0 | 0 |
| | UB | | 1.1 | 1.0 | 1.0 | 2.0 | 67 | | 1.0 | 1.0 | 1.0 | 1.0 | 1.0 |
| Acetylechiumine-*N*-oxide (17) | LB | 100 | 0 | 0 | 0 | 0.0 | 1.0 | 100 | 0 | 0 | 0 | 0 | 0 |
| | UB | | 0 | 0 | 0 | 0 | 1.0 | | 0 | 0 | 0 | 0 | 0 |
| Echimidine (8) | LB | 56 | 9.7 | 0 | 47 | 145 | 1522 | 16 | 6.5 | 3.0 | 25 | 50 | 150 |
| | UB | | 10 | 1.0 | 47 | 145 | 1522 | | 6.7 | 3.0 | 25 | 50 | 150 |
| Echimidine-*N*-oxide (8) | LB | 94 | 1.7 | 0 | 1.0 | 34 | 2031 | 98 | 0 | 0 | 0 | 1.0 | 7.0 |
| | UB | | 2.7 | 1.0 | 1.0 | 34 | 2031 | | 1.0 | 1.0 | 1.0 | 1.0 | 7.0 |
| Echiumine (17) | LB | 71 | 1.1 | 0 | 6.0 | 16 | 56 | 45 | 1.1 | 1.0 | 4.0 | 8.0 | 35 |
| | UB | | 1.8 | 1.0 | 6.0 | 16 | 56 | | 1.6 | 1.0 | 4.0 | 8.0 | 35 |
| Echiumine-*N*-oxide (17) | LB | 98 | 0 | 0 | 0 | 1.0 | 13 | 100 | 0 | 0 | 0 | 0 | 0 |
| | UB | | 1.0 | 1.0 | 1.0 | 1.0 | 13 | | 1.0 | 1.0 | 1.0 | 1.0 | 1.0 |
| Echiuplatine (17) | LB | 84 | 2.3 | 0 | 7.0 | 59 | 381 | 73 | 1.0 | 0 | 3.0 | 11 | 115 |
| | UB | | 3.1 | 1.0 | 7.0 | 59 | 381 | | 1.7 | 1.0 | 3.0 | 11 | 115 |
| Echiuplatine-*N*-oxide (17) | LB | 99 | 0 | 0 | 0 | 0 | 22 | 100 | 0 | 0 | 0 | 0 | 0 |
| | UB | | 0 | 0 | 0 | 0 | 22 | | 0 | 0 | 0 | 0 | 0 |
| Echivulgarine (17) | LB | 89 | 1.6 | 0 | 6.0 | 40 | 427 | 84 | 0.4 | 0.0 | 2.0 | 7.0 | 11 |
| | UB | | 2.5 | 1.0 | 6.0 | 40 | 427 | | 1.2 | 1.0 | 2.0 | 7.0 | 11 |
| Echivulgarine-*N*-oxide (17) | LB | 97 | 0.2 | 0 | 0 | 3.0 | 45 | 100 | 0 | 0 | 0 | 0 | 1.0 |
| | UB | | 1.1 | 1.0 | 1.0 | 3.0 | 45 | | 1.0 | 1.0 | 1.0 | 1.0 | 1.0 |
| Heliotrine (8) | LB | 99 | 0.1 | 0 | 0 | 2.0 | 235 | 100 | 0 | 0 | 0 | 0 | 2.0 |
| | UB | | 0.1 | 0 | 0 | 2.0 | 235 | | 0 | 0 | 0 | 0 | 2.0 |
| Heliotrine-*N*-oxide (14) | LB | 99 | 0.1 | 0 | 0 | 0 | 68 | 100 | 0 | 0 | 0 | 0 | 0 |
| | UB | | 0.1 | 0 | 0 | 0 | 68 | | 0 | 0 | 0 | 0 | 0 |
| Lasiocarpine (14) | LB | 99 | 0.1 | 0 | 0 | 0 | 268 | 100 | 0 | 0 | 0 | 0 | 0 |
| | UB | | 0.1 | 0 | 0 | 0 | 268 | | 0 | 0 | 0 | 0 | 0 |
| Lycopsamine (8) | LB | 49 | 9.5 | 1.0 | 41 | 120 | 1448 | 36 | 5.9 | 4.0 | 21 | 43 | 126 |
| | UB | | 10 | 3.0 | 41 | 120 | 1448 | | 6.8 | 4.0 | 21 | 43 | 126 |
| Lycopsamine-*N*-oxide (14) | LB | 77 | 7.2 | 0 | 25 | 187 | 1066 | 100 | 0 | 0 | 0 | 0 | 0 |
| | UB | | 8.0 | 1.0 | 25 | 187 | 1066 | | 1.0 | 1.0 | 1.0 | 1.0 | 1.0 |
| Retrorsine (8) | LB | 79 | 1.7 | 0 | 9.0 | 28 | 278 | 81 | 1.0 | 0 | 6.0 | 12 | 18 |
| | UB | | 3.1 | 2.0 | 9.0 | 28 | 278 | | 3.2 | 3.0 | 6.0 | 12 | 18 |
| Retrorsine-*N*-oxide (14) | LB | 99 | 0.4 | 0 | 0 | 1.0 | 929 | 100 | 0 | 0 | 0 | 0 | 0 |
| | UB | | 0.4 | 0 | 0 | 1.0 | 929 | | 0 | 0 | 0 | 0 | 0 |
| Senecionine (8) | LB | 73 | 3.6 | 0 | 19 | 62 | 320 | 72 | 1.8 | 0 | 10 | 18 | 25 |
| | UB | | 4.9 | 3.0 | 19 | 62 | 320 | | 3.7 | 3.0 | 10 | 18 | 25 |
| Senecionine-*N*-oxide (14) | LB | 99 | 0.4 | 0 | 0 | 3.0 | 1305 | 100 | 0 | 0 | 0 | 0 | 0 |
| | UB | | 0.4 | 0 | 0 | 3.0 | 1305 | | 0 | 0 | 0 | 0 | 0 |
| Seneciphylline (8) | LB | 78 | 1.9 | 0 | 9.0 | 32 | 316 | 81 | 1.2 | 0 | 7.0 | 18 | 42 |
| | UB | | 3.3 | 2.0 | 9.0 | 32 | 316 | | 3.4 | 3.0 | 7.0 | 18 | 42 |
| Seneciphylline-*N*-oxide (14) | LB | 98 | 0.3 | 0 | 0 | 2.0 | 1441 | 100 | 0 | 0 | 0 | 0 | 0 |
| | UB | | 1.3 | 1.0 | 1.0 | 2.0 | 1441 | | 1.0 | 1.0 | 1.0 | 1.0 | 1.0 |
| Senkirkine (8) | LB | 97 | 0.1 | 0 | 0 | 2.0 | 36 | 97 | 0 | 0 | 0 | 1.0 | 2.0 |
| | UB | | 1.1 | 1.0 | 1.0 | 2.0 | 36 | | 1.0 | 1.0 | 1.0 | 1.0 | 2.0 |

The sum of the PAs in the respective group of eight, fourteen and seventeen is presented in Table 5. The sum of the eight PAs are first presented for all samples, but also recalculated to match the number of samples analysed only for the fourteen or seventeen alkaloids to visualise the impact of including a further six or nine alkaloids in the overall sum. The number of samples with any PAs above the LOD or LOQ is higher and the overall average level is lower when comparing retail honey to bulk honey. The maximum levels found in retail honey are only 10 % or less of the levels found in bulk honey. The eight PAs in common for the two submissions comprised between 75 and 90 % of the total upper bound sum of PAs in the respective alkaloid grouping. The sum of PA levels shown in Table 5 for the eight PAs in the total number of samples and for the PA sum in the groups of 14 and 17 alkaloids tested will be used for the exposure calculations.

**Table 5:** Summary of the number of reported results (N), lower (LB) and upper (UB) bound mean, maximum and respective percentile (P) levels for groupings of 8 (8 PA), 14 (14 PA) and 17 (17 PA) pyrrolizidine alkaloids (PAs) in bulk and retail honey in µg/kg and the proportion of left-censored samples (LC) with no PAs detected or quantified. The sums of the 8 alkaloids in the total number of samples and of the 14 and 17 alkaloids in the respective groups are used for calculating exposure.

| Group | Type of honey | N | LC % | | Mean | P50 | P95 | P99 | Max |
|---|---|---|---|---|---|---|---|---|---|
| **8 PA** | Bulk honey | 13280 | 29 | LB | 28 | 6.0 | 121 | 296 | 2206 |
| | | | | UB | 36 | 15 | 125 | 305 | 2212 |
| | Retail honey | 1324 | 10 | LB | 16 | 10 | 57 | 96 | 169 |
| | | | | UB | 26 | 20 | 62 | 97 | 176 |
| **14 PA** | Bulk honey | 8383 | 23 | LB | 37 | 9.0 | 155 | 413 | 3298 |
| | | | | UB | 44 | 15 | 161 | 418 | 3301 |
| | Retail honey | 208 | 15 | LB | 13 | 7.0 | 46 | 71 | 150 |
| | | | | UB | 20 | 15 | 50 | 75 | 158 |
| **8 PA** in the group of 14 | Bulk honey | 8383 | 25 | LB | 29 | 8.0 | 116 | 292 | 2206 |
| | | | | UB | 34 | 13 | 119 | 295 | 2211 |
| | Retail honey | 208 | 15 | LB | 13 | 7.0 | 46 | 71 | 150 |
| | | | | UB | 18 | 13 | 48 | 73 | 156 |
| **17 PA** | Bulk honey | 4897 | 34 | LB | 34 | 5.0 | 157 | 381 | 2334 |
| | | | | UB | 52 | 24 | 168 | 397 | 2352 |
| | Retail honey | 1116 | 7.5 | LB | 20 | 12 | 68 | 113 | 267 |
| | | | | UB | 36 | 28 | 76 | 121 | 278 |
| **8 PA** in the group of 17 | Bulk honey | 4897 | 36 | LB | 27 | 4.0 | 131 | 307 | 1522 |
| | | | | UB | 39 | 17 | 137 | 313 | 1536 |
| | Retail honey | 1116 | 8.5 | LB | 17 | 10 | 61 | 96 | 169 |
| | | | | UB | 27 | 20 | 66 | 97 | 176 |

Honey can be used as an ingredient in other foods like cereals and fine bakery ware. It would also be possible for animal products like meat, milk and eggs to be indirectly contaminated with PAs through intake of contaminated feed. No data on PA levels in such products were available to EFSA to include in the exposure assessment.

#### 4.2.4.2. Feed

Levels of PAs in 351 feed materials, sampled between 2006 and 2010 as part of the framework of the Dutch monitoring program for animal feeds, are presented in Appendix B. The total PA levels found for the samples tested in the seven feed categories are summarised in Table 6. Rather than summing all individual entries for each left-censored PA when calculating overall statistics, the compounds were merged into four groups of structurally related PAs (senecionine-, lycopsamine-, heliotrine- and

monocrotaline-type PAs) and a group level detection limit value of 4.5 µg/kg was used and added to actual reported levels to derive the upper bound result. This diversion from normal procedures was adopted due to the large range of PAs tested and the likelihood that if none in a group exceeded the detection limit no PAs would be present from that group. This was considered plausible because in most samples only a limited number of PAs were detected in significant amounts and abundance. In 55 % of samples, concentrations of PAs were below the LOD (4.5 µg/kg).

**Table 6:** The number of reported results (N), lower (LB) and upper (UB) bound, maximum and respective percentile (P) levels of Pyrrolizidine alkaloids for seven feed categories in µg/kg.

| Feed category | N | LC% | | Mean | P50 | P95 | P99 | Max |
|---|---|---|---|---|---|---|---|---|
| Cereal grains | 6 | 83.3 | LB | 4.9 | | | | 30 |
| | | | UB | 23 | | | | 48 |
| Oil seeds and fruits | 56 | 76.8 | LB | 13 | 0 | 59 | | 343 |
| | | | UB | 31 | 18 | 77 | | 361 |
| Legume seeds | 1 | 100 | LB | 0 | | | | |
| | | | UB | 18 | | | | |
| Tubers and roots | 1 | 100 | LB | 0 | | | | |
| | | | UB | 18 | | | | |
| Other seeds and fruits | 3 | 66.7 | LB | 12 | | | | 36 |
| | | | UB | 30 | | | | 54 |
| Forages and roughage | 252 | 50.8 | LB | 272 | 0 | 984 | 4507 | 22753 |
| | | | UB | 290 | 18 | 1002 | 4525 | 22771 |
| Other plants and algae | 32 | 37.5 | LB | 316 | 11 | | | 3209 |
| | | | UB | 334 | 29 | | | 3226 |

LC%: percentage of left-censored samples; P50/95/99: $50^{th}$/$95^{th}$/$99^{th}$ percentile; Max: maximum level.

The majority of feeds analysed were categorised as forages and roughages (Table 6). Only these contained a sufficient number of samples to be included in any exposure assessment, but even for this category the variation between different types of feed was considerable. Fresh grass and grass silages (n = 60) were practically free of PAs, with only two samples having total PA concentrations of 28 µg/kg. Similarly, the grass hay samples were virtually free of PAs, although levels of 392 and 22,753 µg/kg were recorded for two samples. High-temperature dried grass generally contained very low amounts of PAs, although one sample contained 154 µg/kg, while a further four samples had levels of PAs between 60 and 100 µg/kg.

In contrast, a high proportion of samples of lucerne (alfalfa) were contaminated with PAs. For 10 samples, PA concentrations were below the LOD, while in 12 samples the total PA content exceeded 1000 µg/kg, with the highest concentration of 6216 µg/kg in a sample of lucerne pellets. The overall mean for the 99 samples of lucerne was 424 µg/kg.

Data were also provided for 67 non-forage feeds, including cereal grains (maize, wheat), oilseeds and fruits. For 50 feeds, PAs were absent or below the LOD. Concentrations in other feeds were generally low (<60 µg/kg) with the exception of two samples of linseed (136 and 343 µg/kg).

Levels of PAs in herbal mixtures used as feed (in the feed category 'other plants and algae') were also reported for 32 samples. In 12 of these, PAs were absent or below the LOD, while in a further 12 samples concentrations were < 100 µg/kg. However in three samples, levels were in excess of 1000 µg/kg, with a maximum of 3200 µg/kg.

## 4.3. Food and feed processing

### 4.3.1. Food processing

As defined by Council Directive 2001/110/EC[19] relating to honey, no pollen or other ingredient of honey is to be removed, unless that it is inevitable when foreign materials are removed. When the removal of foreign materials is undertaken by filtering, and such process leads to the removal of a significant quantity of pollen, appropriate indications have to be reported on the label for the correct information of the consumers.

Model filtering experiments using defined amounts of PA-plant pollen demonstrated a fast release of the pollen-PAs into the honey. After 6 weeks up to 50 % (rising to 75 % after 12 weeks) of the pollen-PAs are found in the honey after the pollen was removed by filtering. In addition, no difference was noted for filtered vs. unfiltered honeys, an indication that the current methods of PA-analysis in honey in use, only detect the already liberated PA-amounts from the pollen together with the PA-amount from the nectar, but will not include the amount of PAs remaining in the pollen of the sample (Kempf et al., 2011a). This means that, considering the fast PA transfer from pollen into honey, filtering would not significantly reduce the level of PAs in honey.

The same study has demonstrated that PAs can be detected in all sorts of food that contain honey as an ingredient. 60 samples were analysed and PAs were detected in mead, candy and fennel honey. Owing to the chemical stability of PAs it seems that the degree of downstream contamination, at least as demonstrated for honey as ingredient, is less influenced by degradation of the PAs through food processing procedures than by diluting of the original PA concentration (Kempf et al., 2011a). As a result, one would conclude that avoiding PA-contaminated ingredients would be the most effective way to reduce PA-contamination of foodstuff.

Pollen supplements used for human consumption are dried to reduce the water content to assure long term storage. It was demonstrated that heating/drying of PA-plant pollen significantly decreased the amount of PANOs but the main degradation is the reduction to the parent free base PAs, thereby maintaining the total level of PAs (Boppré et al., 2008).

Recently, PAs were detected in qurut, a dried cheese from goat's milk typical for Central Asia, demonstrating that PAs were transferred from the feed into goat's milk and 'surviving' all processing steps (Kakar et al., 2010). As demonstrated, ordinary preparation of herbal beverages from PA-containing plant parts will result in beverages containing PAs and PANOs of the used plant material (Oberlies et al., 2004). Methods or studies to alter or reduce the PA-content of herbal infusions are not available.

For borage oil it was demonstrated that the refining process lowers the total PA-content by a factor of about 30,000 and no PAs were detected in the end product (LOD of 20 µg/kg) (Wretensjö and Karlberg, 2003).

### 4.3.2. Feed processing

The stability of PAs in feed products is largely dependent on the residual moisture content. Candrian et al. (1984) investigated the stability of PAs in silage (dry matter (DM) content 19-33 %) and dried hay. The PAs were added in the form of (dried) alpine ragwort (*Senecio alpinus*) in a range of concentrations (0.2 to 100 %). After 114 days in a laboratory-scale silo the content of PAs was reduced to 4.5 % of the initial concentration when only ragwort plants were ensiled. Degradation of PAs was attributed to the fermentation process inside the silo. Degradation was less pronounced at lower contamination levels.

[19] Council Directive 2001/110/EC of 20 December 2001 relating to honey. OJ L 10, 12.1.2002, p. 47-52.

Reduction of the PA content in grass silage containing 3.5 % alpine ragwort was only 54 % after 114 days. In dried hay no detectable loss of PAs occurred during the course of the experiment.

A rapid decline in PA concentrations under composting conditions was observed by Crews et al. (2009). Fresh ragwort plants (*J. vulgaris*) were stored in black bin bags under outdoor conditions for up to 14 weeks. PA concentrations declined rapidly during the first two weeks (87 % reduction), followed by a slower reduction from week 2 to 8. No PAs were detectable after 10 weeks of storage. It was found that PANOs were degraded more rapidly than the corresponding PAs.

From a pilot scale experiment with a composting heap containing bags with chopped ragwort it was concluded that decomposition of PAs was relatively efficient (compared to other plant toxins studied) (Hough et al., 2010). After 4 weeks of composting, levels had dropped below the LOD. However, since this was aerobic decomposition – rather than anaerobic conservation, in the case of silage – it is not clear whether the same would occur in silage.

It was noticed by Mulder et al. (2009) that artificial dehydration and pelleting in an industrial setting of fresh lucerne contaminated with common groundsel (*Senecio vulgaris*) resulted in a change of the ratio between PAs and PANOs. In air dried reference material the proportion of tertiary amines was less than 20 %, while in the dehydrated material the proportion on average was close to 50 %. Additional pelleting appeared to increase the relative tertiary amine content even more, up to 80 %, on average. A similar trend was observed for artificially dried and pelleted grass contaminated with common groundsel. It is not known whether the change in tertiary amine/*N*-oxide proportion is due to reduction of PANOs to the corresponding PAs or that (part of) the PANOs degrade under the conditions applied.

### 4.4. Identification of PAs of relevance for food and feed

**Important PA-plants and PAs currently identified as major sources for PAs in food and feed**

*Senecio* species (tribe Senecioneae, Asteraceae) have been associated with clinical intoxications in livestock and humans. Senecionine-type PAs are dominating and present in highest concentrations in the flowering parts. Recently, the transfer of PAs from *Senecio*-plants (so far mainly *Jacobaea vulgaris*) into honey and contamination of salads with plant parts of *Senecio vulgaris* was reported (BfR 2007; Deinzer et al., 1977; Kempf et al., 2011a). The PA-pattern found in *S. vulgaris* (contamination of salads - BfR 2007) is dominated by seneciphylline, senecionine, retrorsine, integerrimine and spartioidine (tertiary PAs and *N*-oxides). For *J. vulgaris* different chemotypes are described (Witte et al., 1992). A jacobine chemotype characterized by high levels of jacobine, jaconine, jacoline and jacozine and an erucifoline chemotype dominated by erucifoline and acetylerucifoline. Both chemotypes may contain substantial amounts of senecionine and seneciphylline. Jacoline was the major PA from *Jacobaea vulgaris* that was transferred in to milk according to a carry-over study simulating the use of contaminated feed (Hoogenboom et al., 2011).

Lycopsamine-type PAs and amabiline/supinine-type PAs (missing the 7-hydroxy functionality at the necine-base backbone) are a major group of frequently occurring PAs. This group comprises a diverse range of mono and diester PAs and can (co)-occur as structurally related (stereo)isomers, which complicates identification and quantification. For example, lycopsamine can co-occur with one or more of its isomers intermedine, indicine, rinderine and echinatine. These PAs are representative for many species of *Echium*, *Anchusa, Borago, Symphytum* (all Boraginaceae) and *Eupatorium* (Asteraceae), and may serve as markers for the undesirable presence of these plant species (and/or their seed) in food and feed. *Boraginaceae* species containing PAs are used in herbal remedies (*Eupatorium* spp.), food (Borago) and represent an attractive source for nectar and pollen for foraging bees (*Eupatorium, Borago*). In particular, *Echium* spp. have been shown to be a major source for PA-contamination of retail honeys and pollen supplements (Kempf et al., 2008, 2010b). *Echium* spp. contain numerous open-chain mono and diester PAs, and major compounds are echimidine, vulgarine, echivulgarine (Kempf et al., 2008, 2010b; Dübecke et al., 2011).

*Heliotropium* spp. (Boraginaceae) are a known source of PA-contamination of Australian honey (Kempf et al., 2010a) and grains therefore representing a potential hazard for food/feed chains. The latest documented outbreak of acute PA-intoxication of humans was traced back to grain contaminated with seeds of *Heliotropium popovii* (Kakar et al., 2010). PAs indicating the presence of *Heliotropium* are heliotrine, europine, lasiocarpine and indicine or lycopsamine-isomers.

*Crotalaria* spp. (Leguminosae) are another known source of PAs. The genus is represented by alkaloids such as monocrotaline, fulvine, trichodesmine and retusamine. The genus comprises more than 500 species mainly distributed throughout the tropics and subtropics. The genus *Crotalaria* is known to produce high amounts of PAs stored mainly in its seeds. These seeds may contaminate grain for human consumption and has been associated in the past as a source of intoxications in poultry, pigs and cattle as well (Fletcher et al., 2011). So far, there is no indication that *Crotalaria* spp. nectar/pollen contains PAs, which is also reflected in solely non-detected results for monocrotaline in honey samples (Dübecke et al., 2011).

**PAs relevant for food**

Based on the available literature and the occurrence data on PAs in honey provided to EFSA, some conclusions regarding the most relevant PAs in food can be made, and these are summarized below. However, it has to be taken into account that the vast majority of honey data reflect only bulk honey imported to Germany and to a much smaller extend retail honeys obtained from German or Dutch markets. Furthermore, it needs to be considered that only expected PAs and/or available reference PAs were monitored.

The occurrence data on PAs in honey provided to EFSA showed that lycopsamine/echimidine-type PAs contribute the largest part of the PA content in both bulk honeys and retail honeys. Together they make up 80 % of the total in bulk honeys and 79 % in retail honeys. The PAs that contribute most significantly are echimidine (22.5 % in bulk honey, 33.7 % in retail honey) and lycopsamine (22.0 % in bulk honey and 30.6 % in retail honey). Lycopsamine-*N*-oxide contributes significantly to bulk honey as well (16.7 %), but was not detected in retail honey. Minor contributions are made by echimidine-*N*-oxide (3.9 % in bulk honey, not detected in retail honey), echiuplatine (5.3 % in bulk honey, 5.2 % in retail honey) and echivulgarine (3.7 % in bulk honey, 2.1 % in retail honey).

Senecionine-type PAs comprise a minor but still relevant part (approximately 20 %) of the average PA content of contaminated honey (bulk honeys as well as retail honeys). The most important compound is senecionine (8.4 % of the total in bulk honey, 9.3 % in retail honey), followed by seneciphylline (4.4 % in bulk honey, 6.2 % in retain honey) and retrorsine (3.9 % in bulk honey, 5.2 % in retail honey). The corresponding *N*-oxides contribute less. The actual contribution of senecionine-type PAs to honey may be in fact higher, because not all relevant PAs are incorporated in routine analysis.

Heliotrine-type PAs make only a very small contribution to the average PA content of honey. Only heliotrine and lasiocarpine have been found on rare occasions. Recently, *Heliotropium*- PAs were associated with HVOD outbreaks in Afghanistan (Kakar et al., 2010).

In addition, cheese from goat's milk (qurut) contained trichodesmine, a PA found in *Crotalaria* spp. (Kakar et al., 2010).

Based on the available literature and the occurrence data submitted on PAs in honey, the CONTAM Panel identified the following PAs (including the tertiary amine as well as the corresponding *N*-oxide forms) as important markers for contamination of food:

- *Senecio* spp. (Asteraceae): acetylerucifoline, erucifoline, integerrimine, jacobine, jacoline, jaconine, jacozine, retrorsine, senecionine, seneciphylline.

- *Echium* spp. (Boraginaceae): echimidine, echivulgarine, vulgarine.
- Other Boraginaceae and *Eupatorium* spp. (Asteraceae): lycopsamine and isomers.
- *Heliotropium* spp. (Boraginaceae): europine, heliotrine, lasiocarpine and lycopsamine-isomers.
- *Crotalaria* spp. (Fabaceae): fulvine, monocrotaline, retusamine, trichodesmine.

**PAs relevant for feed**

The majority (60 %) of the animal feed data provided to EFSA were from forage and roughage samples and mostly from production sites in The Netherlands. Oil seed samples (16 % of the total) were often imported from outside of the EU. The country of origin of the samples in the other plant category (mainly samples of (mixtures) of herbal additives) was often unknown. The number of samples analysed in the other feed categories was too small to draw any conclusions on the presence and relevance of PAs.

Senecionine-type PAs and PANOs account for approximately 85 % of the total PA content in the feed samples. Forages and roughages constitute the major category in which senecionine-type-PAs are found. Of particular relevance are seneciphylline and seneciphylline-*N*-oxide (10.6 and 10.0 % of the total amount, respectively). Senecionine and its *N*-oxide both contribute for another 6.8 %. Furthermore, retrorsine and its *N*-oxide are of importance as they contribute 5.8 and 4.8 %, respectively. Smaller, but still relevant contributions come from erucifoline (4.8 %), jaconine (4.5 %) and integerrimine-*N*-oxide (3.2 %). Many other senecionine-type PAs and PANOs are detected with lower abundances as well.

Lycopsamine-type PAs contribute approximately 16 % to the average PA content in the positive feed sample results submitted to EFSA. However, in the oil seed samples (mainly soya, originating from outside the EU) they contribute 64 % to the total. The two most abundant PAs in animal feed are an acetylechimidine isomer (4.4 %), and an echimidine isomer (2.7 %).

Heliotrine-type PAs are of importance for the other plants category (herbal feed additives). In fact they are the dominant group (69.6 %). Overall, most important single compounds are europine-*N*-oxide (4.1 %) and heliotrine-*N*-oxide (2.5 %).

The animal feeds were monitored for a limited set of monocrotaline PAs. Only monocrotaline was found with low abundance in some oil seed samples, but not in any of the other animal feed categories.

It should be noted that in feed the larger part of the PAs may be present as PANOs, hence adequate measures should be taken to include PANOs in the analytical methods. Therefore, an unequivocal PA-detection and rough quantification of PA-contamination in food/feed requires a more comprehensive set of reference compounds (especially PANOs) which are not available now.

Based on the available literature and the occurrence data submitted on PAs in feed, the CONTAM Panel identified the following PAs (including the tertiary amine as well as the corresponding *N*-oxide forms) as important markers for contamination of feed:

- *Senecio* spp. (Asteraceae): erucifoline, integerrimine, jacobine, jaconine, retrorsine, senecionine, seneciphylline.
- Boraginaceae and *Eupatorium* spp. (Asteraceae): acetylechimidine and isomers, echimidine and isomers, lycopsamine and isomers.
- *Heliotropium* spp. (Boraginaceae): europine, heliotrine, lasiocarpine.
- *Crotalaria* spp. (Fabaceae): fulvine, monocrotaline, retusamine, trichodesmine.

# 5. Food and feed consumption

## 5.1. Food consumption

### 5.1.1. EFSA's Comprehensive European Food Consumption Database

During 2010, the EFSA Comprehensive European Food Consumption Database (Comprehensive Database) was built from existing national information on food consumption at a detailed level. Competent organisations in the European Union Member States provided EFSA with data from the most recent national dietary survey in their country, at the level of consumption by the individual consumer. Survey results for children were mainly obtained through the EFSA Article 36 project 'Individual food consumption data and exposure assessment studies for children' through the EXPOCHI consortium (EFSA, 2011a). Results from a total of 32 different dietary surveys carried out in 22 different Member States are included in the Comprehensive Database as published (EFSA, 2011a).

Although the food consumption data in the Comprehensive Database are the most complete and detailed currently available in the EU, it should be pointed out that different methodologies were used between surveys to collect the data and thus the information is not suitable for direct country-to-country comparisons.

### 5.1.2. Food consumption data for different age and consumer groups

The CONTAM Panel considered that both acute and chronic exposure to pyrrolizidine alkaloids have to be assessed. Therefore, as suggested by the EFSA Working Group on Food Consumption and Exposure (EFSA, 2011b), all dietary survey results were used to calculate acute dietary exposure (Table 7) while dietary surveys with only one day per subject were excluded when considering chronic exposure (Tables 8 and 9) since they are not adequate for this task. Three representative age groups were selected for the analysis, toddlers from 1 to 3 years of age, other children from 3 to10 years and adults from 18 to 65 years. Since there was little difference in consumption patterns between adults on the one hand and adolescents, elderly and very elderly population groups on the other, there was no need to present consumption separately for those latter groups. There was no representative information available for honey consumption among infants so this age group could not be covered.

Honey consumption for each individual day of honey consumption and the average weight of those consumers from Table 7 were used for calculating acute exposure. For toddlers the mean daily honey portion size for honey consumption days varied between 0.6 g and 17 g and the 95$^{th}$ percentile daily portion size between 2.5 g and 40 g. For other children the corresponding mean consumption varied between 2 g and 18 g and the 95$^{th}$ percentile between 8 and 50 g, and mean for adults between 1.8 g and 36 g and 95$^{th}$ percentile between 5.3 g and 100 g, respectively.

**Table 7:** Number of consumption days, mean and 95th percentile (P95[a]) portion in g per day for all individual days of honey consumption and mean body weight for subjects in kg.

| | Toddlers | | | | Other children | | | | Adults | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **Country** | **Days** | **Weight** | **Mean** | **P95** | **Days** | **Weight** | **Mean** | **P95** | **Days** | **Weight** | **Mean** | **P95** |
| Austria | | | | | | | | | 300 | 68.0 | 17 | 40 |
| Belgium | 1 | 11.0 | 3.0 | | 46 | 17.3 | 14 | 27 | 88 | 71.9 | 30 | 67 |
| Bulgaria | 55 | 12.6 | 8.7 | 20 | 82 | 16.5 | 11 | 25 | 43 | 71.5 | 19 | 30 |
| Czech Republic | | | | | 93 | 25.2 | 17 | 40 | 236 | 75.0 | 22 | 60 |
| Denmark | | | | | 157 | 27.3 | 2.0 | 8.0 | 1492 | 75.2 | 1.8 | 5.3 |
| Estonia | | | | | | | | | 35 | 71.2 | 36 | 78 |
| Finland | 13 | 9.8 | 0.6 | 2.5 | 119 | 21.7 | 11 | 33 | 324 | 75.9 | 8.1 | 26 |
| France | | | | | 104 | 23.9 | 13 | 25 | 1087 | 67.4 | 18 | 50 |
| Germany | 37 | 12.6 | 5.7 | 20 | 249 | 22.6 | 11 | 27 | 2272 | 75.0 | 24 | 60 |
| Greece | | | | | 327 | 21.9 | 11 | 22 | | | | |
| Hungary | | | | | | | | | 217 | 72.4 | 29 | 60 |
| Ireland | | | | | | | | | 302 | 75.7 | 23 | 60 |
| Italy | 11 | 12.6 | 17 | 40 | 45 | 23.3 | 14 | 25 | 381 | 67.9 | 19 | 40 |
| Latvia | | | | | 11 | 30.6 | 18 | 30 | 130 | 76.9 | 26 | 70 |
| The Netherlands | 18 | 14.1 | 9.0 | 20 | 37 | 19.6 | 13 | 40 | 43 | 67.9 | 27 | 70 |
| Poland | 5 | 11.0 | 13 | | 31 | 26.1 | 17 | 40 | 109 | 68.0 | 22 | 50 |
| Slovakia | | | | | | | | | 76 | 73.0 | 30 | 100 |
| Slovenia | | | | | | | | | 49 | 73.1 | 26 | 40 |
| Spain | 1 | 14.4 | 10 | | 61 | 29.4 | 16 | 50 | 194 | 66.7 | 16 | 30 |
| Sweden | | | | | 88 | 26.8 | 13 | 31 | 268 | 71.0 | 18 | 42 |
| United Kingdom | | | | | | | | | 432 | 74.2 | 18 | 49 |
| ALL COUNTRIES | | 12.5 | 8 | 20 | | 23.2 | 11 | 28 | | 72.9 | 18 | 50 |

(a): Note that the 95th percentile is not reliable when the number of subjects is less than 60 and is given in the table as an indication only

Habitual consumption of honey for the chronic exposure calculation was estimated by averaging consumption over the number of days in the survey period for each individual of honey consumers only as presented in Table 8. It is acknowledged that such calculations can be heavily influenced by the number of days included in the survey depending on the frequency of consumption. The proportion of honey consumers varied between 2.8 % and 13.9 % for toddlers, 3.3 % and 24.8 % for other children and 4.8 % and 23.7 % for adults in the different countries. The habitual mean consumption of honey per day varied between 0.3 g and 13 g for toddlers, 0.4 g and 13 g for other children and 0.6 g and 18 g for adults. The corresponding 95th percentile consumption varied between 0.8 g and 27 g for toddlers, 1.1 g and 37 g for other children and 1.9 g and 53 g for adults.

**Table 8:** Number of subjects (N), mean and 95th percentile (P95[(a)]) of the average honey consumption for **honey consumers only** for the survey period in g per day and mean body weight for subjects in kg.

| Country | Toddlers | | | | Other children | | | | Adults | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | N | Weight | Mean | P95 | N | Weight | Mean | P95 | N | Weight | Mean | P95 |
| Belgium | 1 | 11.0 | 1.0 | | 29 | 17.6 | 7.4 | 23 | 72 | 71.8 | 18 | 36 |
| Bulgaria | 49 | 12.6 | 4.9 | 11 | 72 | 16.6 | 6.5 | 15 | | | | |
| Czech Republic | | | | | 79 | 25.0 | 9.8 | 23 | 211 | 74.9 | 13 | 30 |
| Denmark | | | | | 103 | 26.6 | 0.4 | 1.1 | 668 | 74.5 | 0.6 | 1.9 |
| Finland | 11 | 9.8 | 0.3 | 0.8 | 63 | 20.7 | 2.2 | 11 | 211 | 76.0 | 6.2 | 26 |
| France | | | | | 50 | 23.7 | 3.9 | 13 | 337 | 67.4 | 8.3 | 25 |
| Germany | 25 | 12.7 | 2.8 | 7.7 | 164 | 22.5 | 5.7 | 18 | 1682 | 75.0 | 16 | 41 |
| Greece | | | | | 200 | 21.9 | 6.1 | 14 | | | | |
| Hungary | | | | | | | | | 155 | 72.6 | 14 | 37 |
| Ireland | | | | | | | | | 96 | 76.2 | 10 | 36 |
| Italy | 5 | 12.5 | 13 | 27 | 18 | 24.0 | 12 | 23 | 186 | 67.7 | 13 | 37 |
| Latvia | | | | | 10 | 30.6 | 9.3 | 15 | 109 | 77.1 | 16 | 40 |
| The Netherlands | 15 | 14.1 | 5.4 | 10 | 32 | 20.0 | 7.8 | 20 | 36 | 73.4 | 16 | 53 |
| Spain | 1 | 14.4 | 5.0 | | 39 | 29.3 | 13 | 37 | 105 | 66.6 | 10 | 27 |
| Sweden | | | | | 54 | 27.2 | 5.4 | 16 | 85 | 71.0 | 8.0 | 24 |
| United Kingdom | | | | | | | | | 226 | 73.8 | 5.0 | 20 |

(a): Note that the 95th percentile is not reliable when the number of subjects is less than 60 and is given in the table as an indication only.

Average daily honey consumption was also estimated among all participants in the different surveys and presented in Table 9. For toddlers, mean and 95th percentile honey consumption varied from 0.03 g to 1.8 g and 0 g to 20 g, respectively. For other children, mean and 95th percentile honey consumption varied from 0.09 g to 2.0 g and 0 g to 10 g, respectively. For adults, mean and 95th percentile honey consumption varied from 0.13 g to 2.6 g and 0 g to 20 g, respectively.

**Table 9:** Number of subjects (N), mean and 95th percentile (P95[a]) of the average honey consumption for **all consumers** for the survey period in g per day and mean body weight for subjects in kg.

| | Toddlers | | | | Other children | | | | Adults | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **Country** | **N** | **Weight** | **Mean** | **P95[b]** | **N** | **Weight** | **Mean** | **P95** | **N** | **Weight** | **Mean** | **P95** |
| Belgium | 36 | 13.8 | 0.03 | 0 | 625 | 17.9 | 0.34 | 0 | 1304 | 72.0 | 1.0 | 2.8 |
| Bulgaria | 428 | 12.1 | 0.56 | 5.0 | 433 | 16.5 | 1.1 | 7.5 | | | | |
| Czech Republic | | | | | 389 | 26.1 | 2.0 | 13 | 1666 | 75.6 | 1.6 | 10 |
| Denmark | | | | | 490 | 26.6 | 0.09 | 0.48 | 2822 | 74.5 | 0.13 | 0.75 |
| Finland | 497 | 10.0 | 0.01 | 0 | 933 | 20.7 | 0.15 | 0.21 | 1575 | 77.5 | 0.83 | 6.5 |
| France | | | | | 482 | 23.2 | 0.41 | 2.3 | 2276 | 69.6 | 1.2 | 8.0 |
| Germany | 261 | 11.5 | 0.27 | 1.9 | 660 | 24.0 | 1.4 | 9.0 | 10419 | 76.5 | 2.6 | 20 |
| Greece | | | | | 839 | 22.4 | 1.4 | 9.3 | | | | |
| Hungary | | | | | | | | | 1074 | 73.3 | 2.0 | 13 |
| Ireland | | | | | | | | | 958 | 75.3 | 1.0 | 5.7 |
| Italy | 36 | 12.7 | 1.8 | 20 | 193 | 26.1 | 1.1 | 10 | 2313 | 69.7 | 1.0 | 6.7 |
| Latvia | | | | | 189 | 31.2 | 0.49 | 2.5 | 1306 | 77.3 | 1.3 | 10 |
| The Netherlands | 322 | 14.0 | 0.25 | 0 | 957 | 19.8 | 0.26 | 0 | 750 | 75.1 | 0.77 | 0 |
| Spain | 17 | 14.5 | 0.30 | 0 | 555 | 27.0 | 0.89 | 6.4 | 1391 | 69.3 | 0.78 | 5.4 |
| Sweden | | | | | 1473 | 26.1 | 0.20 | 0 | 1210 | 73.5 | 0.56 | 2.9 |
| United Kingdom | | | | | | | | | 1724 | 76.0 | 0.66 | 2.9 |

(a): Note that the 95th percentile is not reliable when the number of subjects is less than 60 and is given in the table as an indication only.

(b): As can be seen when comparing results from Tables 8 and 9 the number of consumers of honey in some countries are less than 5 % and thus the 95th percentile consumption amount will be zero.

The data in Tables 7-9 were transformed into honey consumption amount per kg body weight and summarised in Table 10 to be used for the exposure calculation.

**Table 10:** Summary of minimum and maximum honey consumption for toddlers, other children and adults recorded in the different surveys expressed for daily portion size (acute exposure), daily consumption for honey consumers only and for all consumers.

| | Toddlers | | Other children | | Adults | |
|---|---|---|---|---|---|---|
| | **Mean** | **P95** | **Mean** | **P95** | **Mean** | **P95** |
| | Daily portion size on consumption days (g/kg b.w.) | | | | | |
| Minimum | 0.061 | 0.255 | 0.073 | 0.293 | 0.024 | 0.070 |
| Maximum | 1.349 | 3.175 | 0.809 | 2.041 | 0.506 | 1.370 |
| | Honey consumers only daily consumption (g/kg b.w.) | | | | | |
| Minimum | 0.031 | 0.082 | 0.015 | 0.041 | 0.008 | 0.026 |
| Maximum | 1.040 | 2.160 | 0.500 | 1.307 | 0.251 | 0.722 |
| | All consumers daily consumption (g/kg b.w.) | | | | | |
| Minimum | 0.001 | 0.000 | 0.003 | 0.000 | 0.002 | 0.000 |
| Maximum | 0.142 | 1.575 | 0.077 | 0.498 | 0.034 | 0.261 |

## 5.2. Feed consumption

In contrast to the situation for the human population (Section 5.1), there is no comprehensive database on feed consumption by livestock in the European Union (EU), and therefore examples of feed consumption from published texts have been used for this opinion. It must be stressed however that livestock feeding practices vary widely throughout the EU and that these only provide general estimates of the amount and type of feed consumed by different livestock.

Farm animals in the EU consume a large variety of feeds within a wide range of feeding systems. For ruminants (cattle, sheep and goats) and horses, daily rations consist predominantly of forages, fed either fresh – usually grazed *in situ* - or conserved. For many, forages are the sole feed. However, rations for highly productive animals are supplemented with other feeds such as cereal grains, oilseed meals and by-products of human food production, which are fed when the availability of forages is limited or the supply of nutrients from forages is insufficient to meet the needs of the animal. Examples of feed intake levels are given in Table 11.

**Table 11:** Example intakes levels of ruminant livestock and horses.

| | Live weight (kg) | Growth rate or productivity | DM.[(a)] intake (kg per day) | Forages as a % of total DM intake | Reference |
|---|---|---|---|---|---|
| Dairy cows, lactating | 650 | 40 kg milk per day | 21.4 | 60 | AFRC, 1993 |
| Fattening cattle[(b)] | 400 | 1,000 g per day | 9.6 | 80 | AFRC, 1993 |
| Fattening cattle: cereal beef | 400 | 1,400 g per day | 8.4 | 15 | AFRC, 1993 |
| Sheep: lactating ewes | 80 | Feeding twin lambs | 2.8 | 65 | AFRC, 1993 |
| Goats: milking[(c)] | 60 | 4 kg per day | 3.3 | 60 | NRC, 2007b |
| Goats: fattening | 40 | 200 g per day | 1.5 | 60 | NRC, 2007b |
| Horses (active) | 450 | - | 9.0 | 50 | NRC, 2007a |

(a): DM.: dry matter.
(b): Housed castrate cattle, medium maturing breed.
(c): Months 2-3 of lactation.

PA-containing plants are usually unpalatable, and grazing animals tend to avoid them unless the amount of pasture available for grazing is severely restricted. In dried plants, however, the smell and taste associated with the PAs appears to be less noticeable (Gardner et al., 2006; EFSA, 2007), and animals are therefore unable to exercise the same selectivity as they do when grazing. Therefore, the intake of PAs from forages is mainly likely to arise with housed animals. Since PA contamination of conserved forages is highly variable, and that where it occurs it is the result of poor weed control, the CONTAM Panel concluded that it is not reasonable to estimate typical intakes of PAs from forages.

Horses will generally consume 2-3.5 % of their body weight in feed (DM) each day, of which a minimum of 1 % should be as forage (pasture or hay) (NRC, 2007a). Mature horses with minimal activity may be maintained on forage alone, but for growing and active horses supplementation with cereal grains, cereal by-products (e.g. oats, barley, and wheat bran) and vegetable proteins is necessary. For a mature horse (450 kg live weight) doing a moderate level of activity, NRC (2007a) estimate a DM intake of 9 kg per day, of which half may be as a forage feed. Dried lucerne (alfalfa), either in the form of hay or pellets, is an important feed for horses.

In contrast, the daily rations for non-ruminants (including pigs and poultry) consist predominantly of cereal grains, vegetable proteins and by-products of human food manufacture, and are usually fed in the form of compound feeds. Cereals represented 47 % of all feeds used in industrial compound feed production in the EU, while oilseed cakes and meals and co-products from human food production

represent a further 28 % and 11 %, respectively (FEFAC, 2009). Since pigs and poultry consume approximately two-thirds of all industrial compound feed produced, it would be reasonable to assume that these proportions are broadly typical for the majority of diets fed to these livestock. Production systems vary widely throughout the EU, and the choice and proportions of the various ingredients in the diet is influenced by many factors, including the nutritional requirements of the animal or bird, the price and availability of feeds, the feeding facilities on the farm and market requirements (e.g. organic production). As a result, it is not possible to provide examples of 'normal' diets for pigs or poultry, but typical examples are given in Table 12.

**Table 12:** Examples of feed intakes for pigs and poultry used for the estimations of exposure for non-ruminant livestock.

| | | Live weight (kg) | Feed intake (kg per day) | Feed intake (% b.w.) |
|---|---|---|---|---|
| Pigs | Piglets | 7 | 0.32 | 4.60 |
| | Pigs - growing | 41 | 1.62 | 4.00 |
| | Pigs - fattening | 85 | 3.1 | 3.65 |
| | Breeding sows - gestating | 210 | 3.2 | 1.52 |
| | Breeding sows - lactating | 230 | 7.3 | 3.17 |
| Broilers | Starter | 0.2 | 0.03 | 15.96 |
| | Grower | 1.3 | 0.15 | 11.08 |
| | Finisher ration 1 | 2.3 | 0.20 | 8.70 |
| Layers | Starter (4 weeks) | 0.3 | 0.026 | 10.20 |
| | Grower (18 weeks) | 1.3 | 0.068 | 5.35 |
| | Breeding (week 20) | 1.4 | 0.082 | 5.89 |
| | Laying hen (32 weeks) | 1.9 | 0.11 | 5.95 |

Commercial rabbit production takes place in at least 14 EU countries, but principally in Italy, France and Spain. Annual rabbit meat production in Italy is about 230,000 tonnes, corresponding to 100,000,000 animals/year. The EU accounts for about 55 % of the world's rabbit meat production.[20] Rabbits are reared predominantly for their meat, with rabbit skins a secondary output. Young rabbits are normally kept with the mother to around 4-5 weeks old, then moved with siblings to a fattening cage, where they will stay until 10-12 weeks old before slaughter at about 2 kg b.w.

Because their natural diets consist of predominantly of fibrous feeds, rabbits have developed a strategy of high feed intakes of 65-80 g/kg b.w. in order to meet their nutritional requirements (Carabano and Piquer, 1998). In commercial production they are usually fed a pelleted diet of cereals and vegetable proteins supplemented with minerals, vitamins and trace elements. As with other animals, a wide assortment of feeds is used, depending on their price and availability. Lebas and Renouf (2009) reviewed diet formulations used in experimental studies: in 58 diets, the proportions of cereals, cereal by-products (mostly wheat bran) and oilseed meals (mostly soya bean cakes and sunflower seed cakes) were 18-20 %, 18-20 % and 16 %, respectively. Dried lucerne is a particularly important ingredient, and has been included at levels of up to 65 % (Lebas and Renouf, 2009) in experimental diets. In a typical French commercial rabbit compound, it was included at approximately 20 %,[21] although levels of 50 % or more have been recommended for commercial rabbit diets (McNitt et al., 2000).

[20] Source : http://faostat.fao.org
[21] Gidenne T. (2011) INRA UMR1289 Tandem, personal communication.

# 6. Exposure assessment in humans and animals

## 6.1. Exposure assessment of pyrrolizidine alkaloids in humans

There can be several sources of dietary PA exposure in humans. However, the only information available to EFSA is related to exposure through honey consumption. The following exposure assessment will thus focus only on this source of exposure.

### 6.1.1. Mean and high dietary exposure to pyrrolizidine alkaloids

Calculation of dietary exposure to PAs through consumption of retail honey is presented in Table 13. The acute scenario focuses on the amount of honey consumed during one day of reported honey consumption. Acute exposure was calculated by multiplying the lower and upper bound mean and 95$^{th}$ percentile levels of 8 PAs for all samples, and 14 and 17 PAs, respectively, for the two separate submissions from Table 5 with the minimum and maximum mean and 95$^{th}$ percentile portion size, for honey across the countries for honey consumption days only from Table 10. The chronic scenarios focus on long-term consumption by averaging honey consumption over all survey days for honey consumers or the average amount of honey consumed over all survey days for all survey participants. Chronic or habitual consumption was calculated by multiplying the lower and upper bound mean levels of 8 PAs for all samples, and 14 and 17 PAs, respectively, for the two separate submissions from Table 5 with the minimum and maximum mean consumption recorded across the countries over the survey days (not only honey consumption days) for consumers only of honey during any of the survey days and for the whole survey population (all consumers) from Table 10.

*Retail honey*

Table 13 summarises the range of results of the calculations by giving the minimum lower bound and the maximum upper bound for the three population categories according to the four potential consumption scenarios. The highest acute exposure to PAs was seen in toddlers with a calculated daily intake providing from 0.80 to 48.6 ng/kg b.w. and from 3.32 to 114 ng/kg b.w. when applying the country range of minimum and maximum mean and 95$^{th}$ percentile consumption, respectively, and mean lower and upper bound PA concentrations. Consuming honey at the 95$^{th}$ percentile concentration level could potentially increase acute exposure two to three times compared to the mean concentration, with the highest value of 254 ng/kg b.w. calculated for toddlers consuming 40 g of honey in one day.

For the chronic scenario, PA exposure for the mean consumption and concentration scenario for toddlers could reach a high of 37.4 ng/kg b.w. per day in honey consumers only. However, it is probably closer to the result of 5.10 ng/kg b.w. per day calculated for consumption distributed over all survey participants in the respective age group, based on the uncertainty associated with interpolating a few survey days to long-term consumption and a rather low number of honey consumers. An all-consumer scenario is most often considered to be a better reflection of chronic exposure for foods not consumed regularly by a large proportion of consumers.

**Table 13:** Calculated exposure to PAs through consumption of **retail honey** in ng/kg b.w. per day in toddlers, other children and adults.

| | Toddler consumption | | Other children consumption | | Adult consumption | |
|---|---|---|---|---|---|---|
| | **Mean** | **P95** | **Mean** | **P95** | **Mean** | **P95** |
| **Mean concentration** | | | | | | |
| | **Acute scenario - Daily portion size on consumption days** | | | | | |
| Min LB | 0.80 | 3.32 | 0.95 | 3.81 | 0.31 | 0.92 |
| Max UB | 48.6 | 114 | 29.1 | 73.5 | 18.2 | 49.3 |
| **P95 concentration** | | | | | | |
| | **Acute scenario - Daily portion size on consumption days** | | | | | |
| Min LB | 2.80 | 11.7 | 3.40 | 13.5 | 1.10 | 3.20 |
| Max UB | 108 | 254 | 64.7 | 163 | 40.4 | 110 |
| **Mean concentration** | | | | | | |
| | **Chronic scenario – Honey consumers only daily consumption** | | | | | |
| Min LB | 0.40 | 1.06 | 0.20 | 0.54 | 0.10 | 0.33 |
| Max UB | 37.4 | 77.8 | 18.0 | 47.0 | 9.03 | 26.0 |
| **Mean concentration** | | | | | | |
| | **Chronic scenario - All consumers daily consumption** | | | | | |
| Min LB | 0.01 | 0 | 0.04 | 0 | 0.02 | 0 |
| Max UB | 5.10 | 56.7 | 2.76 | 17.9 | 1.22 | 9.41 |

*Bulk honey*

Most honey would be blended before retail, but a proportion is sold as speciality honey from one producer only. To show the potential impact on exposure of consuming such honey, similar scenarios as for retail honey are presented in Table 14 for bulk honey for comparison purposes.

The theoretical exposure calculated for consumption of unblended (bulk) honey was in general about 50-100 % higher than the results of the calculations for retail honey or sometimes slightly higher than that. However, this calculation is mainly based on occurrence results for honey imported from countries outside Europe and such honey would usually be blended before retail.

**Table 14:** Calculated exposure to PAs through consumption of **bulk honey** in ng/kg b.w. per day in toddlers, other children and adults.

| | Toddler consumption | | Other children consumption | | Adult consumption | |
|---|---|---|---|---|---|---|
| | **Mean** | **P95** | **Mean** | **P95** | **Mean** | **P95** |
| **Mean concentration** | | | | | | |
| | **Acute scenario - Daily portion size on consumption days** | | | | | |
| Min LB | 1.71 | 7.14 | 2.05 | 8.21 | 0.67 | 1.97 |
| Max UB | 70.2 | 165 | 42.1 | 106 | 26.3 | 71.2 |
| **P95 concentration** | | | | | | |
| | **Acute scenario - Daily portion size on consumption days** | | | | | |
| Min LB | 7.40 | 30.9 | 8.90 | 35.5 | 2.90 | 8.50 |
| Max UB | 232 | 546 | 139 | 51 | 87.0 | 236 |
| **Mean concentration** | | | | | | |
| | **Chronic scenario – Honey consumers only daily consumption** | | | | | |
| Min LB | 0.86 | 2.29 | 0.42 | 1.16 | 0.23 | 0.71 |
| Max UB | 54.1 | 112.3 | 26.0 | 68.0 | 13.0 | 37.5 |
| **Mean concentration** | | | | | | |
| | **Chronic scenario - All consumers daily consumption** | | | | | |
| Min LB | 0.03 | 0 | 0.09 | 0 | 0.05 | 0 |
| Max UB | 7.37 | 81.9 | 3.98 | 25.9 | 1.77 | 13.6 |

LB: lower bound; UB: upper bound; P95: 95$^{th}$ percentile.

To put the exposure results into perspective, regular consumption of honey by adults could include daily sweetening of tea or other beverages with a spoonful (typically 10 g) of honey and the spread of honey on toast (typically 20 g). It could also include honey as a minor ingredient in cereal and other products. Even if such indirect honey consumption reached a daily amount of 23 g together with honey consumption as a sweetener in beverages and on toast it would be captured by the highest consumption amount from Table 8 of 53 g at the 95$^{th}$ percentile as used as input in Table 13.

Although there might be other sources of PA exposure, due to lack of data, the CONTAM Panel was not able to quantify dietary exposure from food other than honey. The CONTAM Panel calculated exposure based on a maximum of 17 PAs analysed for honey samples. It noted that the eight PAs in common to both datasets contributed to 75-90 % of the calculated PA exposure.

### 6.1.2. Importance of other dietary sources of human exposure

The use of herbal dietary supplements is increasing worldwide. Some of these herbal products are known to contain PAs, including 'gordolobo yerba' (Family Asteraceae), borage (Family Boraginaceae), and comfrey (genus *Symphytum;* Fu et al., 2002b). In addition, a renewed focus has been placed on traditional Chinese herbal medicinal products as a means of treating and preventing disease. Approximately 50 Chinese herbal plants, containing over 90 individual PAs, have been reported (Roeder, 2000; Fu et al., 2002b). Comfrey and coltsfoot (*Tussilago farfara*) are reported components in traditional Chinese herbal medicinal products (Fu et al., 2002b). No information on consumption or PA-levels is available. Therefore, no conclusions can be drawn on exposure to PAs via these sources.

Some PA-plants especially Borage herb is used in some regions in central Europe as spice or ingredient for dishes (Grüne Soße, salsa verte, green sauce or Frittata di boragina, spice for cucumber salad). There are no data available on how much of these products are consumed, or on the PA-levels in these dishes.

Products containing pollen may contain substantial amounts of PAs and show a high rate of PA-positive samples. In two recent studies, 31 % and 60 %, respectively, of the pollen products were tested PA-positive and a maximum PA-level of 37,855 µg/kg was reported (average levels were 1846 and 5179 µg/kg, respectively (Kempf et al., 2010b; Dübecke et al., 2011). Pollen product labels, package leaflets or internet sources recommend daily consumption doses of up to 6 teaspoons (corresponding to up to 25 g). Consequently, consumption of pollen containing products can substantially contribute to PA exposure.

Some Boraginaceae seed oils are considered to be of high dietary value because of the content of highly unsaturated or special fatty acids (e.g. γ-linolenic acid, stearidonic acid). Recently, the European Commission has approved refined *Echium plantagineum* seed oil as a novel food ingredient. The total PA-content is limited to 4 µg/kg (Commission Decision 2008/558/EC[9]).

Borage and Echium seed oils are also used and sold as food supplements (mainly in capsules) in drugstores and internet-shops, but again no levels of consumption or PA-levels are known for these products. Therefore, an estimation of the contribution of these products to human PA exposure is not possible.

## 6.2. Exposure assessment of pyrrolizidine alkaloids in animals

### 6.2.1. Estimation of pyrrolizidine alkaloids intake in feed by farm livestock and horses

In its report, EFSA (2007) concluded that it was not possible to estimate PA intake by farm livestock because of the wide variation in the levels of the alkaloids in plants, and the variability in livestock diets, a conclusion that was endorsed by FAO/WHO (2011). EFSA (2007) also noted that no systematic analysis of feed materials had been undertaken in the EU on which to base any estimates of intake or exposure. In the absence of any comprehensive database on feed consumption by livestock in the EU, the CONTAM Panel has been unable to provide any reliable assessment of exposure by livestock. However, for some livestock categories, 'worst case' exposures have been estimated below.

Forages are the main – and often sole feed – of ruminant livestock and horses. As reported in Section 4.2, recent data indicate that the forage with the highest concentration of PAs was lucerne mainly contaminated with *S. vulgaris*, with one sample containing 6.2 mg/kg[22] (4.8 mg/kg as the free base and 1.4 mg/kg as the *N*-oxide), all of which were senecionine-type PAs.[23] The amount of lucerne used in ruminant and horse diets in the EU varies considerably, depending on geographic location, price and availability. It is more widely used in southern Europe, where climatic conditions and soil type favour its cultivation, and here it is an important component of dairy cow diets. In central and Northern Europe, it is less widely grown, and where it is available is used more commonly as a feed for horses. Table 11 gives examples of feed intakes for dairy cows and horses. If the sample of lucerne referred to above was the sole forage in the diet of a high yielding dairy cow, then based on the intake figures given in Table 11, the exposure to PAs would be 89.9 mg per day, or 0.14 mg/kg b.w. Similarly, assuming this was the sole forage consumed by horses for feed intakes given in Table 11, the PA intake would be 31.5 mg per day (0.07 mg/kg b.w.). However, it is important to note that these calculations are based on the highest concentration reported. Levels of PAs are not uniformly high in lucerne feeds, as in over 52 % of the samples reported in Section 4.2.4.2 levels of PAs were less than 0.1 mg/kg, and a mean concentration of 0.424 mg/kg was calculated for all lucerne feeds. For this reason, these exposure estimates represent worst-case scenarios.

Pigs and poultry may be exposed to PAs as a result of accidental contamination of their feeds with weed seeds, and reports of toxicosis as a result of consuming contaminated feeds are discussed in Section 7.4.

[22] Equivalent to 7.0 mg/kg expressed on a DM basis. This figure is used in the calculations of exposure that follow.
[23] One sample of grass hay contained 22.7 mg PA/kg, but in the overall category of grass hay (14 samples) only two had levels >100µg/kg.

Of the 65 non-forage feeds reported (Section 4.2.4.2), PAs were not detected in 51 of them and, with the exception of one sample of linseed (343 µg/kg), detected levels in the remainder were <60 µg/kg. In view of the limited number of samples and the absence of any data on the major ingredients in their diets, the CONTAM Panel concluded that it has not been possible to make an estimate of exposure for pigs and poultry, or for other livestock.

For rabbits, feed intakes of 65-80 g/kg b.w. have been reported (Carabano and Piquer, 1998). Assuming a feed intake of 75 g/kg b.w., the average PA concentration in dried lucerne of 14 mg/kg and an inclusion rate of dried lucerne in the diet of 20 %, the intake of PAs by a 2 kg rabbit would be 0.041 mg per day, or 0.021 mg/kg b.w. per day. For the highest PA concentration in dried lucerne (6.2 mg/kg), exposure would be 0.186 mg per day, or 0.093 mg/kg b.w. per day.

# 7. Hazard identification and characterisation

## 7.1. Toxicokinetics

### 7.1.1. Laboratory animals

*Absorption*

Williams et al. (2002) administered Fischer rats and B6C3F1 mice a single dose of 10 mg/kg riddelliine (92 % riddelliine, 5 % retrorsine, 1.3 % seneciphylline) by gavage and observed a concentration peak in plasma within 30 minutes in both species, suggesting a rapid uptake of the substance by oral exposure. Wang et al. (2011) monitored the plasma concentration of senecionine and adonifoline and their metabolites in rats orally dosed by gavage (doses of 5.7, 11.5 or 22.9 mg/kg b.w. and 16, 32 or 64 mg/kg b.w., respectively) or intravenously (1.5 and 4 mg/kg b.w., respectively. Rapid absorption following oral exposure was observed for both alkaloids, with peak concentrations in plasma achieved within approximately 25 minutes and 70 minutes for senecionine and adonifoline, respectively. Absolute bioavailability values of 8.2 % and 33 % were reported for senecionine and adonifoline, respectively; however, a more extensive metabolic conversion of senecionine to its *N*-oxide was observed. Brauchli et al. (1982) exposed rats to 194 mg/kg of PA mixture obtained from comfrey both via gavage and via the skin. By comparing the excretion through the two routes of exposure, the authors estimated that oral absorption was 20-50 times higher than dermal absorption.

*Distribution*

Studies with several radiolabelled PAs showed distribution mainly in red blood cells, liver, kidney, lung and plasma, whereas low levels were observed to be transferred in the milk, mainly in the skim milk fraction, suggesting that the water soluble metabolites were transferred in this compartment. Binding to biological macromolecules was observed both *in vitro* and *in vivo* (Eastman et al., 1982; Estep et al., 1991; Xia et al., 2006; Chen et al., 2009).

Williams et al. (2002) measured the serum concentrations of riddelliine and those of two stable metabolites, riddelliine-*N*-oxide and retronecine, for the period 0.5-24 hours after the administration in rats and mice of a dose of 10 mg riddelliine/kg bodyweight by oral route. A rapid and extensive conversion of riddelliine to the *N*-oxide derivative was observed in male rats and in mice of both sexes, while female rats showed a lower conversion. In all species, low levels of retronecine were measured in serum. Serum elimination half-times were determined in the following order: riddelliine < retronecine < riddelliine-*N*-oxide for male rats and mice of both sexes. Male rats showed a greater volume of distribution and a greater internal exposure (expressed as the area under the curve (AUC)) to riddelliine-*N*-oxide in comparison to female rats, consistent with the higher sensitivity of male rats to riddelliine toxicity. More recently, Wang et al. (2011) compared the toxicokinetics of senecionine and adonifoline following oral and intravenous (i.v.) administration in rats. The plasma concentrations of the 2 PAs, the

respective PANOs and other hydroxylated metabolites, were monitored. Following oral absorption, rapid metabolite formation was observed for both PAs. Evidence for enterohepatic recirculation of senecionine-*N*-oxide was presented, including measurement of senecionine-*N*-oxide as the major metabolite present in bile and the presence of an apparent re-entry peak for senecionine-*N*-oxide after i.v. injection of senecionine. Evidence for enterohepatic recirculation of senecionine-*N*-oxide was presented, including measurement of senecionine-*N*-oxide as the major metabolite present in bile and the presence of an apparent re-entry peak for senecionine-*N*-oxide after i.v. injection of senecionine (Wang et al., 2011).

*Metabolism*

The metabolic pathways of PAs have been extensively studied (for comprehensive reviews, see Fu et al., 2004; Mei et al., 2010).

Following absorption in the gastrointestinal (GI) tract, PAs are distributed to the liver where the metabolism mainly occurs. In general, three metabolic pathways have been identified for pyrrolizidine alkaloids (see Figure 7):

- Cleavage of the ester bonds on position C7 and C9 are mediated by liver microsomal or cytosolic carboxyl esterases to form necine bases and the corresponding necic acids (see Figure 7).

- Retronecine- and heliotridine-type PAs can undergo *N*-oxygenation of the necine base to give *N*-oxides catalyzed by cytochrome P450 (CYP) and flavin-containing monooxygenases (FMO). As the nitrogen of the otonecine-type PAs is methylated, the *N*- oxygenation pathway is not active for this group.

- Finally, a CYP-mediated oxidative pathway leading to the formation of reactive intermediates can take place. This pathway includes two steps for retronecine- and heliotridine-type PAs: the hydroxylation of the necine base in C3 or C8 position, followed by a spontaneous dehydration leading to the formation of the corresponding 6,7-dihydropyrrolizine (pyrrolic) ester(s). A similar oxidative pathway is also active for otonecine-type PAs, for which the formation of the pyrrolic ester is preceded by an oxidative *N*-demethylation with the elimination of a molecule of formaldehyde and a subsequent ring closure to the corresponding dihydropyrrolizine ester(s).

The different metabolic pathways can lead either to detoxification or to bioactivation determining the toxicity of PAs. The species-, strain- or gender-depending variations in enzymatic activities or expressions, resulting in different overall balances of the detoxification and activation pathways, can influence the higher susceptibility of certain animals (e.g. male rat > guinea pig) to PA toxicity (Huan et al., 1998a; Lin et al., 2002, 2003; Chung and Buhler, 2004; Lin et al., 2007).

Esterase-mediated hydrolysis is considered a detoxification pathway, as the formed necines and necic acids have no toxicological activity and are subjected to Phase II conjugation and excreted via the urine.

Due to the lower hepatic esterase activity, this pathway is relatively less important in rats in comparison to other species such as the guinea pig, for which this pathway accounts for about 92 % of the total metabolism (Dueker et al., 1992; Lin et al., 2002). The difference in activity of the hydrolysis pathway between rats and guinea pigs can explain the different sensitivity of the two species to PA toxicity.

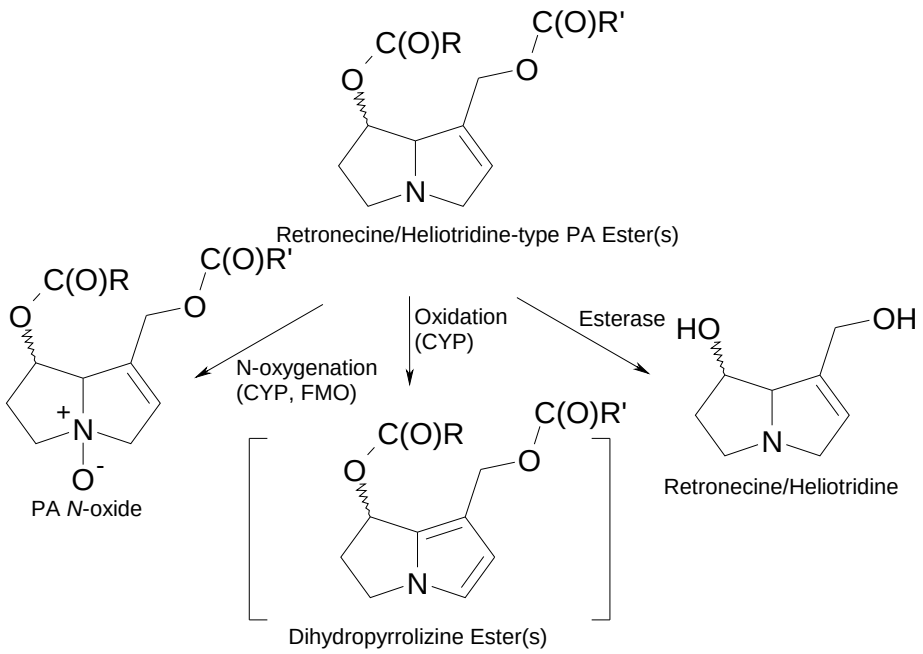


**Figure 7:** Major metabolites of pyrrolizidine alkaloids important for toxicokinetic determinations (note: ester(s) designates PAs and metabolites comprised of either monoesters at the 7-position or diesters at the 7- and 9-positions).

Beside the activity of hepatic esterases, also the structure of the ester functionalities can influence the yield of hydrolysis, as the de-esterification is impaired by steric hindrance in PAs with branched chain ester groups, resulting in a relative stability of these compounds, which are then metabolised by the other routes (Mattocks, 1982).

FMO isoforms are mainly responsible for the *N*-oxygenation of PAs, although also CYP enzymes are known to be involved in this pathway depending on species, strains and tissues (Williams et al., 1989; Fu et al., 2004). While *N*-oxygenation is generally considered as a detoxification pathway, due to the higher water solubility and more rapid excretion in comparison to their parent compounds (Mattocks, 1986; Williams et al., 1989), for PANOs this pathway is metabolically reversible by virtue of hepatic microsomal reductases and does not appear to mitigate subsequent formation of reactive toxic metabolites from PAs (Chou et al., 2003; Wang et al., 2005a).

Metabolic activation of PAs occurs via an oxidative pathway leading to the pyrrolic ester formation and is mainly catalysed by CYP 3A and 2B isoforms, although CYP 2C11 has been implicated in male rat liver (Williams et al., 1989). Due to their electrophilic nature, the dihydropyrrolizine ester(s) are chemically and biologically reactive and can undergo further transformation, particularly in aqueous solution where acid-catalysed polymerisation is facile (Mattocks, 1969). Pyrrolic esters from retronecine-, heliotridine- and otonecine-type PAs can also undergo ester hydrolysis leading to the formation of racemic (±)6,7-dihydro-7-hydroxy-1-hydroxymethyl-5H-pyrrolizine (DHP) (Huan et al., 1998a; Fu et al., 2004; Wang et al., 2005a). While DHP is less reactive than the parent pyrrolic esters, it is also unstable in aqueous solutions and retains considerable alkylating activity (Fu et al., 2004). Pyrrolic esters and DHP react readily with a wide array of cellular components, including nucleophilic sites on proteins and nucleic acids (Fu et al., 2004; Wang et al., 2005b). Finally, pyrrolic esters can also undergo Phase II detoxification by reacting with one or two molecules of glutathione (GSH) to form 7-glutathionyl-6,7-dihydro-1-hydroxymethyl-5H-pyrrolizine (7-GSH-DHP) or 7,9-diglutathionyl-6,7-dihydro-1-hydroxymethyl-5H-pyrrolizine (7,9-diGSH-DHP), respectively (Cheeke, 1988; Lin et al., 1998; Lin et al., 2000; Fu et al., 2004). There are many glutathione-*S*-transferase (GST) isoforms, often with overlapping substrate specificity and again differences in the expression of these enzymes could affect the species-differences in toxicity of PAs. In particular, the lower sensitivity of mice to PA

toxicity in comparison to rats could be explained by the high expression of GST enzymes in the former species.

The extensive binding to GSH can result in cellular GSH depletion, accompanied with a loss in the capacity to scavenge and detoxify reactive oxygen (and nitrogen) species. Hence, increased cellular oxidative stress and lipid peroxidation indirectly caused by exposure to PAs can contribute to the hepatotoxicity (Segall et al., 1985).

In contrast to the 1,2-unsaturated PAs, data suggest that no metabolic activation takes place for fully saturated PAs (e.g., platynecine-type) because reactive pyrrole derivatives cannot form (Mattocks and White, 1971).

Several studies attempted to correlate the formation of reactive pyrrole metabolites to the marked differences in PA toxicity observed in different species (Shull et al., 1976; Cheeke, 1988; Huan et al., 1998a, 1998b; Lin et al., 2002; Fu et al., 2004). Species differences in the formation of pyrrolic metabolites and *N*-oxide from senecionine were studied in vitro in hepatic microsome fractions from hamsters, rats, rabbits, chickens, Japanese quail, sheep and cattle by Huan et al. (1998a). DHP and senecionine-*N*-oxide formation was observed for all the species. However, no strong correlation between the rate of DHP formation and the known species susceptibility to senecionine could be established. The authors showed that, beside the species-specific rate of metabolic activation, the rates of PA hydrolysis and of GHS conjugation play an important role in the overall resistance to PA toxicity. However, Fu et al. (2004) recently noted that no correlation was made between species susceptibility and the formation of tissue-bound pyrroles in the liver in the Huan et al. (1998a) study and stated that such correlation is generally recognised to explain susceptibility to PA toxicity.

Beside the already described pathways, PA detoxification mediated by bacterial flora in the rumen may be an important contribution to the observed resistance of sheep to PA toxicity (Wachenheim et al., 1992a; Anjos et al., 2010), but evidently not occurring to the same extent in other more susceptible ruminants such as cattle (Cheeke, 1988; Wachenheim et al., 1992b) or in horses (Cheeke, 1988). Further details are given in Section 7.4.

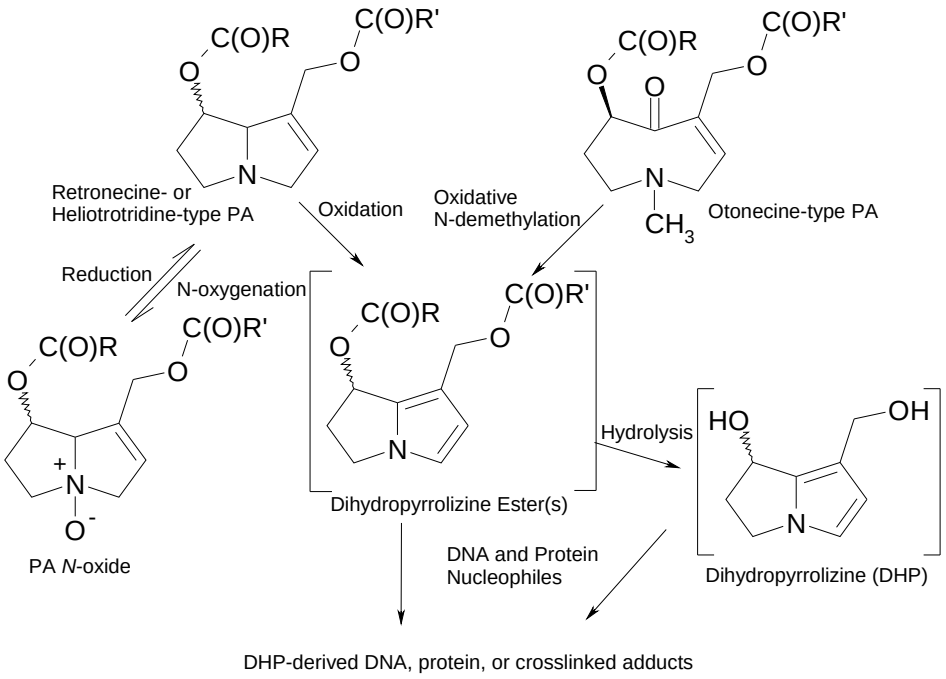


**Figure 8:** Metabolic activation of PAs (note: ester(s) designates PAs and metabolites comprised of either monoesters at the 7-position or diesters at the 7- and 9-positions).

*Excretion*

Studies with several radiolabelled PAs showed an excretion of more of 80 % of the administered substances occurred in urine and faeces by 24 hours after the administration in rats and mice via subcutaneous or intravenous injection (Eastman et al., 1982; Estep et al., 1991).

Male rats administered intraperitoneally with several PAs excreted the substances in urine as unchanged alkaloids, PANOs and pyrrolic metabolites mainly within 24 hours after the treatment (Mattocks, 1968b). On the other hand, only 4 % of administered monocrotaline was excreted as *N*-oxide derivative and 60 % excretion as the unchanged alkaloid were detected in urine of rats administered a 60 mg/kg body weight dose by subcutaneous injection (Estep et al., 1991).

### 7.1.2. Humans

Human metabolism of 1,2-unsaturated PAs was studied in vitro with human liver microsomes. CYP3A4 was identified as the main cytochrome P450 isoform responsible for the metabolism of several 1,2-unsaturated PAs to form pyrrolic esters, DNA adducts and PANOs (Fu et al., 2004). Miranda et al. (1991) studied the metabolism of senecionine by human hepatic microsomes. DHP and senecionine-*N*-oxide were the main metabolites identified, with a marked individual variability on the metabolism rates observed for different liver samples. Specific inhibition of CYP3A4 dramatically reduced the DHP and *N*-oxide formation, indicating the main role of the isoform in the human metabolism of senecionine. Couet et al. (1996) compared the formation of DHP and *N*-oxide derivative in rat and human microsomes for three PAs, monocrotaline, retrorsine and retrorsine-*N*-oxide. DHP resulted as the main metabolite both in rat and human microsomes treated with monocrotaline and retrorsine. Formation of respective PANOs was observed at lower rates both for monocrotaline and retrorsine in human microsomes. Both the parent alkaloid and DHP were detected in human and rat microsomes exposed to retrorsine-*N*-oxide, confirming the reversibility of the *N*-oxide formation. DHP and *N*-oxide were also

observed as the main metabolites formed by liver microsomes from rats and humans of both sexes exposed to riddelliine (Xia et al., 2003). The comparative study showed similar metabolic rates in rat and human microsomes, although a higher formation of riddelliine-*N*-oxide was observed for male rats. Moreover, the same set of DNA adducts were observed for human and rat microsomes, with the human samples showing either similar or 2-3 fold higher quantities in comparison to the mean quantities formed in rats. Similar DNA adduct profiles were obtained for lasiocarpine (a heliotridine-derived diester) and heliotrine (a heliotridine-derived monoester) in male and female human hepatic microsomes (Fu et al., 2004).

### 7.1.3. Summary

1,2-Unsaturated PAs, including riddelliine, are rapidly absorbed via the GI tract and distributed in the body. The study of PA metabolism identified three main pathways leading either to detoxification or to formation of reactive pyrrolic species (DHP and related esters). The latter are considered as the most active species responsible for 1,2-unsaturated PA toxicity via their potential to act as alkylating agents towards proteins and DNA. The balance among the detoxification/activation pathways is likely responsible for the observed different species-, strain- and sex-specific sensitivity to 1,2-unsaturated PA toxicity. In particular, *in vitro* studies with hepatic microsomes suggest that humans are among the sensitive species to 1,2-unsaturated PA toxicity. Similar studies in farm or domestic animals also identified significant differences in the metabolic patterns among species. However, knowledge on the expression of the enzymes in those species is still too limited to allow establishing a conclusive relationship between the metabolism and the sensitivity to 1,2-unsaturated PA toxicity.

## 7.2. Toxicity in experimental animals

Toxicity studies have been conducted with PA-containing powdered seeds or plant extracts and with purified preparations of PAs. This section focuses on the studies conducted with purified PAs, and on characterised plant material providing information on the doses of PAs.

### 7.2.1. Acute toxicity

Acute toxicity was determined for several PAs administered by intraperitoneal (i.p.) or intravenous (i.v.) injection. Available $LD_{50}$s via the two routes are summarized in Tables 15 and 16.

**Table 15:** $LD_{50}$-values of several PAs after i.p. injection in rats.

| PA | $LD_{50}$ (mg/kg) | Observation period | Strain and sex |
|---|---|---|---|
| 7-O-Angeloylheliotridine | 250[a] | <1 (deaths occurred few hours after dosing) | Hooded Wistar, male |
| Cynaustine | 260[b] | 3 days | Strain not specified, male |
| Echimidine | 200[c] | Not reported | Strain not specified, male |
| Echinatine | 350[c] | Not reported | Strain not specified, male |
| Europine | > 1000[c] | Not reported | Strain not specified, male |
| Heleurine | 140[c] | Not reported | Strain not specified, male |
| Heliosupine | 60[c] | Not reported | Strain not specified, male |
| Heliotridine | 1500[a] | Not reported | Hooded Wistar, male |
| Heliotrine | 296[d] | 3 days | Hooded strain (not specified), male |
| | 478[d] | 3 days | Hooded strain (not specified), female |
| Heliotrine-*N*-oxide | > 5000[d] | 8 days | Hooded strain (not specified), male |
| Indicine | > 1000[e] | Not reported | Strain not specified, male |
| Intermedine | 1500[f] | Not reported | Strain not reported, male |
| Jacobine | 138[b] | 3 days | Strain not specified, female |
| Jaconine | 168[b] | 3 days | Strain not specified, female |
| Lasiocarpine | 77[d] | 3 days | Hooded strain (not specified), male |
| | 79[d] | 3 days | Hooded strain (not specified), female |
| Lasiocarpine-*N*-oxide | 547[d] | 3 days | Hooded strain (not specified), male |
| | 181[d] | 3 days | Hooded strain (not specified), female |
| Latifoline | 125[b] | 3 days | Strain not specified, male |
| Lycopsamine | 1500[f] | Not reported | Strain not reported, male |
| Monocrotaline | 175[c] | Not reported | Strain not specified, male |
| | 109[g] | 4 days | Strain not specified, male |
| | 230[g] | 4 days | Strain not specified, female |
| Platyphylline[j] | 252[a] | Not reported | Hooded Wistar, male |
| Retrorsine | 153[g] | 7 days | Strain not specified, female |
| | 34-38[h] | 7 days | Strain not specified, male |
| Retrorsine-*N*-oxide | 250[h] | 7 days | Strain not specified, male |
| Rinderine | 550[b] | 3 days | Strain not specified, male |
| Senecionine | 85[c] | Not reported | Strain not specified, male |
| | 50[g] | 7 days | Strain not specified, male |
| Seneciphylline | 77[b] | 3 days | Strain not specified, male |
| | 83[b] | 3 days | Strain not specified, female |
| Senkirkine | 220[i] | Not reported | ACI, male |
| Spectabiline | 220[b] | 3 days | Strain not specified, male |
| Supinine | 450[c] | Not reported | Strain not specified, male |
| Symphytine | 130[i] | Not reported | ACI, male |
| | 300[f] | Not reported | Strain not specified, male |
| Triacetylindicine | 164[g] | 4 days | Strain not specified, male |

(a): Reported by Downing and Peterson, 1968.
(b): Reported by Bull et al., 1968.
(c): Reported by Culvenor et al., 1969.
(d): Bull et al., 1958.
(e): Schoental, 1968
(f): Cheeke and Shull, 1985.
(g): Mattocks, 1972.
(h): Mattocks, 1971.
(i): Hirono et al., 1979
(j): 1,2-Saturated PA.

**Table 16:** $LD_{50}$ values of several PAs after intravenous (i.v. injection) (as reported by Mattocks, 1986).

| PA | $LD_{50}$ (mg/kg) | Observation period | Species[(a)] |
|---|---|---|---|
| Heliotrine | 274 | 7 days | Rat |
| | 255 | 7 days | Mouse |
| Integerrimine | 78 | 7 days | Mouse |
| Jacobine | 77 | 7 days | Mouse |
| Lasiocarpine | 88 | 5 days | Rat |
| | 85 | 5 days | Mouse |
| | 67.5 | 5 days | Hamster |
| Retrorsine | 38 | 7 days | Rat |
| | 59 | 7 days | Mouse |
| Retrorsine-*N*-oxide | 834 | 7 days | Mouse |
| Riddelliine | 105 | 7 days | Mouse |
| Senecionine | 64 | 7 days | Mouse |
| | 61 | 7 days | Hamster |
| Seneciphylline | 90 | 7 days | Mouse |
| Spartioidine | 80 | 7 days | Mouse |

(a): sex and strain not specified.

A marked variation in the toxicity of different PAs can be observed, with the large macrocyclic diesters (e.g. retrorsine or senecionine) having apparently higher potency in comparison to monoesters (e.g. heliotrine). Wang et al. (2011) recently compared the acute oral toxicity of **senecionine** and **adonifoline** in mice. Senecionine, with a reported $LD_{50}$ of 171 μmol/kg b.w. (corresponding to 57 mg/kg b.w.) was approximately threefold more toxic than adonifoline (reported $LD_{50}$ of 447 μmol/kg b.w., corresponding to 163 mg/kg b.w.). The differences in toxicity were correlated with the different toxicokinetic profiles of the 2 alkaloids (Wang et al., 2011). Similar $LD_{50}$ were reported by Mattocks (1971) for **retrorsine** following i.p. and oral administration (experimental details not specified). **Retrorsine-*N*-oxide** showed a relatively low toxicity when administered by intraperitoneal injection ($LD_{50}$ of 250 mg/kg b.w., however the acute toxicity ($LD_{50}$ of 48 mg/kg b.w) was comparable to the parent PA by oral administration ($LD_{50}$ of 34-38 mg/kg b.w., reported by Mattocks, 1971). This difference was attributed to the reduction of the *N*-oxide to the parent PA mediated by the intestinal microflora in the gut (Mattocks, 1971).

A species-specific response to acute effects of **retrorsine** was noted by White et al. (1973) in mice, hamsters, guinea pigs, fowls and quails exposed via i.p. injection. Retrorsine resulted highly toxic in mice, hamsters and fowls ($LD_{50}$ of 65-69 mg/kg b.w., 81 mg/kg b.w. and 85 mg/kg b.w. were determined for the 3 species, respectively), whereas guinea pigs and quails resulted to be less responsive ($LD_{50}$ of >800 mg/kg b.w. and 279 mg/kg b.w. were determined for the 2 species, respectively). The species sensitivity correlated well with the hepatic levels of formed pyrroles, indicating that a lower metabolic activation is likely responsible of the higher resistance to retrorsine acute toxicity observed in quail and guinea pig.

A number of early publications have reported, with limited detail, that chronic liver lesions are induced by single doses of pyrrolizidine alkaloids, including **lasiocarpine, heliotropine, heliotrine, retrorsine, riddelliine, seneciphylline, senkirkine, hydroxysenkirkine** (e.g. Schoental and Magee, 1959; Schoental, 1979). WHO noted that the liver effects of pyrrolizidine alkaloids are similar whether resulting from one dose, which is not acutely lethal, or multiple low level doses (WHO-IPCS, 1988).

Liver and lung were the main organs affected following acute exposure of laboratory animals to 1,2-unsaturated PAs. Confluent haemorrhagic necrosis in the liver was the most common lesion produced, followed by lesions in the central and sublobular veins of the liver (such as subintimal oedema, necrosis, deposits of fibrin, thrombosis, and occlusion of the lumen). Acute veno-occlusive

disease similar to that observed in human poisoning cases could be experimentally reproduced in monkeys and rats administered a single dose of several 1,2-unsaturated PAs or PANOs (WHO-IPCS, 1988). As shown by Schoental and Bensted (1963), rats receiving a sublethal dose of 30 mg/kg **retrorsine** showed irreversible hepatic lesions possibly leading to chronic liver disease and eventually to hepatocellular carcinoma more than 13 months after administration.

The relative hepatotoxicity of 62 PAs has been investigated by administering single i.p. doses in the range of 0.025-3.2 mmol/kg b.w. (e.g. 7.5-960 mg/kg for a PA with molecular weight of 300) to two-week-old hooded Wistar rats (usually 2 males and 2 females per group), and the livers of surviving animals were examined four weeks later (Culvenor et al., 1976). The 1,2-saturated PA, **platyphylline** caused deaths at 118 mg/kg b.w. within 1 hour, and liver necrosis was not reported (Jago, 1970). Similarly **cynaustraline** caused rapid death (67 mg/kg b.w.) and no hepatotoxicity. Most, but not all of the 1,2-unsaturated PAs resulted in deaths with liver necrosis within 1 week, or in liver megalocytosis, which was observed at autopsy after 4 weeks. On a molar basis, diesters of heliotridine and retronecine were about 4 times more toxic than the respective monoesters, and heliotridine esters were 2-4 times more toxic than retronecine esters. Crotonecine esters were less toxic than retronecine esters. A number of heliotridine esters did not cause liver toxicity under the conditions of the experiment (Culvenor et al., 1976).

Wagenvoort et al. (1974) administered **fulvine** intragastrically at a single dose of 80 mg/kg b.w. Vasoconstriction and medial hypertrophy of pulmonary arteries and right ventricular hypertrophy started to develop one week after the administration.

Pulmonary lesions were observed in dogs and rats administered a single i.v. or subcutaneous injection of **monocrotaline**. Observations included ultra-structural changes in the endothelial cells of the alveolar capillaries, of microvascular leak in the alveolar wall and interstitial oedema (Valdivia et al., 1967a,b; Miller et al.,1978).

### 7.2.2. Repeated dose toxicity

The repeated dose toxicity of pyrrolizidine alkaloids has not been systematically investigated, and the available studies were mainly designed to provide mechanistic information. As a result there is limited information on dose-response relationships. Of the 1,2-unsaturated PAs that have been investigated in experimental animals, the most common toxic effect is hepatotoxicity, characterised by megalocytosis (enlarged hepatocytes containing hyper-chromatic nuclei), and sometimes centrilobular necrosis, fibrosis and bile duct hyperplasia. Pulmonary toxicity is sometimes, but not always, seen with the 1,2-unsaturated PAs, and it most commonly reported with **monocrotaline** and **fulvine**. The structural requirements for toxicity in the lung are the same as those for toxicity in the liver and metabolites produced in the hepatocytes cause toxicity in the lung. Pulmonary toxicity manifests as pulmonary hypertension and can lead to cardiac right ventricular hypertrophy. Acute lesions include alveolar oedema and effects on the alveolar wall. Chronic lesions can include extensive pleural effusion or necrotising pulmonary arthritis (WHO-IPCS, 1988). There may also be abnormal macrophages and a proliferation of mast cells. Initial damage is reported to be to the endothelial cells of the small blood vessels, this is followed by changes in the alveolar wall and then a reduction in the lumen of the small vessels (Mattocks, 1986). Most of the available studies on pulmonary toxicity have involved parenteral administration, and were designed to establish an experimental model for pulmonary hypertension.

Groups of 20 male F-344 rats were fed a diet containing 50 mg/kg **lasiocarpine** (reported to be 'pure crystalline lasiocarpine') for 15 weeks. Following autopsy, the only significant observation in the liver was 'moderately advanced megalocytosis of hepatic parenchymal cells' (Reddy et al., 1976).

**Lasiocarpine** (97 % pure) has been investigated by the National Toxicology Program (NTP) in Fischer 344 rats, with dietary administration for 8 and 104 weeks (NTP, 1978). In the 8-week study, lasiocarpine was administered in the diet at 0, 5, 10, 20, 40, 80 and 160 mg/kg (equivalent to 0, 0.5, 1, 2,

4, 8 and 16 mg/kg b.w. per day) to groups of 15 male and 15 female rats. The rats in the high dose group were culled after 2 weeks due to appreciable weight loss. After 8 weeks, body weight gain was decreased in females and males given 40 and 80 mg/kg lasiocarpine, but not in the lower dose groups.

Based on the above 8-week study, **lasiocarpine** concentrations of 7, 15 and 30 mg/kg diet (equivalent to 0, 0.35, 0.75 and 1.5 mg/kg b.w. per day) were selected for the 104-week study. Groups of 24 male and 24 female rats were used. All high dose females had died by week 69, and high dose males had died by week 88, and a dose-related decrease in survival also occurred at the two lower doses. Nodular hyperplasia was observed in treated rats, but not in controls, indicating a proliferative effect (NTP, 1978). The neoplastic findings of this study are reported in Section 7.2.5 below.

**Riddelliine** (92 % riddelliine, 5 % retrorsine, 1.3 % seneciphylline) has been investigated by the NTP in F344/N rats and B6C3F1 mice exposed by gavage (5 days/week) for 2, 13 and 105 weeks (NTP, 1993; Chan et al., 1994; Chan et al., 2003; NTP 2003). In the 2-week study, groups of five male and five female rats and mice were dosed with 0, 0.33, 1.0, 3.3, 10, or 25 mg per kg body weight. In rats, males were more sensitive than females. Four male rats of the 25 mg/kg dose group died or were culled due to poor condition before the end of the study. Decreased body weight gains were observed in male rats from the 10 and 25 mg/kg groups. At 1.0 mg/kg and above, increased incidence of lesions in the liver (haemorrhagic centrilobular hepatic necrosis, hepatocytic karyomegaly and cytologic alterations), spleen (splenic extramedullary haematopoiesis), lung (pulmonary haemorrhage and oedema) and pancreas (pancreatic oedema) were observed in male rats. Similar effects were observed with lower severity in female rats administered riddelliine at 3.3 mg/kg b.w. per day or greater. Mice were less sensitive; increased liver weights and incidences of hepatic cytomegaly in males and females were the only reported effects (NTP, 1993).

In the 13-week study, groups of 20 male and female rats were given riddelliine doses of 0, 0.1, 0.33, 1.0, 3.3 or 10 mg/kg b.w. per day and mice were given doses of 0, 0.33, 1.0, 3.3, 10 or 25 mg/kg b.w. per day (Chan et al., 1994). Ten animals from each dose group were autopsied at the end of the treatment period, whilst groups of 5 animals were maintained without further treatment for a recovery period of 7 or 14 weeks. There were mortalities in the rats treated at 10 mg/kg b.w. per day, 19 of the 20 males died between days 58 and 92, and 5 females died between days 124 and 173 (during the recovery period). There were dose-related decreased in body weight gain and final body weight, during treatment, with some recovery of weight gain during the recovery period. The liver was the primary target, with the major findings at non-lethal doses being nuclear enlargement and eosinophilia of the cytoplasm that persisted into the recovery periods in both males and females, although the lesions were reported to be less severe in females. The no-observed-adverse-effect-level (NOAEL) was 0.1 mg/kg b.w. per day, 5 days per week (0.07 mg/kg b.w. per day averaged over the week). Mice appeared to be less sensitive than rats: there were no treatment-related deaths and histopathological changes were limited to the high dose group with mild enlargement of centrilobular hepatocytes in both sexes and bile duct hyperplasia in females during the recovery period. Dose-related decreases in body weight and body weight gains were reported in both sexes at 10 or 25 mg/kg b.w. per day, which persisted into the recovery period. The NOAEL was 3.3 mg/kg b.w. per day, 5 days per week (2.4 mg/kg b.w. per day averaged over the week) (Chan et al., 1994).

In the 105 week study, groups of 50 male and female rats given riddelliine doses of up to 1.0 mg/kg b.w. per day and doses of up to 3.0 mg/kg b.w. per day, with only controls and top dose groups for the male rats and female mice (Chan et al., 2003). There was a high rate of mortality in the rats dosed at 1.0 mg/kg b.w. per day. The incidences of several non-neoplastic lesions of the liver (including cytomegaly, focal necrosis, eosinophilic foci, bile duct hyperplasia) were significantly increased in female rats with a LOAEL of 0.033 mg/kg b.w. per day and a NOAEL of 0.01 mg/kg b.w. per day, 5 days per week (0.007 mg/kg b.w. per day averaged over the week). Survival of mice was also decreased in the top dose group, and there was a decrease in mean body weight at 1.0 mg/kg b.w. per day. Non-neoplastic lesions of the liver, kidney, lung and arteries were reported in mice, with a LOAEL

(hepatocyte cytomegaly and karyomegaly) at 0.3 mg/kg b.w. per day and a NOAEL of 0.1 mg/kg b.w. per day. (Chan et al., 2003). The neoplastic findings of this study are reported in Section 7.2.5 below.

### 7.2.3. Developmental and reproductive toxicity

In an early study with limited reported detail, Administration of 25-40 mg **lasiocarpine** in 5-10 doses of 5-10 mg twice weekly or more to lactating rats (200-300 g) orally resulted in liver toxicity and deaths in the suckling pups at doses that had no apparent effects on the dams or their milk production. Similarly **retrorsine** administered orally or by i.p. injection (4-10 mg for 1-14 days) to lactating rats (185-335 g) was more toxic to the pups than to the dams (Schoental, 1959).

Studies in pregnant Sprague-Dawley or hooded rats given **heliotrine** ('purified crystalline') by i.p. injection showed fetal malformations at doses of 100 – 300 mg/kg maternal body weight. These included retarded development, musculoskeletal defects, cleft palate and at high doses cessation of growth, immature fetuses and intrauterine deaths and resorptions. Pups of dams dosed at 50 mg/kg b.w. showed decreased weight and length. Little liver damage was observed in the fetal rats. At doses of 50 mg/kg b.w. and higher, decreased body weight gain was reported in the maternal animals. Litters of dams dosed at 15, 20, 30 and 40 mg/kg b.w. were reported to be normal, however it should be noted that only one dam was treated at each of these doses (Green and Christie, 1961).

When **heliotrine** was administered i.p to Wistar rats at doses of 140-150 mg/kg b.w. per day on gestational days (GD) 10-21, either the dams died within 24h, or the fetuses were resorbed. Administration of heliotrine as a single i.p. dose of 150 mg/kg b.w. on postnatal day (PND) 2, or 10 i.p. doses of 30 mg/kg b.w. over PND 1-28 resulted in liver lesions in the sucklings. Maternal body weight gain was decreased in the 6 days after injection, but no abnormalities were observed at necropsy or histopathological examination (Bhattacharyya, 1965).

**Heliotrine** was administered i.p. to pregnant hooded rats at a dose of 200 mg/kg b.w. on GD 14 and the fetuses were examined on GD 20. Development was less advanced than in controls, and a range of defects was present, the most common being cleft palate, micrognathios and deformation of the ribs and long bones. Effects on the dams were not described (Peterson and Jago, 1980).

**Integerrimine** was administered *i.p.* at doses of 0, 25, 37.5 and 50 mg/kg b.w. (stated to be 0.25, 0.375 and 0.5 of the $LD_{50}$ dose) to pregnant C57BL/6 and C3H/HeJ mice at varying times up to GD8. Injection within the first 3 days resulted in pre- or post-implantation loss in C57BL/6, and also in malformations (microophthalmia, anophthalmia, microcephaly and anencephaly) when dosing was on GD3. Malformations were not observed in C3H/HeJ mice. Effects on the dams were not described (Kvitko and Gimmler, 1986).

Administration of **lasiocarpine** i.p. to Wistar rats at a single dose of 50, 80 or 100 mg/kg b.w. in mid-gestation resulted in rapid death of the animals. Administration of lasiocarpine as a single i.p. dose of 50 mg/kg b.w. on PND 2, or 10 i.p. doses of 10 mg/kg b.w. over PND 1-28 resulted in liver lesions in the sucklings. Effects on the dams were not described (Bhattacharyya, 1965).

Administration of **monocrotaline** i.p. to Wistar rats at a single dose of 100 mg/kg b.w. in mid-gestation, at a single dose of 100 mg/kg b.w. on PND2, or a total of 150 mg/kg b.w. over PND 1-28 resulted in liver lesions in the pups. Effects on the dams were not described (Bhattacharyya, 1965).

Administration of **retrorsine** i.p. to Wistar rats at a single dose of 30 or 40 mg/kg b.w. in mid-gestation, at a single dose of 40 mg/kg b.w. on PND2, or a total of 100 mg/kg b.w. over PND 1-28 resulted in liver lesions in the pups. Effects on the dams were not described (Bhattacharyya, 1965).

A preparation of **riddelliine** (92 % riddelliine, 5 % retrorsine, 1.3 % seneciphylline) was administered by gavage to groups of 20 male and 40 female Fischer F344/N rats at doses of 0, 0.1, 1.0 or 10 mg/kg

b.w. per day and $B_6C_3F_1$ mice at 0, 0.33, 3.3 or 25 mg/kg b.w. per day 5 days per week for 10 weeks before mating for both sexes, continuing through gestation and lactation for the females (Chan et al., 1994). Female rats and mice in the top dose groups had prolonged oestrus. Dam weights were generally lower than controls during pregnancy and lactation and there was a significant decrease in live pup weights and pup survival of mice treated at 25 mg/kg b.w. per day. From the limited detail available on the results of this study, it appears that the fetal NOAELs were 1 mg/kg b.w. per day in rats (0.7 mg/kg b.w. per day averaged over the week) and 3.3 mg/kg b.w. per day in mice (2.4 mg/kg b.w. per day averaged over the week), and it is unclear whether or not the reported effects on the pups were secondary to maternal toxicity (Chan et al., 1994).

In a study available in abstract only, **senecionine** was administered to pregnant rats orally at 20 mg/kg b.w. per day on GD 1-10, or by subcutaneous (s.c.) injection at 2 and 5 mg/kg b.w. per day on GD 4-10. Senecionine treatment resulted in decreased numbers of normal fetuses after oral dosing and in decreased relative liver weights of the fetuses after s.c. injection. Effects on the dams were not described (Nuzzo et al., 1987).

Overall, developmental toxicity of PAs has mainly been observed following parenteral administration, and it is not possible to determine if it is related to maternal toxicity. Therefore, the CONTAM Panel concluded that this effect could not be used in the risk characterisation.

### 7.2.4. Genotoxicity

Extensive *in vitro* and *in vivo* studies have been carried out on the genotoxicity of PAs (Table 17). Owing to the development of advanced analytical methodology which has enabled a detailed study of the mechanism of action, it is now believed that metabolic activation of PAs to pyrrolic ester(s), and the subsequent formation of DNA adducts is the key pathway leading to the genotoxic effects.

*DNA adduct formation.*

The proposed metabolic scheme for the formation of DNA adducts by PAs is shown in Figure 8. Oxidative metabolism of retronecine- and heliotridine- or otonecine-type PAs yields similarly reactive species, the corresponding dihydropyrrolizine mono- or diester. Binding to DNA leads to nucleoside adduct formation, DNA cross-linking, and DNA-protein cross-linking, which presumably induce the cytotoxic, genotoxic, and tumorigenic effects of the PAs (Wang et al., 2005b; Fu et al., 2004). Hydrolysis of the pyrrolic ester(s) yields DHP, which although less reactive than the pyrrolic ester(s), also forms DNA adducts (Fu et al., 2010) and is carcinogenic in experimental animals (Allen et al., 1975; Mattocks and Cabral, 1982).

Initially eight DHP-derived nucleotide adducts were identified using a $^{32}$P-postlabelling/HPLC method (Yang et al., 2001), which were subsequently characterized as two epimeric dGMP adducts and a series of dinucleotide adducts (Chou et al., 2003). Dinucleotide adducts formed with guanine, adenine and thymine, but not with cytosine. More recently, the mononucleotide adducts were structurally characterized by using $^{1}$H-NMR and ESI-MS/MS and a specific and sensitive LC-ESI-MS/MS method was developed to quantify DHP-derived dG and dA adducts (see Figure 9) formed *in vitro* and *in vivo* (Fu et al., 2010). The major nucleoside adducts formed *in vivo* are between the exocyclic amino group of either dG or dA at the 5- and 9-positions of DHP (Fu et al., 2010).

The formation of DNA adducts by PAs is summarised in Table 17. An early study by Candrian et al. (1985) demonstrated that $^{3}$H-labelled **seneciphylline** and **senecionine** bound to DNA, although the adducts were not identified. The $^{32}$P-postlabelling/HPLC method developed by Yang et al. (2001, reviewed in Fu et al., 2004) was later used to identify adducts derived from comfrey root extract, comfrey compound oil, coltsfoot root extract, *Flos farfara* extract, *Ligularia hodgsonnii* extracts, **clivorine**, **heliotrine**, **lasiocarpine**, **monocrotaline**, **retrorsine** and **riddeliine**. The same set of DHP-derived adducts were found from these PAs *in vitro* and *in vivo* using a $^{32}$P-postlabelling/HPLC method,

suggesting that the pyrrolic ester mechanism illustrated in Figure 8 is general for all these structurally related compounds. For riddelliine the amount of DHP adduct formation at different doses correlated with tumorigenic responses suggesting a causal relationship between DNA adduct formation and carcinogenesis.

As there are several reactive sites in the pyrrolic ester(s) molecule, at the C5/C7 and C9 positions, cross-linking can occur between two sites in DNA or between DNA and protein (Table 17 and Figure 9). DNA-protein cross-links have been demonstrated *in vitro* for **dehydromonocrotaline, dehydroriddelliine, dehydrosenecionine, dehydroseneciphylline,** and *in vivo* for **jacobine**. Because the potency of PAs to form DNA-protein cross-links was found to be related to their *in vivo* toxicity in animals, it was postulated that they were mechanistically involved in the toxicity (Kim et al., 1995) DNA - DNA crosslinks are formed by **monocrotaline** and **dehydromonocrotaline**, *in vitro* and *in vivo* (Petry et al., 1984; Pereira et al., 1998). The production of proteinase-sensitive cross-links with DNA and DNA interstrand cross-links by PAs was studied by Hincks et al. (1991) in bovine kidney epithelial cells in the presence of an external metabolising system, and the relative potencies in forming these adducts were: seneciphylline > riddelliine > retrorsine > senecionine > heliosupine > monocrotaline > latifoline > retronecine. The chemical nature of these adducts has not been established.

**Table 17:** Genotoxicity study results for PAs (based on Chen et al., 2010).

| Agent | DNA adducts *in vitro* | DNA adducts *in vivo* | DNA-DNA cross-linking | DNA-protein cross-linking | DNA strand breaks *in vitro* | UDS *in vivo* | MN *in vitro* | MN *in vivo* | CA *in vitro* | CA *in vivo* | SCE *in vitro* | Mutations in bacteria | Mutations in Drosophila | Mutations in rodents |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Clivorine | + | | | | | | | | | | | + | | |
| Heliotrine | + | | | | | | | + | + | | + | +/- | + | |
| Lasiocarpine | + | | | | | | | | + | | | + | | |
| Monocrotaline | + | + | + | + | +/- | | + | + | + | | + | - | + | |
| Retrorsine | + | + | + | | - | + | + | | + | | | + | + | |
| Riddelliine | + | + | + | + | - | + | | +/- | + | | + | +/- | | + |
| Senecionine | | + | + | + | - | + | | | | | | - | + | |
| Seneciphylline | | + | + | + | - | + | | | | | + | (+) | + | |
| Heliosupine | | | + | | - | | | | | | | | | |
| Jacobine | | | + | + | - | | | | | | | | | |
| Latifoline | | | + | | - | | | | | | | | | |
| Isatidine | | | | | + | | + | | +/- | | | - | | |
| Integerrimine | | | | | | | | + | | + | | | + | |
| Fulvine | | | | | | | | | | + | | | | |
| Senkirkine | | | | | | | | | + | | + | + | + | |
| Petasitenine | | | | | | | | | + | | | | | |
| Fukinotoxin | | | | | | | | | | | | + | | |
| Ligularidine | | | | | | | | | | | | + | | |
| LX201 | | | | | | | | | | | | + | | |
| Senecivernine | | | | | | | | | | | | (+) | | |
| 7-acetylintermedine | | | | | | | | | | | | | + | |
| 7-acetyllycopsamine | | | | | | | | | | | | | + | |
| Indicine | | | | | | | | | | | | | + | |
| Indicine-*N*-oxide | | | | | | | | | | | | | + | |
| Intermedine | | | | | | | | | | | | | + | |
| Lycopsamine | | | | | | | | | | | | - | + | |
| Supinine | | | | | | | | | | | | | - | |
| Symlandine | | | | | | | | | | | | | + | |
| Symphytine | | | | | | | | | | | | | + | |
| Dehydroretronecine | | | | | | | | | | | + | + | | |
| Jacoline | | | | | | | | | | | | | + | |

UDS: unscheduled DNA synthesis; MN: micronuclei; CA: chromosomal aberrations; SCE: sister chromatid exchange.
Notes: + = positive result; - = negative result; empty cells = not tested.

**Table 18:** Genotoxicity study results for plant extracts containing PA (based on Chen et al., 2010).

| Agent | DNA adducts *in vitro* | DNA adducts *in vivo* | DNA-DNA cross-linking | DNA-protein cross-linking | DNA strand breaks *in vitro* | UDS *in vivo* | MN *in vitro* | MN *in vivo* | CA *in vitro* | CA *in vivo* | SCE *in vitro* | Mutations in bacteria | Mutations in Drosophila | Mutations in rodents |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Comfrey root extract | | + | | | | | | | | | | | | |
| Comfrey compound oil | | | | | | | | | | | | | | |
| Comfrey | | | | | | | | | | | | | | + |
| Coltsfoot root extract | | + | | | | | | | | | | | | |
| *Flos farfara* extract | | + | | | | | | | | | | | | |
| *Ligularia hodgsonnii* extracts | + | | | | | | | | | | | | | |
| *Crotalaria* seeds | | | | | | | | + | | | | | | |
| Mixture | | | | | | | | + | | | | | | |
| Integerrimine, retrorsine, impurities | | | | | | | | | | | | | | |
| *Crotalaria retusa* | | | | | | | | | | + | | | | |
| *Heliotropium curassavicum* | | | | | | | | | + | | | | | |
| *Senecio inaequidens* | | | | | | | | | | | | (+) | | |
| *S. fuchsia* | | | | | | | | | | | | (+) | | |
| *S. cacaliaster* | | | | | | | | | | | | (+) | | |
| Ragwort | | | | | | | | | | | | + | | |

UDS: unscheduled DNA synthesis; MN: micronuclei; CA: chromosomal aberrations; SCE: sister chromatid exchange.
Notes: + = positive result; - = negative result; empty cells = not tested.

*DNA strand breakage*

No detectable DNA single strand breaks were detected *in vitro* in bovine kidney epithelial cells using the alkaline elution assay following treatment **with heliosupine, latifoline, monocrotaline, retrorsine, riddelliine, senecionine, seneciphylline** (Hincks et al., 1991) or with **jacobine** in rat liver (Petry et al., 1986). The *in vitro* comet assay however indicated that breaks were produced by isatidine in human hepatoma cells (HepG2) (Uhl et al., 2000), and by monocrotaline in the human glioblastoma cell line GL-15 (Silva-Neto et al., 2010).

*Unscheduled DNA synthesis*

Measurements of unscheduled DNA synthesis have been carried out in hepatocytes cultured from rats and mice following PA administration. As indicated in Table 17 positive results were found with **retrorsine, riddelliine, senecionine, seneciphylline** (Chan et al., 1994; Griffin and Segall, 1986; Mirsalis et al., 1987, 1993).

*Chromosomal damage*

PAs are clastogenic (Table 17) (see review by Chen et al., 2010 and references therein). *In vitro* micronuclei (MN) are induced in cultured rat hepatocytes by **isatidine (retrorsine-*N*-oxide), monocrotaline and retrorsine**, and in mouse tissues by *crotalaria* seeds, a crude mixture of integerrimine, retrorsine and impurities, heliotrine, integerrimine, monocrotaline, and riddelliine (which was only weakly positive with a dose of 150 mg/kg b.w. by gavage). Riddelliine was negative in a MN assay of mouse or rat peripheral blood at doses up to 10 mg/kg b.w. for rats and 25 mg/kg b.w. for mice administered by gavage daily for 4 or 13 weeks. Transplacental exposure of mice to heliotrine and monocrotaline resulted in an increased frequency of MN in fetal liver.

Chromosome aberrations (CA) are produced *in vitro* by **isatidine, heliotrine, lasiocarpine, monocrotaline, petasitenine, riddelliine, retrorsine, senkirkine** and by *Heliotropium curassavicum* extracts. *Crotalaria retusa* extracts induced CA in mouse bone marrow cells. CA have been reported in blood cells of children suffering from veno-occlusive disease, and it has been suggested that this is due to fulvine.

Sister chromatid exchange (SCE) was induced *in vitro* in V79 cells by **heliotrine, monocrotaline, riddelliine, seneciphylline and senkirkine**, and in human lymphocytes by **dehydroretronecine**.

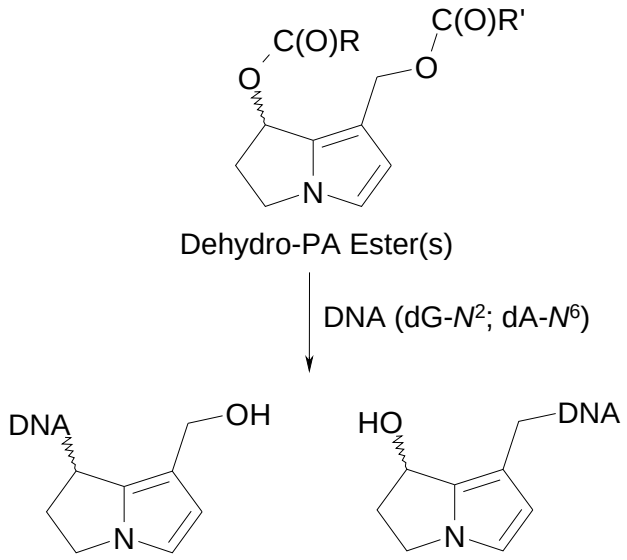


**Figure 9:** PA-derived DNA adducts (note: ester(s) designates PAs and metabolites comprised of either monoesters at the 7-position or diesters at the 7- and 9-positions).

*Mutation*

The mutagenicity of PAs has been clearly demonstrated in bacteria, *Drosophila*, and rodents (see review by Chen et al., 2010 and references therein). Mutation caused by PAs in *Salmonella typhimurium* strains TA1535, TA1537, TA92, TA98 and TA100 have all been found (Table 15). Generally metabolic activation with S9 was used when a positive result was obtained. The reported results do not indicate complete consistency for all PAs. Thus for example *Salmonella typhimurium* T100 (in the presence of S9 metabolic activation, except where indicated) showed positive mutation results for **clivorine**, **dehydroretronecine** (no S9), **fukinotoxin**, **heliotrine**, **lasiocarpine**, **ligularidine**, **riddelliine**, **senkirkine**, and tansy ragwort (*J. vulgaris)*, weakly positive results for retrorsine, senecivernine, seneciphylline, and negative results for isatidine, monocrotaline, senecionine.

In *Drosophila melanogaster*, the sex-linked recessive lethal assay was positive for **heliotrine, monocrotaline, seneciphylline and senkirkine**. In the somatic recombination and wing spot test in *D. melanogaster* 85-90 % of the genotoxic events from integerrimine were due to mitotic recombination activity and 10-15 % due to somatic mutations. 15 PAs and one PANO (indicine-*N*-oxide) were compared by Frei et al. (1992) in the wing spot test, which revealed a 50,000-fold variation in genotoxic potency. The most potent compounds were the macrocyclic diester-type PAs, senkirkine and monocrotaline being the most active.

More recently important mechanistic information has been found from studies of mutagenic activity of PAs in transgenic animals. In Big Blue transgenic rats Mei et al. (2004a, 2004b) explored the mutagenic activity of **riddelliine**. The rats were gavaged with 0.1, 0.3 and 1.0 mg/kg b.w. 5 days a week for 12 weeks. There was a dose-dependent increase in mutant frequency in liver *cII* genes. It is known that liver tumours are produced by the top two doses (NTP, 2003), and that these are mainly developed from endothelial cells in liver (hemangiosarcomas). Further investigation in the transgenic rats showed that endothelial cells had a significantly higher *cII* mutation frequency in treated rats compared to control rats, whereas parenchymal cells showed no difference, indicating that mutation by riddelliine is a key event in the carcinogenesis pathway. Levels of DHP adducts are also significantly higher in endothelial cells than parenchymal cells. The major type of mutations were G:C to T:A transversions and tandem base substitutions of G:G to T:T and G:G to A:T, consistent with a mechanism involving adduct formation at G. Mutations induced by riddelliine were also found in

K-*ras* and *p*53 genes in liver hemangiosarcomas in B6C3F1 mice (Hong et al., 2003). In 58 % of the tumours there was a G:C to T:A mutation in codon 12 of K-*ras*. Dietary comfrey also induced mutations in Big Blue transgenic rats, with a much greater frequency in liver compared to lung, consistent with liver being the major cancer target tissue (Mei et al., 2005; Mei and Chen, 2007; Mei et al., 2010). Again G:C to T:A transversions and tandem base substitutions were the major mutations. This supports the hypothesis that PAs in comfrey are responsible for its carcinogenicity.

### 7.2.5. Carcinogenicity

The main carcinogenic target site for PAs in experimental systems is the liver although tumours have been reported in many other tissues, e.g. lung, kidney, skin, bladder, brain and spinal cord, pancreatic islets and adrenal gland (Chen et al., 2010). There are no human epidemiological data on PA carcinogenesis. The two PAs with most data are riddelliine and lasiocarpine, both of which have been tested for carcinogenicity by the NTP.

**Riddelliine** (refer to purity profile in Section 7.2.2) was studied in a two year carcinogenicity study under NTP in F344 rats and B6C3F$_1$ mice. Liver haemangiosarcomas were seen in 43 of 50 male rats and 38 of 50 female rats at 1 mg/kg b.w. per day and in 3 of 50 female rats at 0.33 mg/kg b.w. per day. A statistically significant increase in the incidence of mononuclear cell leukemia in male and female rats exposed to 1 mg/kg b.w. per day was observed when survival adjusted rates were taken into account (Chan et al. 2003). In male mice, liver haemangiosarcomas were seen in 31 of 50 animals at 3 mg/kg b.w. per day. Female mice showed alveolar and bronchiolar neoplasms in 13 of 50 animals at 3 mg/kg b.w. per day (Chan et al., 2003). The authors of this study concluded that there was clear evidence of carcinogenic effects of riddelliine in F344 rats and B6C3F$_1$ mice (NTP, 2003). IARC have classified riddelliine as Group 2B 'possibly carcinogenic to humans' (IARC, 2002) and the NTP classification is 'reasonably anticipated to be a carcinogen'.

A two-year carcinogenicity study of **lasiocarpine** has been carried out by the NTP in F344 rats. Liver angiosarcoma was seen in 13 of 23 male and 2 of 23 female rats following dietary administration at 30 mg/kg in diet (equivalent to 1.5 mg/kg b.w. per day), in 11 of 23 males and 7 of 24 females at 15 mg/kg in diet (equivalent to 0.75 mg/kg b.w. per day) and in 5 of 24 males and 8 of 22 females at 7 mg/kg in diet (equivalent to 0.35 mg/kg b.w. per day). The authors of this study concluded that this study had shown that lasiocarpine was carcinogenic in F344 rats (NTP, 1978).

Additional studies on lasiocarpine have been carried out using dietary and i.p. administration. Following dietary administration of 50 mg/kg for 55 weeks (equivalent to 2.5 mg/kg b.w. per day), 9 of 20 rats had liver hemangiosarcoma and 7 of 20 had hepatocellular carcinoma (Rao and Reddy, 1978). Following i.p. administration for 56 weeks, 11 of 18 animals surviving at termination had liver tumours of which 10 had hepatocellular carcinoma (Svoboda and Reddy, 1972). IARC have classified lasiocarpine as Group 2B 'possibly carcinogenic to humans' (IARC, 1976).

In a limited rat study in which **clivorine** was administered in drinking water (0.005 %) for 340 days, it induced an increased incidence of haemangioendothelial sarcoma of the liver (Kuhara et al., 1980).

In a limited rat study in which **petasitenine** was administered in drinking water (0.01 %) for 480 days, there was a treatment-related increased incidence of liver haemangioendothelial sarcomas and liver cell adenomas (Hirono et al., 1977).

In a limited study in male rats using i.p. administration of **senkirkine** (22 mg/kg b.w. twice weekly for 4 weeks, and then once per week for 52 weeks), there was a treatment-related increased incidence of liver cell adenomas (Hirono et al., 1979).

In a non-standard study of **symphytine** in male rats using i.p. administration (13 mg/kg b.w. twice weekly for 4 weeks and then once per week for 52 weeks), there was a treatment-related increase in

liver haemangioendothelial sarcoma and a small treatment-related increase in liver cell adenomas (Hirono et al., 1979).

In a limited study in male rats using subcutaneous administration of **monocrotaline** (5 mg/kg b.w. biweekly for 12 months), there were treatment-related increases in a number of tumours, principally liver cell carcinomas and pulmonary adenocarcinoma (Shumaker et al., 1976). The IARC classification of monocrotaline is Group 2B 'possibly carcinogenic to humans'.

Eight male and 14 female rats received 0.03 - 0.05 mg/ml **isatidine** in their drinking water. Seven animals showed no gross hepatic lesions. In the other animals, liver changes varied from simple hyperplasia through trabecular hepatoma to a metastasising carcinoma (in one animal) (Schoental et al., 1954).

In 95 rats treated with a single oral dose of 30 mg/kg b.w. **retrorsine**, 29 animals survived more than one year. A variety of tumours was found in these rats, hepatomas being predominant (5) (Schoental and Bensted, 1963).

### 7.3. Modes of action

The liver is the primary site for toxicity and genotoxicity of 1,2-unsaturated-PA esters based on the following evidence: metabolic activation to DNA-reactive species by hepatic cytochrome P450-mediated formation of reactive pyrrolic metabolites occurs in microsomes from experimental and livestock animals and humans (Fu et al., 2004); the formation of defined DHP adducts at dG and dA sites in liver DNA of PA-treated rats (Fu et al., 2010) occurs preferentially in liver endothelial cells (Chen et al., 2010); the concomitant induction of mutations in transgenic rats, through G:C -> T:A transversions, also occurs primarily in endothelial cells (Chen et al., 2010); and the formation of hemangiosarcomas, which are derived from endothelial cells, and hepatomas, which are derived from epithelial cells, in riddelliine-treated male and female rats and mice , provides strong evidence for a genotoxic mechanism for hepatocarcinogenicity by PAs. Evidence for riddelliine-induced tumor formation in additional organs in rats (mononuclear cell leukaemia) and mice (alveolar/bronchiolar adenoma and carcinoma) is consistent with this mechanism. The observations that PA esters from different structural classes (i.e., retronecine, heliotridine, and otonecine) undergo metabolic activation to a common reactive pyrrolic intermediate (pyrrolic ester(s) or DHP, see Figure 8) and form the same DNA adducts (Fu et al., 2004), suggest that a genotoxic carcinogenic mechanism is applicable for all 1,2-unsaturated-PA esters and their *N*-oxides, which are metabolically converted into PAs. The evidence for common metabolic activation pathways across species, including humans, suggests that a genotoxic carcinogenic mechanism for PAs is generalisable.

The toxicity of 1,2-unsaturated pyrrolizidine esters is determined by the proportion and rate of conversion to the pyrrole, and the chemical reactivity of the pyrrole produced. Most of the toxic effects appear to be mediated via alkylation of macromolecules. In animal studies, hepatic parenchymal cell and sinusoidal endothelial cell injury occur early in the process of pyrrolizidine alkaloid induced disease. The sequence of events is considered to be failure of DNA-mediated RNA synthesis, concurrent with disruption of protein synthesis, failure of pyruvate oxidation, loss of glycogen, structural damage to mitochondria, increased lysosomal activity, failure of mitochondrial nicotinamide adenine dinucleotide (NAD) dependent enzyme synthesis and necrosis (Neuman and Steenkamp, 2009). The zone affected by the necrosis depends on the location of the enzymes responsible for metabolic activation (WHO-IPCS, 1988).

Megalocytosis of hepatic parenchymal cells is considered to occur when cells are stimulated to go through the cell cycle but do not divide. This anti-mitotic effect has been suggested to be a result of cross-linking of actin, which plays a major role in cell division (Stegelmeier et al., 1999). Veno-occlusion is thought to result from damage to the sinusoidal and central vein endothelial cells leading to thickening and then collagenisation. Occlusion of the central vein occurs which is preceded by a

functional blocking of the blood flow. Fibrosis occurs from the central vein through the sinusoids and into the space of Disse (WHO-IPCS, 1988). It has been suggested that oxidative stress associated with liver damage enhances collagen transcription, directly and/or through the activation of hepatic stellate cells responsible for its synthesis (Chojkier, 2003).

In the lung, marked accumulation of platelets in the peripheral pulmonary circulation is observed 12 hours after injection of monocrotaline, whilst pulmonary hypertension is established 2 weeks later. This is considered to be due to hypertrophy of the muscular components of small and medium-size pulmonary arteries, possibly due to increased deposition of the extracellular matrix protein tenascin modulating cell proliferation and migration. Progressive deposition in the arteries of basement membrane proteins, such as fibronectin, collagen type IV and laminin have been reported to be important in the pathogenesis of monocrotaline-induced pulmonary hypertension (Chojkier, 2003).

In contrast to 1,2-unsaturated PAs, 1,2-saturated (platynecine-type) PAs, which do not form reactive pyrroles, apparently exhibit toxicity unrelated to oxidative metabolic bioactivation.

### 7.4. Adverse effects in livestock, fish and companion animals

As reported by EFSA (2007), all livestock species are susceptible to PA toxicity. In general, modern management of feeds and livestock herds has reduced the risk of PA toxicity considerably, but as reported below occasionally intoxications are still reported. Reports of poisoning in livestock, together with the results of studies in experimental animals, suggest that species differ in their susceptibility to PAs. In general, sheep, goats and rabbits appear to be more resistant and tolerate higher PA dosages; in sheep this tolerance is thought to be due to the demonstrated detoxification by PA-destroying rumen microbes (Radostits et al., 2000; Fletcher et al., 2011; Wiedenfeld and Edgar, 2011). In sheep this detoxification has been shown to lead to (probably temporary) acquired resistance by continuous administration of low doses of PAs (Anjos et al., 2010). Rabbits and guinea pigs also appear to be less sensitive to PAs (McLean, 1970), while horses, pigs and poultry, are considered to be more sensitive (WHO-IPCS, 1988).

Depending on the animal species, the individual alkaloids present, the total amount ingested and the time span over which the ingestion has taken place, intoxication with PAs can be acute or chronic. The time that elapses between ingestion and the onset of clinical disease can range from only 24 to 48 hours (Ubiali et al., 2011) to over several days or/and even to months (Fu et al., 2004).

In all animal species and for all ingested plant species (and thereby alkaloids) investigated so far, lesions in the liver have been observed. However, clinical and pathological examination of intoxicated animals, whether from accidental poisonings or from controlled feeding studies, have revealed different overall patterns concerning the lesions observed.

#### 7.4.1. Ruminants

The history about the lower sensitivity to especially PA chronic toxicity reported for small ruminants begins with a paper from Bull et al. (1956) reporting on a relative insensitivity of sheep grazing *H. europaeum* to pyrrolizidine alkaloids contained therein, in comparison with the susceptibility of rats to parental administered PAs (Bull et al., 1956). Dick et al. (1963) provided an explanation for this phenomenon when they demonstrated that PAs were detoxified in sheep rumen content. These authors found e.g. that heliotrine and lasiocarpine underwent a reaction to form *l*-goeensine (7α-hydroxy-1-methylene-8α-pyrrolizidine) and 7α-angeloxy-1-methylene-8α-pyrrolizidine, respectively, compounds which later by Lanigan and Smith was shown to be further degraded to the common product of 7α-hydroxy-1α-methyl-8α-pyrrolizidine (Dick et al., 1963; Lanigan and Smith, 1970). A number of studies on the influence of different antimethanogenic drugs on this rumen reaction followed (Lanigan 1970, 1971, 1972; Lanigan et al., 1978). In 1992 these early studies were followed by a number of

publications from Oregon State University (College of Veterinary Medicine). These showed that gram-positive bacteria were most likely critical members for the degradation of jacobine in the rumen of sheep (Wachenheim et al., 1992a), and that when measuring *in vitro* degradation of *Jacobaea vulgaris* PAs in bovine (cattle), ovine (sheep), and caprine (goat) rumen contents average rates of around 3, 19 and 26 micrograms/ml per hour, respectively were found. The rumen contents used were from animals that had not recently been exposed to PAs. Estimates of numbers of PA-biotransforming bacteria were 1.1 x $10^7$ bacteria/ml rumen contents (bovine), 3.0 x $10^7$ bacteria/ml (ovine) and 2.4 x $10^7$ bacteria/ml (caprine) (Wachenheim et al., 1992b).

These observations on species variation among ruminants with regard to sensitivity to PA toxicity were added still a new aspect by Anjos et al. (2010) in their study on the sensitivity of sheep to seeds of *Crotalaria retusa* (containing 6.84 % of monocrotaline). The authors found that 9 month-old sheep were susceptible to acute intoxication by monocrotaline, with a lethal dose of approximately 205 mg/kg b.w.. The sheep developed strong resistance to acute toxicity of monocrotaline (up to a single dose of 342 mg/kg b.w.) after the daily ingestion of a non lethal dose of 137 mg/kg b.w. for 20 days (Anjos et al., 2010). The authors in their discussion present both the possibility of an adaptation of the ruminal microflora to metabolise monocrotaline, as earlier suggested for other PAs (e.g. by Bull et al., 1956 and Lanigan, 1970), and of the possibility of induced changes in the hepatic metabolism as explanations (Anjos et al., 2010).

Since the publication of the EFSA (2007) opinion, a number of reports have been published on the effects of PA intake by sheep and cattle, but the clinical signs and pathological findings described in the 2007 opinion remain valid.

*Cattle*

Torres and Coelho (2008) reported on a controlled feeding experiment using five 8-month old crossbred calves weighing approximately 140 kg each. The animals were orally administered 0.38 g/kg b.w. of dried leaves of *Senecio brasiliensis* (content of alkaloids not stated) for up to 24 days. Liver needle biopsies were taken every 15 days for up to day 60. One calf died after 45 days and four were evaluated up to day 60. Biopsy samples were investigated by routine light microscopy and transmission electron microscopy. From day 30 progressive liver damage characterized by hepatocellular ballooning, necrosis, apoptosis and megalocytosis, centrilobular, pericellular and portal fibrosis was seen (Torres and Coelho, 2008). As reported by Schmeda Hirschmann et al. (1987), *S. brasiliensis* has been shown to contain between 2.1 and 3.8 g/kg PAs in dried leaves. Thus, the dose tested by Torres and Coelho (2008) could correspond approximately to 0.6-1 mg PA/kg b.w. per day.

Fletcher et al. (2011) reported a series of studies in which PA-containing plants were included in the diets of weaned calves (110-120 kg b.w.) fed for a period of 6 weeks. In the first study diets containing *Crotalaria novae-hollandiae*, and designed to supply 5.5 mg PA/kg b.w. per day did not induce toxicity and produced no clinical symptoms. Similarly, diets containing *Heliotropium amplexicaule* as part of a ration (15 %) and supplying 15 mg PA/kg b.w. per day produced no clinical signs, histopathological changes nor significant variations in biochemical or haematological parameters in calves. In a third study of similar design, *Senecio brigalowensis* was fed, at 15 % of the diet, to supply 2.5 mg PAs/kg b.w. per day. Free PAs were detected in blood (maximum 90 µg/kg) and liver (maximum 400 µg/kg), and these tended to plateau after 2-3 weeks, but again this intake of plant did not cause clinical symptoms or any biochemical, haematological and histopathological changes. The CONTAM Panel noted that the study duration may have been too short, as it may take several months before the first signs of intoxication occur after the exposure has ended. This was demonstrated for instance by a case study in which a calf was treated with *Jacobaea vulgaris* plant material by gavage to supply a dosage of 3.0 mg PAs/kg b.w. per day for 18 days. The calf did not display signs of intoxication for 150 days, after which period its health deteriorated and the calf had to be euthanatized at day 182 (Molyneux et al., 1988).

Overall, the CONTAM Panel concluded that the studies available do not allow the derivation of a NOAEL for cattle.

*Sheep*

Grecco et al. (2011) reported an outbreak of poisoning in sheep on two farms in Brazil where available forage was limited and therefore animals had been eating *Senecio* spp. Morbidities of around 10 % were noted. In addition to the usual described clinical signs and hepatic lesions found upon necropsy, also hepatic encephalopathy (*status spongiosus*) was observed in the brains in all necropsied sheep (Grecco et al., 2011). In contrast to this report in free-range animals, Anjos et al. (2010) reported on an experiment which examined the sensitivity of 9-month old sheep fed with seeds of *Crotalaria retusa* either at a high dose, or at a high dose after an initial period (70 days) of exposure to a low dose. Signs of intoxication and subsequent death occurred from a single dose of around 200 mg of monocrotaline/kg b.w. The studies available do not allow the derivation of any NOAEL for sheep.

### 7.4.2. Pigs

Only one study on adverse effects in pigs has been published since the 2007 EFSA opinion on feed. Following a case of toxicosis in Brazil in which pigs were accidentally fed sorghum grains contaminated with seeds of *Crotalaria spectabilis* (Ubiali et al., 2011) a controlled feeding experiment was undertaken in which a total of 16 piglets (b.w. between 5 and 12 kg) were fed different doses of *C. spectabilis* seeds. The experiment was structured into two studies, namely one giving three pigs either 0.5, 1.25 or 2.5 g of seeds/kg b.w. per day for 10 days, and one where groups of piglets were given single doses of 2.5, 5 or 9.5 g of seeds/kg b.w.. In the first study, liver fibrosis was observed at the lowest dose given (0.5 g of seeds/kg b.w. per day), with clinical symptoms starting around 100 hours after the start of exposure. Liver necrosis was also seen for the highest continuously dosed group (2.5 g of seeds/kg b.w. per day), as well as for the two groups given single doses of 5 and 9.5 g of seeds/kg b.w.. No information is given on the content of PAs in the seeds (Ubiali et al., 2011), and therefore it is not possible to establish any dose response relationship for PAs.

In conclusion, most or all of the reported accidental intoxications and controlled experiments in pigs relate to *Crotalaria* species (*C. retusa* or *C. spectabilis*), with monocrotaline as the main PA. Lesions have been found in the liver, kidneys and lungs. Severe megalocytosis in liver and kidneys has been reported from the inclusion of as little as 0.004 % *C. retusa* seeds with a content of total alkaloids around 4 % (Hooper and Scanlan, 1977; EFSA, 2007). In piglets fed seeds of *C. spectabilis* for ten days, liver fibrosis developed at the lowest dose of 0.5 g of seeds/kg b.w. per day (Ubiali et al., 2011). The studies available do not allow the derivation of any NOAEL for pigs.

### 7.4.3. Poultry

For poultry, the literature reports on poisonings from the consumption of seeds of *C. retusa* (mainly monocrotaline) and *Senecio vernalis* (mainly senecionine). For both types of intoxications pathological liver changes were seen, while for *C. retusa* fed animals enlargement of the spleen was also observed. For laying hens exposed to a diet supplemented with 0, 5, 20 or 40 g/kg ground *S. vernalis* containing 0.14 % PAs (corresponding to total PA intakes of 7, 28 and 56 mg/kg diet) for a period of 210 days, a significant reduction in egg production, elevation in serum γ-GT and a decrease in serum albumin and total protein were observed, as was pathological liver changes for the group on the 2 % and 4 % diet (Eröksüz et al., 2003). This long term study allows deriving a NOAEL for total *S. vernalis* (green parts) alkaloids of 7 mg/kg diet for laying hens.

Since EFSA 2007 one relevant study has been published. Eröksüz et al. (2008) conducted a feeding study including 160 Japanese quail (80 male and 80 female) fed *ad labium* with conventional grower and layer diets until they were 74 days old, and which were divided into 4 groups (3 test groups and

1 control group). The test groups were fed isonitrogenic and isocaloric diets containing 30 % plant material (aerial parts; i.e. leaves, stems, and flowers) of *Heliotropium dolosum* (HD group), *Senecio vernalis* (SV group), or *Heliotropium circinatum* (HC group); the control (C group) received 0 %. The feeding study lasted for 6 weeks in order to evaluate parental and progenial toxicity, along with the transference of alkaloid residues to their eggs. The pyrrolizidine alkaloid content in the feed was 390 mg/kg in the HD group, 450 mg/kg in the HC group, and 420 mg/kg in the SV group. The PAs were determined in *H. dolosum.* Total PAs and tertiary base content of the aerial parts were 0.13 % and 0.06 %, respectively. Alkaloids in fraction A (free tertiary bases) and fraction B (tertiary bases and *N*-oxides) were identified as europine (8.37 % and 25.95 %), heliotrine (1.14 % and 4.81 %), lasiocarpine (76.31 % and 43.97 %), echimidine (3.76 % and 6.78 %), and heliosupine (10.42 % and 18.49 %) (Eröksüz et al., 2008).

No apparent exposure-related clinical signs or death occurred in any of the groups. During the 6-week study period, mean cumulative feed consumption in both male and female test groups was significantly less than that of the controls. At the end of the sixth week, the HD group proved to have had the lowest feed consumption rate; the feed consumption rate was also lower in the HC and SV groups than in the control (Eröksüz et al., 2008). Upon necropsy the severity and incidence of microscopical lesions were somewhat more pronounced in females than in males, and were most prominent in the HD group. The main histological change in the liver and bile duct was periportal or irregular oval cell proliferation in clumps or in cords. Bile duct hyperplasia and fibrotic liver changes, together with bile pigment deposits, were generally seen the HD group (Figure 2). Megalocytosis in hepatocytes was not detected in any of the test groups; however, mild cytomegaly was observed in all test groups. The cytomegalic cells had pale nuclei with marginated chromatin and prominent nucleoli of normal size. Proximal tubules in the kidneys also showed mild cytomegaly in the HD and HC groups (Eröksüz et al., 2008). Egg production and hatchability significantly decreased in all test groups, as compared to the control group. In spite of the occurrence of specific biochemical and histopathological changes in parental quail, no remarkable changes were observed in their progeny on post-hatching days 0, 10, 20, 30, or 40 (Eröksüz et al., 2008).

Since this quite detailed study showed pathological liver changes in all dosed groups no NOAELs can be derived.

#### 7.4.4. Rabbits

No new information has become available since the EFSA 2007 feed opinion. No information is available to allow the derivation of a NOAEL.

#### 7.4.5. Fish

No new information has become available since the EFSA 2007 feed opinion.

#### 7.4.6. Companion animals

Numerous outbreaks of PA-intoxication of horses have been reported (Pearson, 1991; Creeper et al., 1999; EFSA, 2007; Crews and Anderson, 2009). The last, published after the EFSA 2007 opinion, investigated the cause of diarrhea seen in four out of 13 horses on a farm in England, all horses having access to the same batch of hay. The authors found clear chemical evidence of contamination of the hay with common ragwort (*J. vulgaris*). Chemical analysis reported a total PA content of 10 mg/kg hay. The horses had had access to the contaminated hay batch for 6 months when action due to the observed diarrhea was taken and blood tests showed the four horses to have raised liver enzyme activities (Crews and Anderson, 2009). No NOAEL can be derived from this or other studies on horses.

### 7.4.7. Transfer from feed to live-stock products of animal origin

*Milk*

A number of studies were reviewed by EFSA (2007) which showed that PAs consumed in feed are excreted in milk. More recently, Hoogenboom et al. (2011) studied the possible transfer of PAs from contaminated feed to milk in cows. For three weeks, cows were fed a ration with increasing amounts (50-100-200 g per day) of dried, ground, ragwort (*J. vulgaris*). Ragwort was administered in equal amounts in the morning and in the evening by gavage to the rumen. Milk was collected and sampled twice a day. For milk, a dose-related occurrence of PAs was found. Jacoline was the major component in milk despite being a minor component in the ragwort material. Practically no *N*-oxides were observed in milk, notwithstanding the fact that they constituted over 80 % of the PAs in ragwort. The overall carry-over of the PAs was estimated to be around 0.1 %, but for jacoline it was 4 %. The results obtained were in line with an older study described by Dickinson et al. (1976), who treated cows with very high rations of dried ragwort (4 kg per day). A carry-over rate of 0.1 % was estimated in this study with jacoline being the major PA transferred.

In Section 6.2 it was estimated that the PA intake of a high yielding dairy cow, for which the sole forage in the ration was the dried lucerne with the highest level of PA reported to EFSA, would be approximately 90 mg per day, and that all of these would have been Senecio-type PAs. In the study of Hoogenboom et al. (2011), none of the Senecio-type PAs consumed was detected in milk, and therefore in this example it might be assumed that transfer to milk would be negligible.

*Meat*

Data on PA-transfer from feed to meat and other tissues of livestock is rather limited. In a recent study (Fletcher et al., 2011), weaned calves were put on a ration containing 15 % of *Crotalaria novae-hollandiae, Heliotropium amplexicaule* (blue heliotrope) or *Senecio brigalowensis* for 6 weeks. Total PA content in muscle and liver tissue as well as in blood was determined with LC-MS and GC-MS. Of the three groups, calves receiving *Crotalaria*-diet (to supply a dose of 5.5 mg PA/kg b.w. per day) showed the highest total PA-content in muscle and liver tissue (up to 250 µg/kg and 2500 µg/kg, respectively). For calves fed with *Senecio*-diet (to supply a dose of 2.5 mg PA/kg b.w. per day), maximum liver tissue PA-levels plateaued at 400 µg/kg during the trial, but decreased to 40 µg/kg by the end of the trial. In the tissues of calves receiving *Heliotropium* in the -diet (to supply a dose of 15 mg PA/kg b.w. per day), PAs were not detected with a detection limit of 1 µg/kg. It was noted by the authors that the concentrations of the PAs detected in the tissues did not reflect well the original composition in the plant material, indicating that PAs are metabolised by hydrolysis or oxidation processes at different rates and excreted. Based on these observations, together with the results of an abattoir survey for PA residues in meat from cattle known to originate from fireweed (*Senecio* spp.) infested areas, the transfer of PAs to animal tissues appears to be low. Persistent PA-adducts with proteins and/or DNA were analysed as well (no details given), but the significance of possible release following consumption of edible animal tissue is not investigated and still a matter of debate (Fletcher et al., 2011).

*Eggs*

Limited information is available regarding the transfer of PAs from contaminated feed to eggs. In a case of accidental poisoning of laying hens by wheat-based feed contaminated with seeds of *Heliotropium europaeum*, the wheat as well as some eggs were analysed for PA content (Edgar and Smith, 2000). The wheat contained a total of 26 mg/kg PAs, mostly heliotrine, europine and lasiocarpine. The mean PA content of eggs taken during the contamination period was 156.5 µg/kg (average of two measurements). However, it was noticed that a significant part of the PA content found in the eggs was due to the presence of PAs not related to *H. europaeum*, but that were typical for contamination with *Echium plantagineum* (possibly due to the use of an earlier feeding batch contaminated with *E. plantagineum*). Since the relative contributions of the various PAs were not

given, nor were feed consumption and egg production data provided, it is not possible to estimate the feed-to-egg carry-over.

Eröksüz et al. (2003) studied the toxicity of *Senecio vernalis* (PA content 1.4 g/kg DM) to laying hens and the possible transfer of PAs to eggs. Although the hens were fed substantial amounts of *S. vernalis* (up to 4 % in the diet, corresponding to 56 mg PA/kg feed) for several weeks, no PAs could be detected in the eggs above the LOD. The LOD of the GC/MS method used was not specified.

In 2008, Eröksüz et al. conducted a feeding study of Japanese quail with aerial parts of *Heliotropium dolosum* (HD group, PA content 1.3 g/kg DM), *Heliotropium circinatum* (HC group, PA content 1.5 g/kg DM) and *Senecio vernalis* (SV group, PA content 1.4 g/kg DM). The transfer of PA to eggs was also studied using a GC/MS method. Three groups of birds were fed with diets containing 390, 450 and 420 mg/kg total PA, for the HD, HC and SV group, respectively, for six weeks. The observed PA-content of the eggs was 8.66 µg/kg for the HD group, 20.5 µg/kg for the HC group and 3.21 µg/kg for the SV group. It was noticed that not all PAs present in the diet were transferred to the eggs; the transfer of europine was relatively efficient, while lasiocarpine was not transferred (above the LOD). Based on the data provided in the paper on the feed consumption and egg production of the birds, the CONTAM Panel has calculated the following transfer ratios: 0.35 % for the HD group, 1.08 % for the HC group and 0.22 % for the SV group.

### 7.5. Observations in humans

In humans, poisoning with 1,2-unsaturated PAs is described as acute hepatic veno-occlusive disease (HVOD), characterized by rapidly developing and progressing symptoms of upper abdominal discomfort, dragging pain in the right hypochondrium, ascites, and sometimes oliguria and oedema of the feet. Nausea and vomiting may be present. Jaundice and fever are rare. There is generally gross, tender, smooth hepatomegaly, often accompanied by massive pleural effusion and sometimes slight splenomegaly and minimal ankle oedema.

The liver toxicity of 1,2-unsaturated PAs is well known due to human case reports in consequence of the treatment with herbal medicines and infusions (e.g. 'bush teas' drunk in the West Indies) prepared from *Crotalaria*, *Gynura, Heliotropium*, *Symphytum* or *Senecio* species and to large outbreaks of human poisoning including deaths, e.g. by grain crops contaminated with seeds of *Heliotropium* species in India, USSR and Afghanistan (Mattocks, 1986; WHO-IPCS, 1988) and later in Tadjikistan (Prakash et al., 1999). In central India an outbreak of HVOD, in which 42 % of the 67 recorded cases were fatal, was probably caused by the consumption of cereals mixed with seeds of a *Crotalaria* species (Tandon et al., 1976). Afghanistan has recently been afflicted by HVOD associated with the contamination of wheat with the seeds of weeds locally called 'charmac' or 'charmak' (*Heliotropium popovi*) (WHO, 2001; IRIN, 2008; Kakar et al., 2010).

The acute disease is associated with high mortality, and a subacute or chronic onset may lead to liver cirrhosis. Death often occurs after oesophageal haemorrhage. Megalocytosis (enlarged hepatocytes containing hyper-chromatic nuclei), a typical effect of PA-toxicity in animals, is not seen in humans (WHO-IPCS, 1988). Although all age groups are affected, children are described to be particularly vulnerable to the effects of 1,2-unsaturated PAs (Ridker and Mc Dermott, 1989). The liver is usually the target organ but, in an epidemic caused by contamination of the staple cereal with the seeds of *Trichodesma,* the brain and the nervous system were mainly affected. As well as HVOD some PAs (e.g. monocrotaline, fulvine) induce pulmonary arterial hypertension and right ventricular hypertrophy leading to classical *cor pulmonale* (Mattocks, 1986; WHO-IPCS 1988, 1989a, 1989b; Huxtable, 1989; Wiedenfeld et al., 2008; Edgar et al., 2011).

Chromosome aberrations have been reported in the blood cells of children affected by HVOD, which was believed to have been caused by fulvine (WHO-IPCS, 1988). However, epidemiological studies to assess the carcinogenic role of pyrrolizidine alkaloids for man are not available.

The 1,2-unsaturated PA, indicine-*N*-oxide derived from *Heliotropium indicum* has been found to have antitumour activity, and has been used in clinical trials as a chemotherapeutic agent to treat human leukaemia and solid tumours (e.g. Cook et al., 1983; WHO-IPCS, 1988). Taking into consideration that treated patients suffered from severe diseases and that the drug was administered intravenously corresponding results were considered of low relevance for this assessment and are not reported here.

### 7.5.1. Selected reports on outbreaks of diseases and cases of poisonings associated with the intake of pyrrolizidine alkaloids

Literature on human cases of poisoning and poisoning outbreaks have been summarised by several reports (Mattocks, 1986; WHO-IPCS 1988, 1989a, 1989b; Huxtable, 1989; FAO/WHO, 2011; Wiedenfeld and Edgar, 2011). Thus only selected data are presented here giving some information on the relationship between ingested dose levels and observed toxic effects.

#### 7.5.1.1. Outbreaks

Several authors report about on outbreak of HVOD in 1975 in central India (Tandon et al., 1976; Krishnamachari et al., 1977). Staple millet *(Panicum miliare)* was found to be contaminated with seeds of *Crotalaria* species. In the seeds pyrrolizidine alkaloids closely similar to monocrotaline and fulvine were identified. The total alkaloid content was estimated to be 5.3 g/kg of seeds, expressed as monocrotaline. The levels of contamination of the millet with the seeds were reported to be 0 - 3.4 g/kg in the unaffected and 0 - 19 g/kg in the affected households. Assuming a daily average intake of 400 g staple millet/adult and a maximum level of contamination of the millet of up to 20 g/kg, the authors calculated that the estimated amount of alkaloid ingested was up to 40 mg per day. (Krishnamachari et al., 1977). In the report of the WHO-IPCS it was calculated that this dose corresponds to 0.66 mg PA/kg b.w. per day for a 60-kg adult, crotananine and cronaburmine being the principal alkaloids (WHO-IPCS, 1988).

In an outbreak in north-western Afghanistan a large number of patients with massive ascites and emaciation were observed. Clinicopathological study showed that these were typical cases of HVOD. The outbreak was caused by consumption of bread made from wheat contaminated with seeds of *Heliotropium popovii*. Examination of 7200 inhabitants from the affected villages showed evidence of liver disease in 22.6 % of the cases, which was advanced in 15 %. Clinical improvement was observed in thirteen cases after 3 to 9 months of supportive hospital treatment, and in three cases liver biopsies showed almost complete disappearance of initial abnormalities. According to the analyses in 2 laboratories the seeds contained pyrrolizidine alkaloids at concentrations reported to be 7.2 and 13.2 -14.9 g/kg. Heliotrine and one or two other compounds similar in character to lasiocarpine were identified, the alkaloids occurring mainly as the *N*-oxides (75-100 %). Samples of wheat from several villages contained an average of 0.03 % seeds[24]. The authors estimated that an adult consumed at least 700 g flour per day, containing approximately 2 mg of toxic alkaloids (Mohabbat et al., 1976). In the report of the WHO-IPCS it was calculated that this dose corresponds to 0.033 mg PA/kg b.w. per day for a 60-kg adult, heliotrine being the principal alkaloid (WHO-IPCS, 1988).

Regarding the latest outbreak of HVOD in February 2008 in Western Afghanistan, which was associated with the consumption of bread made from wheat contaminated with the seeds of *Heliotropium popovi* (local name: charmac or charmak), an investigation using a case-control design has been performed. Sixty-seven cases of HVOD were compared with 199 community controls. Consumption of bread was strongly associated with the disease (adjusted odds ratio: 35.8 [95 % CI: 7.6–168.2]). Wheat flour samples from affected households had levels (median) of 0.16 mg

[24] There is some uncertainty about the estimate, since Mohabbat et al. (1976) also stated 'The alkaloid content in samples of the wheat flour varied from 0.500 - 0.186 % …'.

heliotrine/kg, 5.4 mg heliotrine-*N*-oxide/kg and 0.045 mg lasiocarpine/kg. PA levels in samples from control households were two-fold lower than those from affected households. A secondary, though minor, source of intake of 1,2-unsaturated PAs was likely qurut (whey) produced from the milk of goats which have been ingested PAs containing plants while grazing. In qurut, heliotrine, lasiocarpine and heliotrine-*N*-oxide were also found. Furthermore, trichodesmine, although not present in the charmac samples, was detected in the qurut, suggesting that the animals are ingesting other plants than charmac while grazing. Compared to wheat flour there was 1000 times less PAs in milk and whey. In water samples PAs were not detected (Kakar et al., 2010).

#### 7.5.1.2. Case Reports

Poisonings were reported in 2 infants in Arizona in the USA, a 6-month-old girl (body weight: 6 kg) and a 2-month-old boy (body weight: unknown), following ingestion of infusions of a herb, called Gordolobo Yerba by the Mexican-American population and identified as *Senecio longilobus* (Stillman et al., 1977; Fox et al., 1978; Huxtable, 1980). HVOD was diagnosed in the girl. She presented with acute hepatocellular disease and portal hypertension, and extensive hepatic fibrosis developed over two months. PAs were found in the plant from which the infusion she had drunk had been prepared. They were largely identified as riddelline and *N*-oxides of retrorsine, seneciphylline, and senecionine (Huxtable, 1980) and occurred in the herb in a concentration of 3 g free alkaloid and 10 g *N*-oxides/kg. It was estimated that, the girl received a total dose of between 70 and 147 mg of PAs including their *N*-oxides during a period of 2 weeks (Stillman et al., 1977; Huxtable, 1980). In the report of the WHO-IPCS it was calculated that this dose corresponded to 0.8 – 1.7 mg PA/kg b.w. per day for the 6-kg girl, riddelliine and retrorsine being the principal alkaloids (WHO-IPCS, 1988).

The boy was administered an infusion of the same herb for 4 days, after which he became progressively more ill and lethargic (Fox et al., 1978; Stillman et al., 1977). Administration of the infusion had been stopped one day before admission on which he was diagnosed as a case of Reye's syndrome, but subsequently developed jaundice and possibly ascites. The infant's conditions deteriorated with onset of seizures, bradycardia and periods of apnea. He died on the sixth day of hospitalisation. At autopsy, extensive centrilobular haemorrhagic necrosis of the liver was seen. The basal ganglia showed kernicterus (Fox et al., 1978). The sample of herb contained a PA concentration of 5 g/kg and a PANO concentration of 10 g/kg (Huxtable, 1980; Fox et al., 1978). It was calculated that the infant had most probably consumed a total of 66 mg of mixed alkaloids over the 4-day period. Assuming the body weight of the boy to be 5.5 kg, in the report of the WHO-IPCS it was calculated that this dose corresponds to 3 mg PA/kg b.w. per day, riddelliine and retrorsine being the principal alkaloids (WHO-IPCS, 1988).

During the period 1974-1977, 3 cases of HVOD were diagnosed in India. The patients had taken the same herb, *Heliotropium eichwaldii*, containing heliotrine in the form of the *N*-oxide in the concentration of 1-2 % by weight. Approximate intake of heliotrine amounted to 200 mg per day (for a 60-kg adult corresponding to 3.3 heliotrine mg/kg b.w. per day, as calculated in the report of the WHO-IPCS (1988)) in patients No. 1 and 2, and to 500 mg per day in patient No. 3. The period of treatment was 20 days in patient No. 1, 50 days in patient No. 2 and was not known for patient No. 3. Patients 1 and 2 were presented with fulminant hepatic failure characterized by sudden onset of jaundice, abdominal pain, ascites, gastrointestinal bleeding and hepatic encephalopathy. Death occurred in the 2$^{nd}$ week (patient No.1) and within 12 weeks (patient No. 2). Cirrhosis of the liver was diagnosed in patient No. 3. In this case the liver biopsy showed centrilobular haemorrhagic necrosis with reticulin collapse (Datta et al., 1978).

HVOD had been diagnosed as described by Kumana et al. (1983, 1985) in 4 young Chinese women with psoriasis who took herbal infusions, the toxic component of which has since been identified as *Heliotropium lasiocarpum* (Culvenor et al., 1986). The body weights of patients No 1, 2, 3 and 4 were indicated to be 51, 61, 49 and 42 kg, respectively. 45, 30, and 19 days after starting the treatment, abdominal ascites and hepatomegaly developed in patients No. 1, 2 and 3, respectively. Patients No. 1

and 3 stopped drinking the tea when symptoms began and they experienced clinical and biochemical remission. The condition of patient No. 2, who continued taking the herbal remedy for 16 days after the onset of symptoms, deteriorated and she died of hepatic failure. Patient No. 4 stopped taking the herbal tea after 21 days. When assessed 77 days later, she had mild hepatomegaly only. A detailed analysis of the alkaloid intake was carried out for each case. The ingested cumulative doses of alkaloid (base and *N*-oxide) consumed by patients No. 1, 2, 3, and 4 were calculated to be 1350 mg over 45 days, 1380 mg over 46 days, 570 mg over 19 days, and 630 mg over 21 days, respectively (Kumana et al., 1983, 1985; Culvenor et al., 1986). In the report of the WHO-IPCS it was calculated that these doses correspond to 0.59, 0.49, 0.60, and 0.71 mg PA/kg b.w. per day for the patients No 1, 2, 3 and 4, respectively, heliotrine being the principal alkaloid (WHO-IPCS, 1988).

In a case report from the USA, HVOD was diagnosed in a 49-year old woman. The patient had portal hypertension associated with obliteration of the smaller hepatic venules. A liver biopsy specimen showed centrilobular necrosis and congestion. Analysis of food supplements that the woman regularly consumed showed the presence of PAs. According to the authors the major source was a powder purporting to contain ground comfrey (*Symphytum* spp.) root: For 4 months before admission, she had taken 2 capsules of 'comfrey-pepsin' with each meal. For 6 months before admission she had daily consumed a commercially available herbal tea. The herbal tea and the capsules were analysed for PAs using monocrotaline as a standard. PAs and PANOs were found, but the compounds were not precisely identified. Based on the analysis of the PA content, the authors calculated that during the 6 months before she was hospitalised, the patient had consumed a total of at least 85 mg of PAs, corresponding to 15 µg/kg b.w. per day. The authors noted that the total PA consumption was relatively low and that it was possible that the patient had other sources of exposure and that probably she had been consuming PA-containing supplements for longer than established in the clinical history (Ridker et al., 1985).

A female neonate was referred to intensive care at the age of 5 days with jaundice, massive hepatomegaly and ascites. When the infant was 27 days old, an open liver biopsy was done. The patient died 11 days later. Biopsy findings showed centrilobular fibrosis, neovascularisation and iron deposition associated with widespread circumferential connective tissue occlusion of the small and medium size hepatic veins suggesting a diagnosis of HVOD. The mother had had daily consumption of a herbal tea because of different plants, including *Tussilago farfara* (coltsfoot), containing senecionine (including its *N*-oxide) at 0.60 mg/kg dry weight. No estimate of the dose to the mother or the fetus was given. A liver biopsy specimen was not obtained from the mother but her physical appearance and blood tests showed no abnormalities (Roulet et al., 1988). In comments responding to this article it was assumed that the herb was not *Tussilago* but *Petasites* because the typical leading alkaloid of *Tussilago*, senkirkine, was not found by (Roulet et al., 1988; Sperl et al., 1995).

HVOD was diagnosed in an 18-month-old boy who had regularly consumed a herbal tea mixture since the 3rd month of life. The boy developed portal hypertension with severe ascites. Histology of the liver showed centrilobular sinusoidal congestion with perivenular bleeding and parenchymal necrosis without cirrhosis. The child was given conservative treatment only and he recovered completely within 2 months. The tea contained peppermint and what the mother thought was coltsfoot (*Tussilago farfara*). Macroscopic and microscopic analysis of the leaf material indicated that *Adenostyles alliariae* had been erroneously gathered by the parents in place of coltsfoot. Seneciphylline and the corresponding *N*-oxide were identified as the major components by thin-layer chromatography, mass spectrometry and nuclear magnetic resonance (NMR) spectroscopy. It was calculated that the child had consumed at least 60 µg/kg b.w. per day of the PA mixture over 15 months (Sperl et al., 1995).

A pregnant 28 year old woman was admitted to hospital in the 27$^{th}$ week of gestation because of fetal ascites. During the 32nd week a male infant was delivered by emergency caesarean section but died 12 hours later. At autopsy no internal or external malformations were detected. Liver histology showed HVOD. Teas used by the family were found to be free of PAs. However, a herbal mixture from Turkey, of which 2 g per day were used for daily cooking during the entire pregnancy, contained

6 mg/kg lycopsamine, 3.5 mg/kg 'interrimine'[25] and 3 mg/kg of their O7-acetyl derivatives. The patient had identified *Heliotropium* and *Symphytum* in the herbal mixture. The metabolites dehydrolycopsamine and dehydrointegerrimine[26] were reportedly found in the fetal liver, although the high reactivity of such intermediates makes this finding unlikely. Neither the maternal nor the fetal dose of total PAs resulting from the use of this mixture was estimated by the authors (Rasenack et al., 2003).

In China two patients were hospitalised with complaints of abdominal distensions after repeated oral intake of decoctions of *Gynura segetum* at a dose of 3 g per day as traditional medicine (period of treatment: three months in one case; unknown in the other). In both patients physical examination revealed hepatomegaly. In one case it was accompanied by ascites and HVOD was diagnosed (Dai et al., 2006). *Gynura segetum* (Lour.) Merr. contains senecionine (WHO-IPCS, 1988).

In a 62-year-old woman HVOD was diagnosed in a Chinese hospital. The woman had ingested a self-made remedy, prepared under heating and containing three slices of root of *Gynura segetum* (about 2 g per day) and rice wine, for 3 months before admission. (Dai et al., 2007).

The CONTAM Panel noted that the outbreaks and case reports have not been followed up for long-term effects and that there are no epidemiological studies on the association of exposure to PAs and development of cancers.

### 7.6. Biomarkers

Measurement of pyrrolic species covalently bound to hepatic and serum proteins as a potential biomarker for PA metabolic activation associated with hepatotoxicity in experimental animals and humans has been reported (Lin et al., 2011). This potential methodology is relatively non-specific in nature, involving chemical release of the pyrrole moiety from adducted proteins in solution, derivatisation with dimethylaminobenzaldehyde, and either colorimetric or LC-MS/MS detection. Pyrrole–protein adducts in the serum of rats treated with a hepatotoxic Chinese herb containing senecionine and seneciphylline decreased after dosing from 100 % on day 1 to 65 % (week 1), 15 % (week 2), 11 % (week 3), to undetectable (week 4). Future studies will be needed to provide information about the identities of individual adducted proteins, likely involving tandem mass spectrometric- or antibody-based detection technologies, to provide the sensitivity and specificity required for use in human biomonitoring applications.

### 7.7. Dose response assessment

#### 7.7.1. Dose response data in animals

The CONTAM Panel performed dose-response modelling of the data on neoplastic changes from the 105-week gavage study on **riddelliine** (Chan et al., 2003; NTP, 2003) and from the 104-week feeding study on **lasiocarpine** (NTP, 1978). In both studies, liver haemangiosarcoma was selected as the key effect.For both substances the BMR was chosen as 10 % extra risk, the default response level for quantal response data as recommended in EFSA (2009) on the use of the BMD approach. The outcomes of the dose-response analyses are shown in Appendices C and D for riddelliine and lasiocarpine, respectively.

[25] The CONTAM Panel noted that 'interrimine' cited as in the original report may be a misspelling of intermedine, since no compound bearing this name is described in the scientific literature.

[26] The CONTAM Panel noted that integerrimine is not a constituent of *Heliotropium* or *Symphytum* species. Thus it cannot be excluded that dehydrointermedine is meant since acetyl intermedine was one of the compounds present in the herbal mixture.

In the **riddelliine** study, the female rat was selected as the most sensitive species and sex. The administered doses (0, 0.01, 0.033, 0.1, 0.33 and 1 mg/kg b.w. per day, 5 days /week) were averaged over the week by multiplying them by 5/7, leading to average doses of 0, 0.007, 0.024, 0.071, 0.236 and 0.714 mg/kg b.w. per day. Incidence of liver haemangiosarcoma is reported in Table 19.

**Table 19:** Incidence of liver haemangiosarcoma in female rats exposed to riddelliine (doses averaged over the week).

| | **Dose groups (mg/kg b.w. per day)** | **control** | **0.007** | **0.024** | **0.071** | **0.236** | **0.714** |
|---|---|---|---|---|---|---|---|
| Females | Tested animals | 50 | 50 | 50 | 50 | 50 | 50 |
| | Incidence of liver haemangiosarcoma | 0 | 0 | 0 | 0 | 3 | 38 |

The models providing acceptable fits resulted in $BMD_{10}$ values ranging from 0.208 to 0.363 mg/kg b.w. per day, and $BMDL_{10}$ values ranging from 0.180 to 0.299 mg/kg b.w. per day.

In the **lasiocarpine** study, the administered concentrations in feed were equivalent to external doses of 0, 0.35, 0.75 and 1.5 mg/kg b.w. per day. The incidence of liver haemangiosarcoma is reported in Table 20 for male and female rats.

**Table 20:** Incidence of liver haemangiosarcoma in male and female rats exposed to lasiocarpine.

| | **Dose groups (mg/kg b.w. per day)** | **control** | **0.35** | **0.75** | **1.5** |
|---|---|---|---|---|---|
| Males | Tested animals | 23 | 24 | 23 | 23 |
| | Incidence of liver Angiosarcoma | 0 | 5 | 11 | 13 |
| Females | Tested animals | 24 | 22 | 24 | 23 |
| | Incidence of liver Angiosarcoma | 0 | 8 | 7 | 2 |

In males the models providing acceptable fits resulted in $BMD_{10}$ values ranging from 0.103 to 0.260 mg/kg b.w. per day, and $BMDL_{10}$ values ranging from 0.002 to 0.200 mg/kg b.w. per day. The CONTAM Panel noted that at the lower end of these ranges the difference between the BMD and the BMDL was 1-2 orders of magnitude, indicating an excessive level of uncertainty, and selected the $BMDL_{10}$ of 0.07 mg/kg b.w. per day, which was the lowest $BMDL_{10}$ from those models with an acceptable outcome (Multistage unrestricted model, see Appendix D), as reference point.

In females, only one model gave a statistically acceptable modelling of the incidence data, with a calculated $BMD_{10}$ and $BMDL_{10}$ of 0.095 mg/ kg b.w. per day and 0.062 mg/kg b.w. per day, respectively. The CONTAM Panel noted that a poor dose-response relationship is obtained for this analysis, due to the high mortality rate observed in the mid and high dose female groups (see Section 7.2.2). The results of the BMD calculations reflected this poor correlation and indicated a low confidence of the dose response analysis. The CONTAM Panel therefore did not use these data for the risk characterisation.

**The $BMDL_{10}$ of 70 μg/kg b.w. per day calculated from the male rat data of the lasiocarpine study was selected as the reference point for the MOE calculation.**

# 8. Risk characterisation

## 8.1. Human health risk characterisation

There are few toxicological data on the majority of the PAs. Toxicity of 1,2,-unsaturated PAs in experimental animals is characterised by hepatotoxicity, developmental toxicity, genotoxicity and carcinogenicity. Some also exhibit pulmonary toxicity. The toxicity is dependent on metabolism to a reactive pyrrole. Whilst a number of 1,2-saturated PAs exhibit acute toxicity, they cannot form the reactive pyrrole that is responsible for genotoxicity and longer term toxicity. Therefore the CONTAM Panel decided to base the risk characterisation on the 1,2-unsaturated PAs. Of those that have been tested, all have shown some evidence of genotoxicity. Similarly, whilst only lasiocarpine and riddelliine have been adequately tested for carcinogenicity, a number of other 1,2-unsaturated PAs have shown indications of carcinogenic potential. Furthermore, based on the potential for metabolism to the DNA-reactive pyrrole, including in humans, it is likely that there is a common mode of action for all 1,2-unsaturated PAs of relevance to humans.

From a number of outbreaks of disease and case reports, PAs are also known to cause hepatotoxicity in humans, specifically HVOD. Those 1,2-unsaturated PAs that cause pulmonary toxicity in experimental animals (e.g. monocrotaline and fulvine) have similarly been reported to have pulmonary effects in humans. The doses of PAs resulting in disease in these reports are generally not well characterised. The WHO-IPCS (1988) considered it prudent to conclude that a PA dose equivalent to 10 µg heliotrine/kg b.w. per day may lead to disease in humans. In terms of equivalent doses of heliotrine the total doses in the known outbreaks or cases of HVOD were estimated to range from 1000 to 120,000 µg/kg b.w. for non-fatal cases However, the reports have not been followed up for possible long term effects such as cancer. Taking into account the relevant mode of action, and that in an animal study a single hepatotoxic dose of a 1,2-unsaturated PA (retrorsine) was found to eventually lead to hepatocellular carcinoma, it is probable that PA exposure resulting in toxicity in humans could also lead to carcinogenicity.

Overall, the CONTAM Panel concluded that the data from experimental animals are relevant to humans and the carcinogenicity data provided the most suitable basis for the risk characterisation. The primary site of PA carcinogenicity is the liver, chronic exposure of rats to lasiocarpine and riddelliine primarily causes liver tumours, specifically haemangiosarcomas and, to a lesser extent, hepatocellular carcinoma. For riddelliine, NTP (2008) concluded that the predominance of haemangiosarcoma was likely to be due to the greater genotoxicity and toxicity in the endothelial cells than in the parenchymal cells, and that the development of hemangiosarcoma may have resulted from endothelial cell DNA adduct formation, proliferation of endothelial cells and mutations. Increased expression of vascular endothelial growth factor (VEGF) also could have contributed by stimulating endothelial cell proliferation. Furthermore the primary toxic effect of riddelliine, venous occlusion, occurs in the same target and the non-cancer effects are likely to involve the same reactive intermediate(s) (NTP, 2008). This mode of action is similarly relevant for other 1,2-unsaturated PAs, and the carcinogenic potency is likely to be related to a combination of the genotoxic potency and the toxicity.

A $BMDL_{10}$ for excess cancer risk of 70 µg/kg b.w. per day was identified for induction of liver haemangiosarcomas by lasiocarpine in male rats, which was lower than the lowest $BMDL_{10}$ of 180 µg/kg b.w. per day for riddelliine. Comparative data for carcinogenic potency of other PAs are not available. Based on the i.p. $LD_{50}$ values, lasiocarpine is amongst the most toxic of the PAs that have been investigated. In the data on PAs submitted to EFSA, lasiocarpine was below the limit of detection or quantification in 99 % of the honey samples. In contrast, some of the PAs, such as lycopsamine, which was one of the most frequently detected PA in honey, are more than an order of magnitude less toxic than lasiocarpine. Therefore basing the risk characterisation for exposure to the combination of 1,2-unsaturated PAs that could be present in honey on the $BMDL_{10}$ for lasiocarpine is a conservative approach, that is likely to encompass concomitant exposure to co-occurring 1,2-unsaturated PAs in honey, including not only those that were measured and for which no data are available on

carcinogenic potency, but also others that could have been present but were not measured. Because the 1,2-unsaturated PAs have the potential to be genotoxic and carcinogenic, the CONTAM Panel concluded that it was not appropriate to establish a health-based guidance value such as a Tolerable Daily Intake (TDI), and decided to adopt the Margin of Exposure (MOE) approach by using the $BMDL_{10}$ for excess cancer risk in male rats treated with lasiocarpine as the reference point for comparison with the estimated dietary exposure. The EFSA Scientific Committee concluded that a MOE of 10,000 or higher, based on a $BMDL_{10}$ from an animal study, is of low concern from a public health point of view (EFSA, 2005).

While there are a number of possible sources of dietary exposure to PAs, the CONTAM Panel was only able to estimate the exposure from honey for which occurrence and consumption data were available. The wide range in estimates of dietary exposure to PAs is largely influenced by different patterns of honey consumption in different countries and to a lesser extent by the large number of non-quantifiable data. Based on the data relating to consumers only of retail honey and mean sum of PAs (Table 13), for adults the estimated exposure is between 0.009 and 0.0001 µg/kg b.w. per day for honey consumers at the mean consumption level and between 0.026 and 0.0003 µg/kg b.w. per day at the 95$^{th}$ percentile (maximum UB and minimum LB across European countries). Compared to the $BMDL_{10}$ of 70 µg/kg b.w. per day, the MOEs are in the range of 7800 - 700,000 and 2700 - 230,000, respectively. The more realistic scenario based on population honey consumption allows for days when honey is not consumed results in estimates of chronic dietary exposure for adults is between 0.0012 and 0.00002 µg/kg b.w. per day at the mean consumption level and between 0.0094 and < 0.00001 µg/kg b.w. per day at the 95$^{th}$ percentile (maximum UB and minimum LB across European countries). The MOEs are in the ranges 58,000 - 3,500,000 and 7400 - >7,000,000, respectively. Taking into account the influence of samples with non-quantifiable levels of PAs, and the conservative nature of using the $BMDL_{10}$ for a potent PA as the reference point, these MOEs are likely to be a low concern.

The highest estimates of chronic dietary exposure to PAs are for toddlers, being between 0.037 and 0.0004 µg/kg b.w. per day for retail honey consumers at the mean consumption level, and between 0.078 and 0.0011 µg/kg b.w. per day at the 95$^{th}$ percentile (maximum UB and minimum LB across European countries). The MOEs are in the range of 1900 - 175,000 and 900 - 66,000, respectively. However, as seen in Table 8, the consumption data are based on small numbers of toddlers. The more realistic scenario based on population honey consumption results in estimates of dietary exposure for toddlers between 0.0051 and 0.00001 µg/kg b.w. per day, and between 0.057 and < 0.00001 µg/kg b.w. per day at the mean and 95$^{th}$ percentile (maximum UB and minimum LB across European countries). The MOEs are in the ranges 14,000 - 7,000,000 and 1200 - >7,000,000, respectively. The CONTAM Panel concluded that there is a possible health concern for those toddlers who are high consumers of retail honey.

For other children, the estimated chronic dietary exposures are intermediate between those of toddlers and adults. The MOEs are in the ranges of 25,000 - 1,800,000 and 3900 - >7,000,000 at the mean and 95$^{th}$ percentile population consumption of honey. Again, there is a possible health concern for those children who are high consumers of retail honey.

For individuals who regularly eat locally produced unblended honey, exposure to PAs could be up to twice that of people who consume retail honey. At high level consumption this could also be a concern.

Because PAs also exhibit acute toxicity in humans and in animals, the CONTAM Panel considered possible health effects of acute dietary exposure, based on 95$^{th}$ percentile consumption of honey and 95$^{th}$ percentile occurrence of PAs in honey. The available acute toxicity data from animal studies mainly involved i.p. administration, which is of limited relevance to dietary exposure. The CONTAM Panel therefore based its evaluation on the lowest known PA dose associated with acute/short term toxicity in humans, i.e. approximately 2 mg/kg b.w. per day in a 6-month old girl exposed over 2

weeks. This dose is 4 orders of magnitude higher than the highest estimates of acute exposure to PAs from honey consumption in Tables 13 and 14, indicating that PAs in honey will not lead to acute toxicity.

In addition to honey, there are other possible sources of dietary exposure to PAs. Based on the limited available data indicating limited carry over from animal feed, meat, milk and eggs are not likely to be major sources, but this requires confirmation.

Exposure to PAs from pollen and herbal dietary supplements can potentially be very much higher than dietary exposure from honey. Certain herbal products are known to have caused human illness. Data on PAs in herbal dietary supplements in the EU market are generally not available. However, if such supplements are prepared from PA-containing plants, then they could present a risk of both acute and chronic effects in the consumer. Furthermore, borage oil and Echium oil marketed as dietary supplements, and salad crops contaminated with PA-plants such as *Senecio vulgaris* (common groundsel), could present a risk to the consumer, but data were not available for the CONTAM Panel to perform exposure assessments or risk characterisation for these sources.

## 8.2. Animal health risk characterisation

All livestock species are susceptible to PA toxicity (EFSA, 2007). However, the susceptibility differs between species. Possible explanations for a lower susceptibility in especially small ruminants include a reduction in the production of the active metabolites by hepatic microsomal enzymes, and/or detoxification of PAs by rumen microflora (Anjos et al, 2010).

Most of the reported cases of intoxication were isolated incidences and occurred as a result of accidental contaminations of feeds with PA-containing plants, or where ruminant livestock have consumed PA-containing plants because of a lack of other forages or feeds with PAs (Edgar and Smith, 2000). In many Member States of the EU, improved weed control and post-harvest cleaning of grain has resulted in a decline in the incidence of PA toxicosis. However, it is reported that the presence of ragwort (*Jacobaea vulgaris*) is increasing in west and central Europe (Leiss, 2011). A number of cases of toxicosis in cattle and horses, which are attributed to ragwort, are reported in the EU each year, but in the absence of any EU register of cases of TA-toxicosis, it is not possible to assess the scale of the risk to these livestock.

*Cattle*

Reported accidental poisonings and experimental studies with cattle have involved *J. vulgaris* (Molyneux et al., 1988; Craig et al., 1991; EFSA, 2007), *S. vernalis* (Skaanild et al, 2001; EFSA, 2007) and *S. brasiliensis* (Torres and Coelho, 2008). A 6-week feeding study with *S. brigalowensis* to supply a PA dose of 2.5 mg/kg b.w. per day did not cause adverse effects in cattle during the runtime of the experiment (Fletcher et al., 2011). However, in view of the limitations of the study, the observed NOAEL cannot be viewed as a safe dose for cattle.

*Sheep and goats*

Sheep and goats appear to be more resistant to PA toxicosis than cattle. In sheep, mortality has been reported as a result of eating *Senecio* spp. (Grecco et al., 2011), while intoxication (also with some mortality) has resulted following intake of *Crotalaria retusa* (monocrotaline). In addition to the usual described clinical signs and hepatic lesions found upon necropsy, hepatic encephalopathy (status spongiosus) was also observed in the brains of all necropsied sheep (Grecco et al., 2011). While none of the cattle studies allow any quantitative assessment of acute toxic doses, for sheep with no previous exposure, symptoms of intoxication and death resulted from a single dose of approximately 205 mg of monocrotaline/kg b.w. (Anjos et al., 2010). However, sheep that were exposed to a non-toxic dose of 137 mg/kg b.w. for 20 days, subsequently became resistant to toxic doses up to 342 mg/kg b.w. (Anjos

et al., 2010). Because of the potential for developing resistance to PAs, it has not been possible to estimate a general NOAEL.

*Pigs*

No NOAEL can be derived for pigs, a species which however is known to be very susceptible to PA intoxication

*Rabbits*

No studies allowed for quantitative considerations (EFSA, 2007) and no further information was retrieved in this opinion.

*Poultry*

A NOAEL for total S. vernalis (green parts) alkaloids of 7 mg/kg diet for laying hens can be derived from the long term study of Eröksüz et al. (2003). Since non-forage feeds as reported for their content of PAs only showed positive results for linseed, no worst case scenario could be calculated for poultry, and the risk was characterized as being low based on the available data.

*Fish*

No studies allow quantitative considerations (EFSA, 2007).

*Companion animals: Horses*

Adverse effects in horses as a result of PA intoxications include impaired liver function and, in some cases, respiratory dyspnoea and damage to the kidney (EFSA, 2007). A long delay between the exposure and the onset of clinical signs seems to be characteristic. No studies allowed for quantitative considerations.

## 9. Uncertainty analysis

The evaluation of the inherent uncertainties in the assessment of exposure to pyrrolizidine alkaloids has been performed following the guidance of the opinion of the Scientific Committee related to Uncertainties in Dietary Exposure Assessment (EFSA, 2006). In addition, the report on 'Characterizing and Communicating Uncertainty in Exposure Assessment' has been considered (WHO-IPCS, 2008). According to the guidance provided by the EFSA opinion (2006) the following sources of uncertainties have been considered: Assessment objectives, exposure scenario, exposure model, and model input (parameters).

### 9.1. Assessment objectives

The objectives of the assessment were clearly specified in the terms of reference. The CONTAM Panel assessed the new occurrence data that were collected by EFSA between October 2010 and beginning of 2011. Due to lack of occurrence data for PAs in various food commodities, an assessment on the risk to human health related to the presence of pyrrolizidine alkaloids in food was not possible. As occurrence data for PAs were only reported for honey, the CONTAM Panel performed a risk assessment for chronic and acute human dietary exposure to honey across the European population. In its risk assessment the CONTAM Panel derived margins of exposure based on the $BMDL_{10}$ for excess cancer risk for induction of liver haemangiosarcomas by lasiocarpine in male rats and chronic dietary exposure to PAs of European consumers of different age classes. Overall, the uncertainty of the assessment objectives is considered low.

Due to the extremely limited data on occurrence of PAs in feed, the CONTAM Panel was not able to perform a meaningful exposure assessment for livestock, fish and companion animals.

## 9.2. Exposure scenario/Exposure model

*Food*

In response to EFSAs request to submit occurrence data on pyrrolizidine alkaloids in food, no replies were received even until the extension of the deadline in January 2011. Thus, the industry was contacted with the assistance of the Confederation of the Food and Drink Industries of the European Union (now FoodDrinkEurope) and two submissions were received covering the presence of a range of pyrrolizidine alkaloids in honey imported to the European Union as well as in retail honey. Overall, results for 14,604 samples of honey were reported to EFSA of which 13,280 samples concerned bulk honey and 1324 samples covered retail honey ready for consumption. Results for a total of 64 different pyrrolizidine alkaloids (with another 10 identified only as an iso-form since the specific structure was not identified) were reported, but not all samples were analysed for the whole range of substances. There were a considerable number of pyrrolizidine alkaloids with no sample results above the LOD. Moreover, it was noted that the number of samples with any pyrrolizidine alkaloids above the LOD or LOQ increases and the average level decreases when comparing retail honey to bulk honey. The maximum levels found in retail honey are only 10 % or less of the levels found in bulk honey.

There is uncertainty over possible regional differences in pyrrolyzidine alkaloid contamination of honey, and the CONTAM Panel recognised that the data set is not representative of PA occurrence in honey on the EU market, especially as both data submissions were each received from only one Member State.

As regards consumption data, there is uncertainty due to the low number of honey consumers, potential “brand loyalty” and the extrapolation of short-term food consumption data to long-term exposure.

Overall, there is considerable uncertainty regarding the total dietary exposure to PAs in the risk assessment.

*Feed*

A total of 351 sample results were reported for a variety of different feed samples covering 64 different PAs. However, not all samples were analysed for the whole range of substances. As all data were submitted by one Member State, the results cannot be regarded as being representative for contamination of feed with PAs across Europe. The CONTAM Panel was only able to perform a worst case scenario for exposure to PAs for horses, dairy cows and rabbits taking the highest amount of PAs in lucerne into account. The animal risk assessment is hampered by limited representative feed consumption data across Europe, limited information on PA levels related to outbreaks or poisoning cases and scarce data on adverse effects in livestock, fish and companion animals.

## 9.3. Model input (parameters)

There are no prescribed fixed official methods or defined performance criteria for the analysis of pyrrolizidine alkaloids and laboratories can use any method of analysis, provided it can be demonstrated in a traceable manner that they fulfil the requirements according to ISO 17025. This may have added to the uncertainty in the analytical results. The limited number of defined reference compounds, isotope labelled internal standards and certified reference materials is a limitation when the method performance for the analytical procedures for analysis of pyrrolizidine alkaloids in food and feed is assessed. This adds considerably to the overall uncertainty in the analytical results.

### 9.4. Other uncertainties

The CONTAM Panel decided to base the risk characterisation on the 1,2-unsaturated PAs. Of those that have been tested, all have shown some evidence of genotoxicity. Similarly, whilst only lasiocarpine and riddelliine have been adequately tested for carcinogencity, a number of other 1,2-unsaturated PAs have shown indications of carcinogenic potential. Furthermore, based on the potential for metabolism to the DNA-reactive pyrrole, including in humans, it is likely that there is a common mode of action for all 1,2-unsaturated PAs of relevance to humans.

Overall, the CONTAM Panel concluded that the data from experimental animals are relevant to humans and the carcinogenicity data provided the most suitable basis for the risk characterisation. A $BMDL_{10}$ of 70 µg/kg b.w. per day for excess cancer risk for induction of liver haemangiosarcomas by lasiocarpine in male rats was used as a reference point. A possible source of uncertainty in the calculation of the $BMDL_{10}$ is related to the fact that no reliable calculation was possible for induction of liver haemangiosarcomas in female rats, due to the high mortality observed in female rats exposed to lasiocarpine, which affected the quality of the dataset used for the BMD modelling. Overall, a low degree of uncertainty is associated to the $BMDL_{10}$ calculation. Lasiocarpine is amongst the most toxic PAs tested, and the carcinogenic potency of many other PAs, such as those present in food, is likely to be lower. Basing the risk characterisation of PAs on the $BMDL_{10}$ for lasiocarpine is a conservative approach.

### 9.5. Summary of uncertainties

**Table 21:** Summary of qualitative evaluation of the impact of uncertainties on the risk assessment of the dietary exposure of pyrrolizidine alkaloids in honey

| Sources of uncertainty | Direction |
|---|---|
| Uncertainty in analytical results | +/-[(a)] |
| High number of samples with PAs below LOD and LOQ | +/- |
| Limited number of analytical standard compounds | - |
| Influence on contamination of retail honey due to different geographic, seasonal and botanical origins | +/- |
| Extrapolation of occurrence data from one European country to whole Europe | +/- |
| Lack of data on occurrence of PAs in food other than honey | - |
| Exposure assessment based on low number of honey consumers | +/- |
| Influence of 'brand loyalty' | +/- |
| Limited data on toxicity of individual pyrrolizidine alkaloids | +/- |
| Use of the data from lasiocarpine as a reference point for PAs | + |
| Limited data on feed consumption across Europe | +/- |
| Limited information on PA levels related to outbreaks or poisoning cases in animals | +/- |
| Occurrence data on PAs in feed only from one Member State | +/- |

(a): + = uncertainty with potential to cause over-estimation of exposure/risk; - = uncertainty with potential to cause under-estimation of exposure/risk

The CONTAM Panel considered that the impact of the uncertainties on the risk assessment of human and animal exposure to pyrrolizidine alkaloids through consumption of food and feed is substantial.

# CONCLUSIONS AND RECOMMENDATIONS

## CONCLUSIONS

### *General*

- Pyrrolizidine alkaloids (PAs) include more than 600 individual compounds in estimated 6000 plant species.
- These alkaloids occur principally in two forms, namely tertiary base PAs and PA-N-oxides (PANOs).

### *Methods of analysis*

- Because PAs and PANOs are metabolically interconvertible and both are toxicologically important, it is necessary that both species are included in the analytical determinations.
- Commercial sources of certified standards, including isotopically labelled compounds, are limited or nonexistent. In addition, the large number of naturally occurring PAs/PANOs makes a comprehensive coverage of all individual PAs/PANOs that can occur worldwide impossible. A few individual marker PAs are available which allow a screening for most of the relevant plant species involved in known contamination of food and feed.
- At present, the lack of certified standards hampers an unequivocal quantification.
- Current methods in use to analyse the PA content of food and feed comprise gas chromatography – mass spectrometry (GC-MS) in selected ion monitoring (SIM) mode and liquid chromatography – tandem mass spectrometry (LC-MS/MS) methods. The current LOQs are 10 µg/kg for total PA-content by GC-MS and approximately from 0.1 to 5 µg/kg per individual PA in LC-MS/MS.
- So far, none of the methods mentioned have been validated by inter-laboratory studies. In addition, no certified matrix reference materials or proficiency studies are available for the determination of PAs and PANOs.

### *Occurrence and effect of processing*

- The CONTAM Panel identified the following PAs (including the tertiary amine as well as the corresponding *N*-oxide forms) of particular importance for food and feed:
    - Senecionine-type PAs: acetylerucifoline, erucifoline, integerrimine, jacobine, jacoline, jaconine, jacozine, retrorsine, senecionine, seneciphylline. These PAs occur particularly in the *Senecioneae* (Asteraceae family), but are also found in *Crotalaria* spp. (Fabaceae family).
    - Lycopsamine-type PAs: acetylechimidine and isomers, echimidine and isomers, echivulgarine, lycopsamine and isomers, vulgarine. These PAs occur in the Boraginaceae family and in the *Eupatorieae* (Asteraceae family).
    - Heliotrine-type PAs: europine, heliotrine, lasiocarpine. These PAs occur in *Heliotropium* spp. (Boraginaceae family).
    - Monocrotaline-type PAs: fulvine, monocrotaline, retusamine, trichodesmine. These PAs occur in *Crotalaria* spp. (Fabaceae family).

- Data on PA occurrence in food are available only for honey, and were only reported from one Member State. In the absence of data from other countries, these were used in the risk assessment.

- Eight of the PAs analysed in honey (echimidine, echimidine-*N*-oxide, heliotrine, lycopsamine, retrorsine, senecionine, seneciphylline and senkirkine) constituted around 75-90 % of total PAs measured.

- The mean lower bound (LB) and upper bound (UB) concentrations for the 8 PAs in bulk honey are 28 and 36 µg/kg, respectively. The corresponding values in retail honey are 16 and 26 µg/kg, respectively.

- The number of samples with quantified levels of any PA is higher and the overall PA levels is lower when comparing retail honey to bulk honey. The maximum PA levels found in retail honey were only 10 % or less of the levels found in bulk honey.

- Any herbal product or preparation (food, tea, salad, vegetables, etc.) that, intentionally or unintentionally, contains PA-plant material will inevitably contain PAs and PANOs, but no or insufficient data is available to take this into account for a risk characterisation.

- Foodstuff containing honey as an ingredient also contains PAs, but due to the lack of data, these cannot be taken into account in calculating total PA-exposure.

- Pollen used as food supplements may contain PAs at concentrations 30 to 100 times higher than corresponding honey samples.

- PA levels in 351 feed samples, mainly forages and roughages, were reported to EFSA by one Member State. Fresh grass and grass silages were practically free of PAs, with only two samples having total PA concentrations of 28 µg/kg. Similarly, the grass hay samples were virtually free of PAs, although levels of 392 and 22,753 µg/kg were recorded for two samples. High-temperature dried grass generally contained very low amounts of PAs, although one sample contained 154 µg/kg, while a further four samples had levels of PAs between 60 and 100 µg/kg.

- In contrast, a high proportion of samples of lucerne (alfalfa) were contaminated with PAs. For 10 samples, PA concentrations were below the LOD, while in 12 samples the total PA content exceeded 1000 µg/kg, with the highest concentration of 6216 µg/kg in a sample of lucerne pellets. The overall mean for the 99 samples of lucerne was 424 µg/kg.

- Data were also provided for 67 non-forage feeds, including cereal grains (maize, wheat), oilseeds and fruits. For 50 feeds, PAs were absent or below the LOD. Concentrations in other feeds were generally low (<60 µg/kg) with the exception of two samples of linseed (136 and 343 µg/kg).

- Levels of PAs in herbal mixtures used as feed (in the feed category 'other plants and algae') were also reported for 32 samples. In 12 of these, PAs were absent or below the LOD, while in a further 12 samples concentrations were < 100 µg/kg. However, in three samples levels were in excess of 1000 µg/kg, with a maximum of 3200 µg/kg.

- PAs are largely biodegraded when forages are conserved as silage. A rapid decline in PA concentrations under composting conditions was also observed, with PANOs being degraded more rapidly than the corresponding PAs.

***Human exposure***

- In an acute scenario, it seems possible that PA exposure in toddlers could reach up to 114 ng/kg body weight (b.w.) during one day at a mean PA concentration in retail honey and more than double this at the 95$^{th}$ percentile PA concentration. Calculated acute exposures for all other age groups are lower or much lower.

- In a chronic scenario for honey consumers only, it was estimated that mean PA exposure might vary between 0.40 and 37.4 ng/kg b.w per day for toddlers, but would be less for all other age groups. At the 95$^{th}$ percentile, honey consumption level PA exposure could reach up to 77.8 ng/kg b.w. per day for toddlers.

- Based on the limited number of survey days included in any food consumption survey and a rather low frequency of honey consumers with only 2.8 to 13.9 % reported for toddlers across the countries, chronic exposure to PAs in honey might be better represented by an all consumer scenario. Mean chronic exposure for toddlers in the all consumer scenario varied between 0.01 and 5.10 ng/kg b.w. per day with a maximum 95$^{th}$ percentile at 56.7 ng/kg b.w. per day. Again all other age groups showed lower or much lower estimated exposure.

- Limited data indicate that carry-over of PAs from animal feed into milk and eggs appears to be approximately 0.1 and 1 %, respectively. In muscle and other tissues, mostly poorly characterised protein adducts appear to be present, but the contribution to carry-over has not been quantified.

***Animal exposure***

- Livestock and domestic animals may be exposed to PAs by the consumption of forage and roughage contaminated with plant (parts) of *Senecioneae* and *Boraginaceae* spp. In particular, lucerne (alfalfa; *Medicago sativa*) forage is occasionally contaminated with substantial amounts of PAs, which is most likely due to contamination with Senecio vulgaris. Horses and rabbits may be more exposed due to high consumption of lucerne.

- Herbs and herbal mixtures used as feed that are contaminated with PA-containing plant (or their parts) are another possible source of exposure of livestock to PAs, but these feeds generally represent only a small proportion of the diet. The origin of these additives can be diverse, and PAs from *Senecioneae*, *Boraginaceae* and *Heliotropium* spp. can be present in substantial amounts.

- Overall, the data on PA occurrence in feed are too limited to undertake a reliable estimate of the animal exposure.

***Hazard identification and characterisation***

***Toxicokinetics***

- Data on several PAs show that they are readily absorbed from the GI tract and undergo extensive metabolism in mammals.

- Metabolism of 1,2-unsaturated PAs consists of: esterase hydrolysis, a detoxification pathway; N-oxide formation, neither activation nor detoxification; and cytochrome P450-dependent oxidative conversion to dihydropyrrolizine (pyrrolic) ester(s), highly reactive species that can be deactivated by conjugation to glutathione.

***Toxicity of PAs***

- Toxicity of the 1,2-unsaturated PAs in experimental animals is characterised by hepatotoxicity, developmental toxicity, genotoxicity and carcinogenicity. Some also exhibit pulmonary toxicity. The liver is the primary site for genotoxicity of 1,2-unsaturated-PAs.

- Reactive electrophilic dihydropyrrolizine (pyrrolic) species (DHP) alkylate nucleophilic groups in DNA and protein forming covalent adducts, or crosslinks.

- Following metabolic activation to reactive pyrrolic intermediates, 1,2-unsaturated PAs from different structural classes (i.e., retronecine, heliotridine, and otonecine; di-esters and mono-esters) form a common set of DHP adducts at dG and dA sites in rat liver DNA. These findings suggest that a genotoxic carcinogenic mechanism is applicable for all 1,2-unsaturated-PA esters and their *N*-oxides, which can be metabolically converted into PAs.

- The concomitant induction of mutations compatible with DHP adduct formation in liver cells of transgenic rats and the formation of hemangiosarcomas and hepatomas in riddelliine (retronecine type PA)-treated male and female rats and mice provides strong evidence for a genotoxic mechanism for hepatocarcinogenicity.

- In contrast to 1,2-unsaturated PAs, 1,2-saturated PAs do not undergo metabolic activation to reactive pyrrolic species responsible for hepatotoxicity and genotoxicity. Therefore, the CONTAM Panel decided to base the risk characterisation on the 1,2-unsaturated PAs.

- Human case reports of poisonings due to PA-containing herbal medicines and teas and large outbreaks of human poisonings including deaths associated with grain crops contaminated with PA-containing weeds, have demonstrated the toxicity of 1,2-unsaturated PAs in humans, affecting predominantly liver and lung.

- Poisoning with 1,2-unsaturated PAs in humans is mainly characterised by acute hepatic veno-occlusive disease (HVOD). The acute disease is associated with high mortality, and a subacute or chronic onset may lead to liver cirrhosis.

- The plants reported to be associated with poisonings in humans are *Crotalaria, Gynura Heliotropium, Senecio, Symphytum* and *Trichodesma* species.

- The lowest known doses associated with acute/short-term toxicity in humans are reported to be 3 mg PA/kg b.w. per day (exposure of a boy for 4 day-period, lethal outcome) and 0.8 -1.7 mg PA/kg b.w. per day (exposure of a girl for a 2 week-period, HVOD).

- The lowest known dose associated with long-term toxicity (HVOD) in humans is reported to be 15 µg PA/kg b.w. per day (exposure for a period of 6 months).

- Substantial, long-term follow-up data or epidemiological studies to assess whether exposure to 1,2-unsaturated PAs results in cancer in humans are not available.

- Overall, based on the present knowledge of metabolism, activation, DNA adduct-formation, genotoxicity and carcinogenicity studies, the CONTAM Panel concluded that 1,2-unsaturated PAs may act as carcinogens in humans.

***Adverse effects in livestock, fish and companion animals***

- All animal species are susceptible to PA intoxication with small ruminants and rabbits being the most resistant.

- Since the publication of the EFSA (2007) opinion, a number of reports have been published on the effects of PA intake by livestock and companion animals, although the clinical signs and pathological findings described in the 2007 opinion remain valid.

***Human health risk characterisation***

- The available reports on human poisonings do not provide sufficient reliable information to be used as a basis for establishing a health-based guidance-value.

- Overall, the CONTAM Panel concluded that the data from experimental animals are relevant to humans and the carcinogenicity data provided the most suitable basis for the risk characterisation.

- Because 1,2-unsaturated PAs are genotoxic and carcinogenic, the CONTAM Panel concluded that it was not appropriate to establish a Tolerable Daily Intake (TDI), and decided to apply the Margin of Exposure (MOE) approach. A $BMDL_{10}$ for excess cancer risk of 70 µg/kg b.w. per day was calculated for induction of liver haemangiosarcomas by lasiocarpine in male rats and used as reference point for comparison with the estimated dietary exposure.

- Lasiocarpine is amongst the most toxic of the PAs that have been tested. In the data on PAs submitted to EFSA, lasiocarpine was below the limit of detection or quantification in 99 % of the honey samples. Some PAs, such as lycopsamine, which was one of the most frequently detected PA in honey, are more than an order of magnitude less toxic. Since the toxicity influences the carcinogenicity, the carcinogenic potency of most PAs present in honey is likely to be lower, therefore basing the risk characterisation on the $BMDL_{10}$ for lasiocarpine is a conservative approach, which is likely to also encompass concomitant exposure to co-occurring PAs.

- In relation to PAs in retail honey, the MOEs for adults are in the ranges of 57,000 - 3,500,000 and 7400 - >7,000,000, at the mean and 95$^{th}$ percentile of consumption (based on maximum UB and minimum LB across European countries).

- The EFSA Scientific Committee has concluded that a MOE of 10,000 or higher, based on a $BMDL_{10}$ from an animal study, is of low concern from a public health point of view. Taking this into account, together with the influence of samples with non-quantifiable levels of PAs, and the conservative nature of using the $BMDL_{10}$ for a potent PA as the reference point, these MOEs are likely to represent a low concern.

- For toddlers, the MOEs are in the ranges 14,000 - 7,000,000 and 1200 - >7,000,000, respectively. For other children, MOEs are in the ranges of 25,000 - 1,800,000 and 3900 - >7,000,000 at the mean and 95$^{th}$ percentile population consumption of honey.

- The CONTAM Panel concluded that there is a possible health concern for those toddlers and children who are high consumers of honey.

- For individuals who regularly eat locally produced unblended honey, exposure to PAs could be up to twice that of people who consume retail honey.

- Estimates of acute dietary exposure to PAs in honey are 4 orders of magnitude lower than the lowest known PA dose associated with acute/short term toxicity in humans, indicating that PAs in honey will not lead to acute toxicity.

- Exposure to PAs from pollen and herbal dietary supplements can potentially be very much higher than dietary exposure from honey and is known to have caused human illness. Data on

PAs in herbal dietary supplements are generally not available. However, if such supplements are prepared from PA-containing plants, then they could present a risk of both acute and chronic effects in the consumer.

- Borage oil and Echium oil marketed as dietary supplements, and salad crops contaminated with PA-plants such as *Senecio vulgaris* (common groundsel), could present a risk to the consumer, but data were not available for the CONTAM Panel to perform exposure assessments or risk characterisation for these sources.

***Animal health risk characterisation***

- Even though all animal species are susceptible to both acute and chronic PA intoxication, the risk of PA poisoning in the EU appears to be low. Most poisonings reported recently have been due to accidental exposure, but in the absence of integrated data on the incidence in the EU, it has not been possible to quantify this risk.

## RECOMMENDATIONS

- There is a need for a larger and more diverse set of certified reference standards and reference materials, covering both PAs and PANOs identified as markers of the main PA-containing plant families.

- There is a need for defined performance criteria for the analysis of PAs and PANOs in feed and food.

- Ongoing efforts should be made to collect analytical data on occurrence of PAs and PANOs in relevant food and feed commodities. The PAs monitored should include at least the compounds identified in this opinion as markers for the main PA-containing plant families.

- Data on the occurrence of PAs in other possibly relevant foods such as milk, eggs and meat should be collected.

- Regarding honey, data are needed how the occurrence of certain PAs correlates with the geographical and botanical origin.

- There is a need for toxicological data relating to the PAs most commonly found in honey.

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# APPENDICES

## A. STRUCTURE OF MAIN PAS

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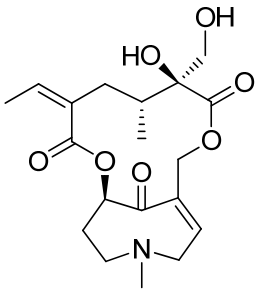

7-O-angeloylheliotridine

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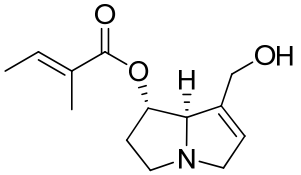

7-O-acetylintermedine

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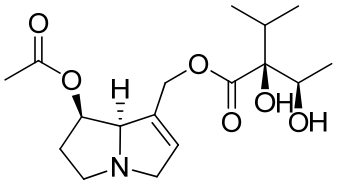

7-O-acetyllycopsamine

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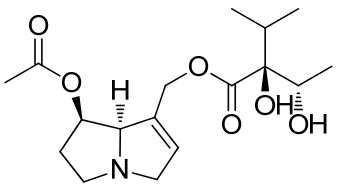

(Z)-Acetylerucifoline

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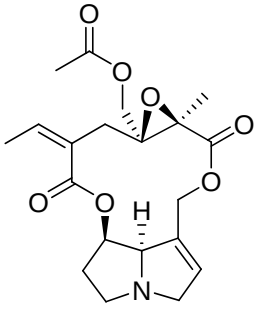

Amabiline

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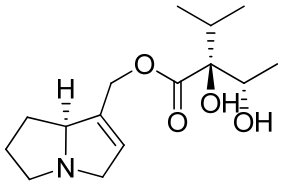

Clivorine

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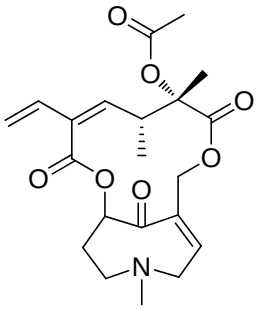

Crotananine

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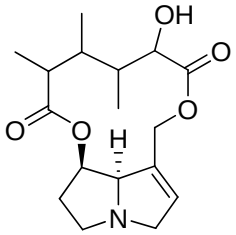

Cronaburmine

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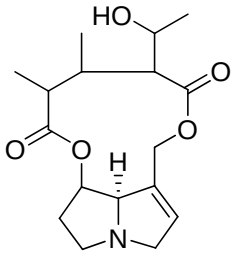

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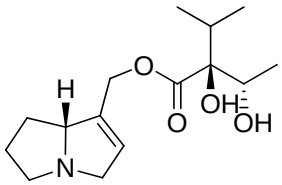

Cynaustraline

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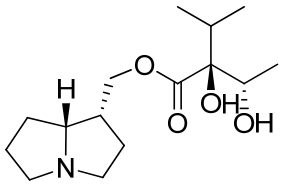

Echimidine

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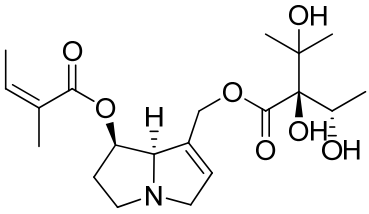

Echinatine

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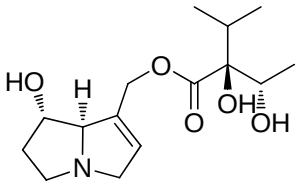

Echivulgarine

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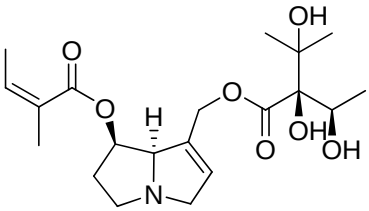

(*E*)-Erucifoline

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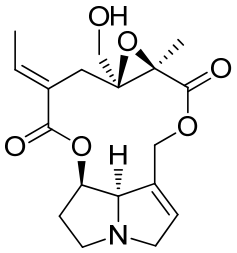

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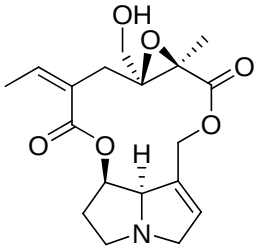

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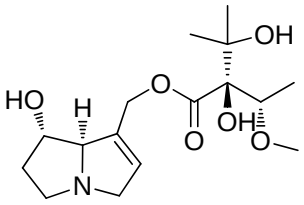

Fukinotoxin

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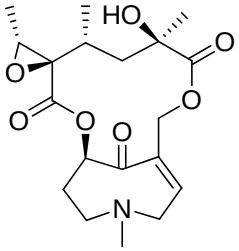

Fulvine

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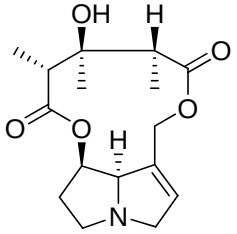

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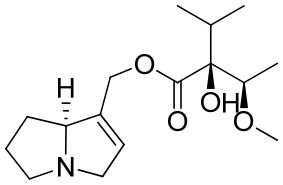

Heliosupine

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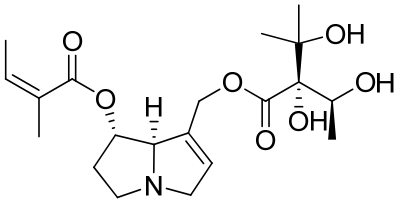

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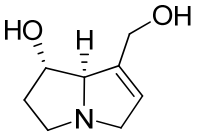

Heliotrine

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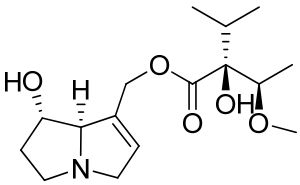

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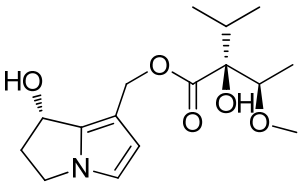

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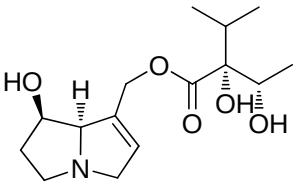

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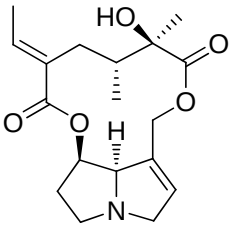

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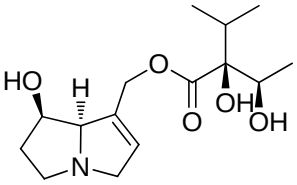


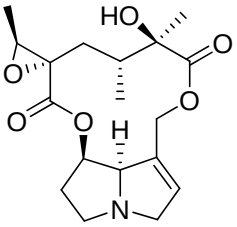
Isatidine

CAS: 15503-86-3

See retrorsine-*N*-oxide

Jacobine

CAS: 6870-67-3



Jacoline

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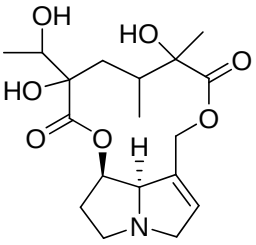

Jaconine

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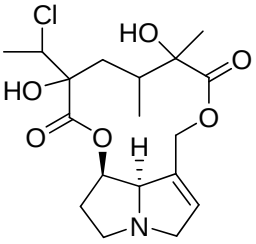

Jacozine

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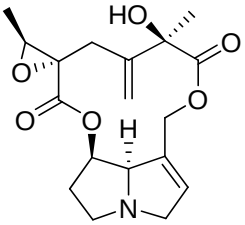
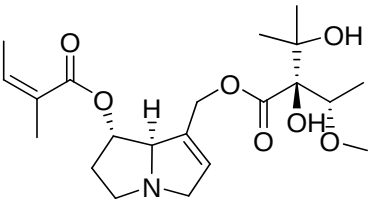


Lasiocarpine

Synonim : 7-Angelyleuropine

CAS: 303-34-4



Latifoline

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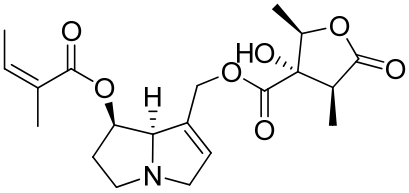

Ligularidine

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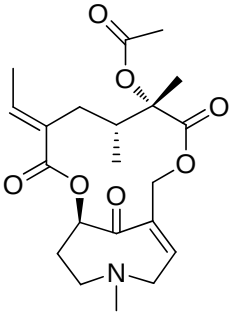

Lycopsamine

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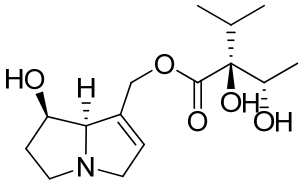

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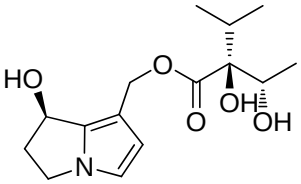

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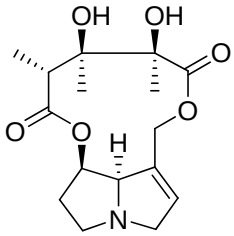
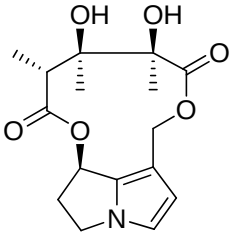


Dehydromonocrotaline



Parsonsine

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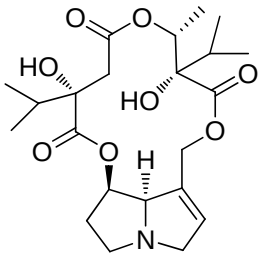

Petasitenine

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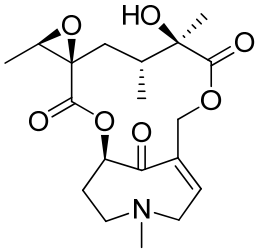

Platyphylline

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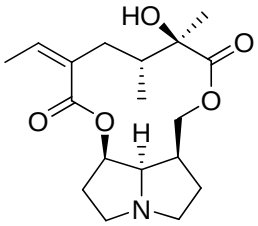

Retronecine

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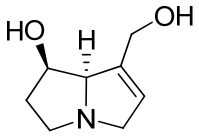

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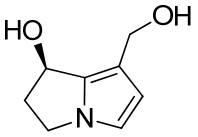

Retrorsine

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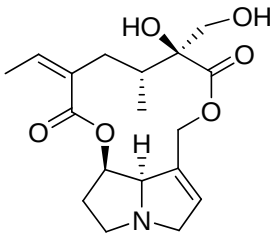
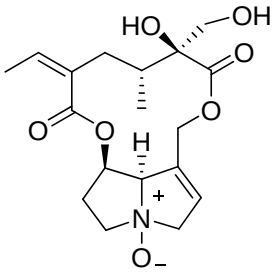


Retrorsine-*N*-oxide

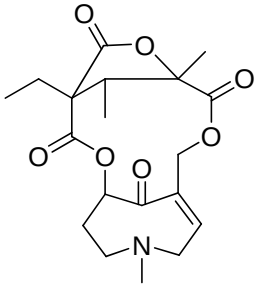
Synonym: isatidine

CAS: 15503-86-3



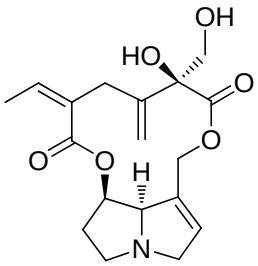
Retusamine

CAS: 6883-16-5

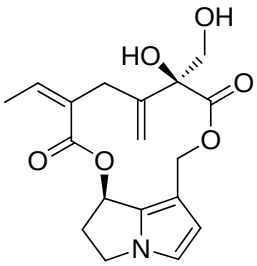


Riddelliine

CAS: 23246-96-0

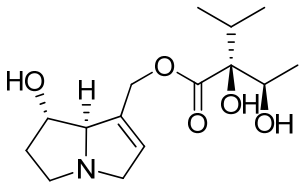


Dehydroriddelline



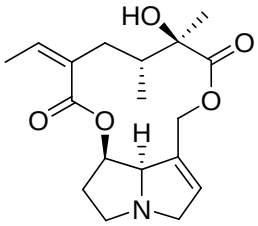
Rinderine

CAS: 6029-84-1

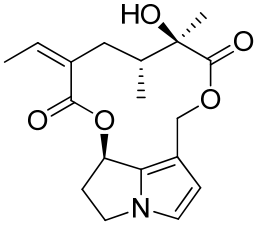


Senecionine

CAS: 130-01-8

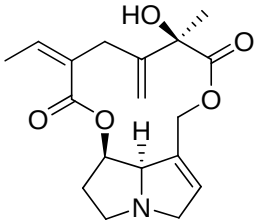


Dehydrosenecionine

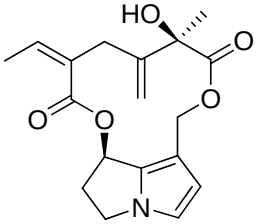


Seneciphylline

CAS: 480-81-9

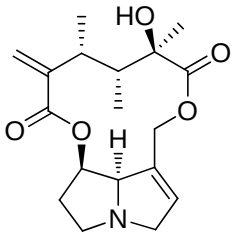


Dehydroseneciphylline



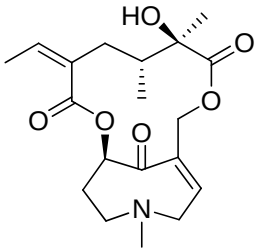
Senecivernine

CAS: 72755-25-0



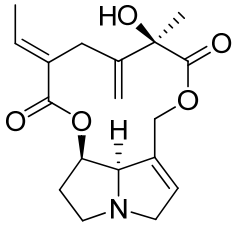
Senkirkine

CAS: 2318-18-5



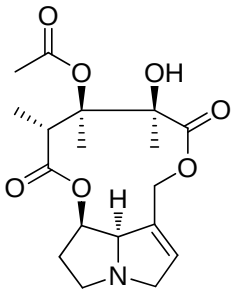
Spartioidine

CAS: 520-59-2



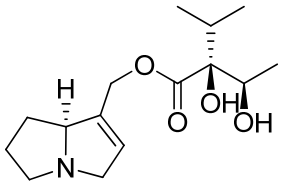
Spectabiline

CAS: 520-55-8



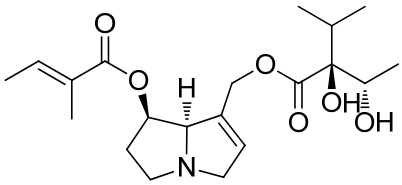
Supinine

CAS: 551-58-6



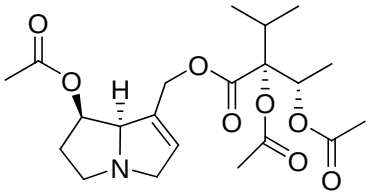
Symphytine

CAS: 22571-95-5



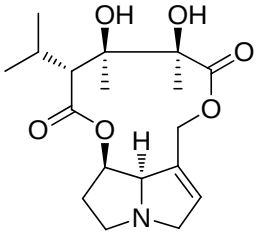
Triacetylindicine

CAS: 39870-08-1



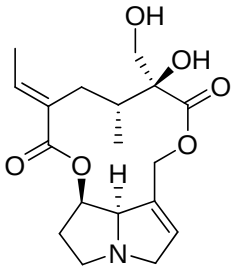
Trichodesmine

CAS: 548-90-3



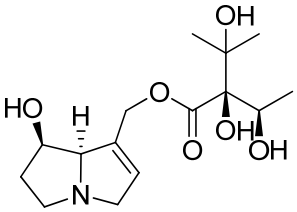
Usaramine

CAS: 15503-87-4




Vulgarine

CAS: 846552-52-1



## B. OCCURRENCE OF PAS IN FEED

Only substances with levels above the LOD are shown in the tables for simplicity and only the lower bound. High percentile levels were only calculated when reporting included a statistically sufficient number of samples. Because of the large range of pyrrolizidine alkaloids analysed for the feed samples and the high number of samples below the LOD, it was considered inappropriate to calculate the total upper bound by adding LOD for all the alkaloids.

Only two alkaloids were present at detectable levels in the few samples analysed for the feed category cereal grains and products derived thereof (Table B1).

**Table B1:** The number of reported results (N), lower bound mean with standard deviation (SD), maximum and respective percentile (P) levels of PAs in ceral grains and products derived thereof in µg/kg.

| Substance | N | Mean | (SD) | P50 | P95 | P99 | Max |
|---|---|---|---|---|---|---|---|
| Echimidine | 6 | 4.0 | (9.0) | 0 | - | - | 23 |
| Iso-lycopsamine-1 | 6 | 1.0 | (3.0) | 0 | - | - | 7.0 |

There were some results above the LOD for the oil seeds and oil fruit feed category, mainly for the lycopsamine-type group as indicated by the 95$^{th}$ percentile levels. The results are presented in Table B2.

**Table B2:** The number of reported results (N), lower bound mean with standard deviation (SD), maximum and respective percentile (P) levels of PAs in oil seeds, oil fruits, and products derived thereof in µg/kg.

| Substance | N | Mean | (SD) | P50 | P95 | P99 | Max |
|---|---|---|---|---|---|---|---|
| Echimidine | 56 | 0 | (2.0) | 0 | 0 | - | 17 |
| Echimidine-*N*-oxide | 56 | 0 | (1.0) | 0 | 0 | - | 11 |
| Integerrimine | 56 | 0 | (1.0) | 0 | 0 | - | 9.0 |
| Integerrimine-*N*-oxide | 56 | 0 | (1.0) | 0 | 0 | - | 9.0 |
| Iso-echimidine | 56 | 0 | (2.0) | 0 | 0 | - | 12 |
| Iso-lycopsamine-1 | 56 | 1.0 | (3.0) | 0 | 10 | - | 21 |
| Iso-lycopsamine-2 | 56 | 0 | (1.0) | 0 | 0 | - | 5.0 |
| Iso-lycopsamine-*N*-oxide-1 | 56 | 4.0 | (19) | 0 | 23 | - | 117 |
| Iso-lycopsamine-*N*-oxide-2 | 56 | 0 | (1.0) | 0 | 0 | - | 9.0 |
| Lycopsamine | 56 | 2.0 | (6.0) | 0 | 17 | - | 31 |
| Monocrotaline | 56 | 0 | (3.0) | 0 | 0 | - | 19 |
| Retrorsine-*N*-oxide | 56 | 1.0 | (5.0) | 0 | 0 | - | 35 |
| Senecionine | 56 | 0 | (3.0) | 0 | 0 | - | 20 |
| Senecionine-*N*-oxide | 56 | 2.0 | (12) | 0 | 0 | - | 91 |
| Seneciphylline | 56 | 0 | (1.0) | 0 | 0 | - | 8.0 |
| Seneciphylline-*N*-oxide | 56 | 1.0 | (3.0) | 0 | 0 | - | 25 |

There were no PAs detected in the individual samples analysed in the feed categories of legumes and products derived thereof or tubers and roots and products derived thereof. In the feed category other

seeds and fruits and products derived thereof one out of three samples showed the presence of echimidine-type substances (Table B3).

**Table B3:** The number of reported results (N), lower bound mean with standard deviation (SD), maximum and respective percentile (P) levels of PAs in other seeds and fruits, and products derived thereof in µg/kg.

| Substance | N | Mean | (SD) | P50 | P95 | P99 | Max |
|---|---|---|---|---|---|---|---|
| Echimidine | 3 | 10 | - | - | - | - | 29 |
| Echimidine-*N*-oxide | 3 | 3.0 | - | - | - | - | 8.0 |

A range of PAs were quantified in some samples from the feed group of forages and roughage and products derived thereof. The highest levels were found for erucifoline-*N*-oxide at 3572 µg/kg, jaconine at 3458 µg/kg and seneciphylline-*N*-oxide at 3310 µg/kg as presented in Table B4.

**Table B4:** The number of reported results (N), lower bound mean with standard deviation (SD), maximum and respective percentile (P) levels of PAs in forages and roughage and products derived thereof in µg/kg.

| Substance | N | Mean | (SD) | P50 | P95 | P99 | Max |
|---|---|---|---|---|---|---|---|
| Acetylerucifoline | 252 | 0 | (2.0) | 0 | 0 | 10 | 29 |
| Acetylerucifoline-*N*-oxide | 252 | 1.0 | (12) | 0 | 0 | 12 | 183 |
| Acetyllycopsamine | 252 | 2.0 | (17) | 0 | 0 | 49 | 212 |
| Acetyllycopsamine-*N*-oxide | 252 | 0 | (1.0) | 0 | 0 | 6.0 | 11 |
| Acetylseneciphylline | 252 | 0 | (1.0) | 0 | 0 | 0 | 8.0 |
| Acetylseneciphylline-*N*-oxide | 252 | 0 | (1.0) | 0 | 0 | 0 | 12 |
| Dehydrojaconine | 252 | 1.0 | (17.0) | 0 | 0 | 0 | 264 |
| Desacetyldoronine | 252 | 0 | (2.0) | 0 | 0 | 9.0 | 27 |
| Doronine | 252 | 0 | (1.0) | 0 | 0 | 0 | 19 |
| Echimidine | 252 | 0 | (0) | 0 | 0 | 0 | 6.0 |
| Echimidine-*N*-oxide | 252 | 0 | (3.0) | 0 | 0 | 17 | 35 |
| Echiumine | 252 | 1.0 | (6.0) | 0 | 0 | 20 | 79 |
| Echiumine-*N*-oxide | 252 | 2.0 | (22) | 0 | 0 | 34 | 342 |
| Erucifoline | 252 | 3.0 | (44) | 0 | 5.0 | 21 | 704 |
| Erucifoline-*N*-oxide | 252 | 15 | (225) | 0 | 0 | 47 | 3572 |
| Floridanine | 252 | 0 | (2.0) | 0 | 0 | 0 | 27 |
| Florosenine | 252 | 0 | (2.0) | 0 | 0 | 0 | 26 |
| Integerrimine | 252 | 8.0 | (38) | 0 | 19 | 200 | 411 |
| Iintegerrimine-*N*-oxide | 252 | 10 | (84) | 0 | 17 | 142 | 1297 |
| Iso-acetylechimidine | 252 | 14 | (104) | 0 | 37 | 275 | 1513 |
| Iso-acetylechimidine-*N*-oxide | 252 | 4.0 | (24) | 0 | 9.0 | 104 | 288 |
| Iso-acetyllycopsamine | 252 | 0 | (2.0) | 0 | 0 | 7.0 | 28 |
| Iso-acetyllycopsamine-*N*-oxide | 252 | 0 | (1.0) | 0 | 0 | 0 | 13 |
| Iso-echimidine | 252 | 8.0 | (54) | 0 | 25 | 239 | 737 |
| Iso-echimidine-*N*-oxide | 252 | 2.0 | (19) | 0 | 5.0 | 43 | 289 |

**Table B4:** Continued.

| Substance | N | Mean | (SD) | P50 | P95 | P99 | Max |
|---|---|---|---|---|---|---|---|
| Iso-lycopsamine-1 | 252 | 0 | (2.0) | 0 | 0 | 11 | 22 |
| Iso-lycopsamine-2 | 252 | 0 | (1.0) | 0 | 0 | 8.0 | 14 |
| Iso-lycopsamine-*N*-oxide-1 | 252 | 0 | (2.0) | 0 | 0 | 10 | 19 |
| Iso-lycopsamine-*N*-oxide-2 | 252 | 0 | (1.0) | 0 | 0 | 0 | 9.0 |
| Jacobine | 252 | 7.0 | (98) | 0 | 5.0 | 25 | 1548 |
| Jacobine-*N*-oxide | 252 | 6.0 | (95) | 0 | 0 | 14 | 1513 |
| Jacoline | 252 | 2.0 | (26) | 0 | 0 | 0 | 409 |
| Jacoline-*N*-oxide | 252 | 1.0 | (10) | 0 | 0 | 0 | 160 |
| Jaconine | 252 | 14 | (218) | 0 | 0 | 26 | 3458 |
| Jaconine-*N*-oxide | 252 | 4.0 | (60) | 0 | 0 | 0 | 945 |
| Jacozine | 252 | 0 | (3.0) | 0 | 0 | 0 | 50 |
| Jacozine-*N*-oxide | 252 | 0 | (5.0) | 0 | 0 | 0 | 83 |
| Lycopsamine | 252 | 4.0 | (20) | 0 | 14 | 128 | 185 |
| Lycopsamine-*N*-oxide | 252 | 2.0 | (10) | 0 | 5.0 | 52 | 126 |
| Onetine | 252 | 0 | (2.0) | 0 | 0 | 0 | 34 |
| Otosenine | 252 | 1.0 | (6.0) | 0 | 0 | 22 | 78 |
| Retrorsine | 252 | 18 | (89) | 0 | 48 | 540 | 840 |
| Retrorsine-*N*-oxide | 252 | 15 | (72) | 0 | 41 | 349 | 735 |
| Riddelliine | 252 | 3.0 | (19) | 0 | 15 | 65 | 263 |
| Riddelliine-*N*-oxide | 252 | 3.0 | (22) | 0 | 7.0 | 47 | 322 |
| Senecionine | 252 | 21 | (122) | 0 | 48 | 632 | 1519 |
| Senecionine-*N*-oxide | 252 | 21 | (181) | 0 | 26 | 376 | 2789 |
| Seneciphylline | 252 | 33 | (162) | 0 | 126 | 1062 | 1944 |
| Seneciphylline-*N*-oxide | 252 | 31 | (221) | 0 | 53 | 630 | 3310 |
| Senecivernine | 252 | 2.0 | (10) | 0 | 14 | 33 | 137 |
| Senecivernine-*N*-oxide | 252 | 1.0 | (3.0) | 0 | 0 | 15 | 27 |
| Senkirkine | 252 | 1.0 | (8.0) | 0 | 0 | 5.0 | 121 |
| Spartioidine | 252 | 7.0 | (33) | 0 | 30 | 195 | 350 |
| Spartioidine-*N*-oxide | 252 | 3.0 | (15) | 0 | 14 | 79 | 139 |
| Usaramine | 252 | 0 | (2.0) | 0 | 0 | 16 | 16 |
| Usaramine-*N*-oxide | 252 | 1.0 | (6.0) | 0 | 0 | 18 | 88 |

There were also some findings of PAs in the feed group other plants, algae and products derived thereof with some high levels of europine-*N*-oxide at 1219 µg/kg and heliotrine-*N*-oxide at 1135 µg/kg as presented in Table B5.

**Table B5:** The number of reported results (N), lower bound mean with standard deviation (SD), maximum and respective percentile (P) levels of PAs in other plants, algae and products derived thereof in µg/kg.

| Substance | N | Mean | SD | P50 | P95 | P99 | Max |
|---|---|---|---|---|---|---|---|
| Acetyllycopsamine | 32 | 0 | (3.0) | 0 | - | - | 16 |
| Acetyllycopsamine-*N*-oxide | 32 | 4.0 | (13) | 0 | - | - | 67 |
| Echiumine | 32 | 1.0 | (4.0) | 0 | - | - | 24 |
| Erucifoline-*N*-oxide | 32 | 0 | (1.0) | 0 | - | - | 7.0 |
| Europine | 32 | 3.0 | (12) | 0 | - | - | 66 |
| Europine-*N*-oxide | 32 | 101 | (307) | 0 | - | - | 1219 |
| Heliotrine | 32 | 9.0 | (29) | 0 | - | - | 117 |
| Heliotrine-*N*-oxide | 32 | 61 | (217) | 0 | - | - | 1135 |
| Heleurine-*N*-oxide | 32 | 20 | (82) | 0 | - | - | 438 |
| Integerrimine | 32 | 1.0 | (3.0) | 0 | - | - | 18 |
| Integerrimine-*N*-oxide | 32 | 3.0 | (13) | 0 | - | - | 76 |
| Iso-acetylechimidine | 32 | 1.0 | (3.0) | 0 | - | - | 18 |
| Iso-acetylechimidine-*N*-oxide | 32 | 3.0 | (17) | 0 | - | - | 99 |
| Iso-acetyllycopsamine-*N*-oxide | 32 | 3.0 | (12) | 0 | - | - | 70 |
| Iso-echimidine | 32 | 1.0 | (2.0) | 0 | - | - | 11 |
| Iso-echimidine-*N*-oxide | 32 | 5.0 | (12) | 0 | - | - | 57 |
| Iso-lycopsamine-1 | 32 | 1.0 | (4.0) | 0 | - | - | 20 |
| Iso-lycopsamine-2 | 32 | 1.0 | (3.0) | 0 | - | - | 16 |
| Iso-lycopsamine-*N*-oxide-1 | 32 | 25 | (87) | 0 | - | - | 480 |
| Iso-lycopsamine-*N*-oxide-2 | 32 | 4.0 | (16) | 0 | - | - | 77 |
| Lasiocarpine | 32 | 3.0 | (11) | 0 | - | - | 55 |
| Lasiocarpine-*N*-oxide | 32 | 22 | (70) | 0 | - | - | 312 |
| Lycopsamine | 32 | 1.0 | (6.0) | 0 | - | - | 29 |
| Lycopsamine-*N*-oxide | 32 | 21 | (80) | 0 | - | - | 444 |
| Retrorsine | 32 | 0 | (3.0) | 0 | - | - | 16 |
| Retrorsine-*N*-oxide | 32 | 4.0 | (18) | 0 | - | - | 100 |
| Riddelliine-*N*-oxide | 32 | 0 | (1.0) | 0 | - | - | 7.0 |
| Senecionine | 32 | 2.0 | (9.0) | 0 | - | - | 52 |
| Senecionine-*N*-oxide | 32 | 6.0 | (29) | 0 | - | - | 164 |
| Seneciphylline | 32 | 1.0 | (6.0) | 0 | - | - | 35 |
| Seneciphylline-*N*-oxide | 32 | 6.0 | (29) | 0 | - | - | 161 |
| Senecivernine | 32 | 0 | (3.0) | 0 | - | - | 15 |
| Senecivernine-*N*-oxide | 32 | 2.0 | (12) | 0 | - | - | 68 |
| Senkirkine | 32 | 0 | (1.0) | 0 | - | - | 5.0 |
| Spartioidine | 32 | 0 | (1.0) | 0 | - | - | 6.0 |
| Spartioidine-*N*-oxide | 32 | 1.0 | (5.0) | 0 | - | - | 29 |

## C. $BMDL_{10}$ CALCULATION – RIDDELLIINE (NTP, 2003)

Liver haemangiosarcoma was selected as the key effect. As riddelliine was administered by gavage on 5 days/week in the study, the administered doses (0, 0.01, 0.0033, 0.1, 0.33 and 1 mg/kg b.w. per day) were corrected by a factor 5/7 to take into account the experimental administration rate. This led to corrected doses of 0, 0.007, 0.024, 0.071, 0.236, 0.714 mg/kg b.w. per day.

BMDL was calculated by means of the software BMDS v2.1.2 (US EPA). All models for dichotomous (quantal) data were selected for the analysis at the default benchmark response (BMR) of 10 % (95 % confidence level) advised by the EFSA guidance on the use of benchmark dose (EFSA, 2009). The models allowing for restrictions (e.g. Log Logistic or Multistage) were run both with and without the selected default restrictions.

### C1. Incidence data

| | Dose groups (mg/kg b.w. per day) | control | 0.007 | 0.024 | 0.071 | 0.236 | 0.714 |
|---|---|---|---|---|---|---|---|
| Females | Tested animals | 50 | 50 | 50 | 50 | 50 | 50 |
| | Incidence of liver haemangiosarcoma | 0 | 0 | 0 | 0 | 3 | 38 |

### C2. BMR: 0.1 (extra risk)

**C3. Model acceptability criteria:** All quantal dose response models in the US EPA's benchmark dose software[27] BMDS 2.1.2 were used. Acceptability of a model was assessed using the log-likelihood value associated with the fitted model (when tested versus the full model). In accordance with the Scientific Opinion of the EFSA (EFSA, 2009) a goodness-of-fit was judged as sufficient if the fit showed a p-value not smaller than 0.05 (i.e. $p \geq 0.05$), using the likelihood ratio test.

[27] http://www.epa.gov/ncea/bmds/about.html

## C4: Table of BMDL results.

### Females

| Models | Restriction | N. parameters | Log-likelihood | P-value | Accepted | $BMD_{10}$ (mg/kg b.w. per day) | $BMDL_{10}$ (mg/kg b.w. per day) |
|---|---|---|---|---|---|---|---|
| Null model | – | 1 | -119.66 | – | – | – | – |
| Gamma | Power ≥1 | 3 | -38.9238 | 0.9999 | Yes | 0.277134 | 0.215468 |
| Weibull | Power ≥1 | 3 | -39.0021 | 0.9986 | Yes | 0.290296 | 0.21798 |
| LogLogistic | Slope ≥1 | 3 | -38.9478 | 0.9997 | Yes | 0.278321 | 0.216329 |
| LogProbit | Slope ≥1 | 3 | -38.9039 | 1 | Yes | 0.269899 | 0.215028 |
| Multistage | Betas ≥1 | 2 | -41.1158 | 0.6549 | Yes | 0.207973 | 0.180434 |
| Logistic | – | 3 | -38.9478 | 0.7179 | Yes | 0.362769 | 0.298919 |
| Multistage-Cancer | – | 2 | -41.1158 | 0.6549 | Yes | 0.207973 | 0.180434 |
| Probit | – | 3 | -39.6299 | 0.9078 | Yes | 0.327038 | 0.270182 |
| Quantal-Linear | – | 2 | -54.791 | 0.0003 | No | 0.0947957 | 0.0735869 |
| Gamma | No restr. | 3 | -38.9238 | 0.9999 | Yes | 0.277133 | 0.215468 |
| Weibull | No restr. | 3 | -39.0021 | 0.9986 | Yes | 0.290296 | 0.21798 |
| LogLogistic | No restr. | 3 | -38.9478 | 0.9997 | Yes | 0.278321 | 0.216329 |
| LogProbit | No restr. | 3 | -38.9039 | 1 | Yes | 0.269899 | 0.215028 |
| Multistage | No restr. | 3 | -36.2356 | 0.1656 | Yes | 0.283329 | * |
| Full model | – | 6 | -38.9024 | – | – | – | – |

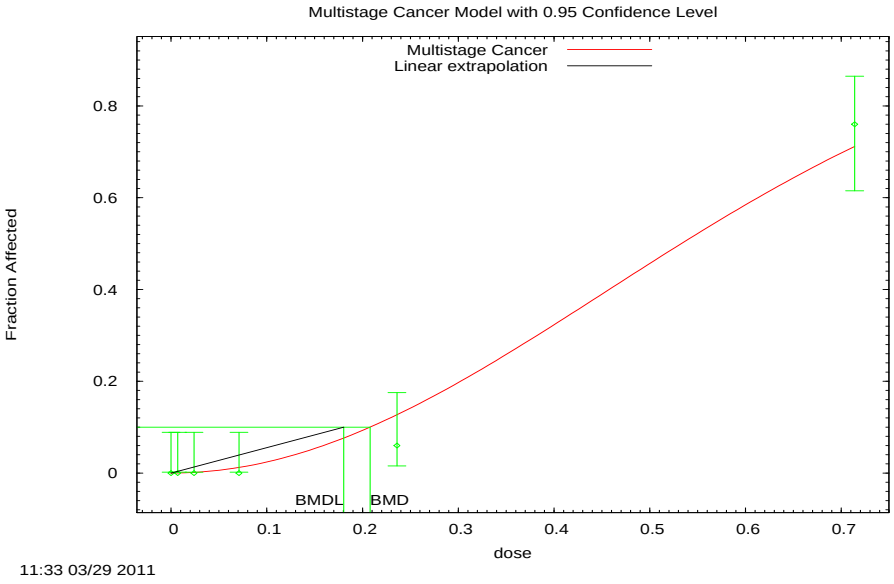
*No $BMDL_{10}$ was calculated for this model.

### Comments on results

### Female rats

All dichotomous models satisfied the log-likelihood acceptability criterium when compared to the null model. However, the Quantal-linear model did not pass the goodness-of-fit criterium and was thus considered as not acceptable for the $BMDL_{10}$ derivation.

The calculated $BMDL_{10}$ were in the range in the range 0.18 – 0.30 mg/kg b.w. per day. No differences were observed in models allowing for restrictions when default restrictions were applied. The lowest $BMDL_{10}$ of 0.18 mg/kg b.w. per day was calculated by the Multistage model (with restriction $\beta>0$) and the Multistage-cancer model (plot reported below).



## D. $BMDL_{10}$ CALCULATION – LASIOCARPINE (NTP, 1978)

Liver haemangiosarcoma was selected as the key effect. The conversion of doses from mg/kg in food to mg/kg b.w. per day for male rats was carried out by considering an average weight and a daily food intake of 400 g and 20 g, respectively (default values from WHO-IPCS EHC 70 and 240). For females, a mean body weight of approximately 200 g and a daily food consumption of 10 g were assumed. This led to doses of approximately 0, 0.35, 0.75 and 1.5 mg/kg b.w. per day for male and female rats.

BMDL was calculated by means of the software BMDS v2.1.2 (US EPA). All models for dichotomous (quantal) data were selected for the analysis at the default benchmark response (BMR) of 10 % (95 % confidence level) advised by the EFSA guidance on the use of benchmark dose (EFSA, 2009). The models allowing for restrictions (e.g. Log Logistic or Multistage) were run both with and without the selected default restrictions. $BMDL_{10}$ were calculated separately for male and female rats.

### D1. Incidence data

| | Dose groups (mg/kg b.w. per day) | control | 0.35 | 0.75 | 1.5 |
|---|---|---|---|---|---|
| Males | Tested animals | 23 | 24 | 23 | 23 |
| | Incidence of liver Angiosarcoma | 0 | 5 | 11 | 13 |
| Females | Tested animals | 24 | 22 | 24 | 23 |
| | Incidence of liver Angiosarcoma | 0 | 8 | 7 | 2 |

### D2. BMR: 0.1 (extra risk)

**D3. Model acceptability criteria:** All quantal dose response models in the US EPA's benchmark dose software[28] BMDS 2.1.2 were used. Acceptability of a model was assessed using the log-likelihood value associated with the fitted model (when tested versus the full model). In accordance with the Scientific Opinion of the EFSA (EFSA, 2009) a goodness-of-fit was judged as sufficient if the fit showed a p-value not smaller than 0.05 (i.e. $p \geq 0.05$), using the likelihood ratio test.

[28] http://www.epa.gov/ncea/bmds/about.html

## D4. Table of BMDL results

### Males

| Models | Restriction | N. parameters | Log-likelihood | P-value | Accepted | $BMD_{10}$ (mg/Kg b.w. per day) | $BMDL_{10}$ (mg/Kg b.w. per day) |
|---|---|---|---|---|---|---|---|
| Null model | – | 1 | -57.7117 | – | – | – | – |
| Gamma | Power ≥1 | 2 | -44.5048 | 0.7685 | Yes | 0.157756 | 0.117057 |
| Weibull | Power ≥1 | 2 | -44.5048 | 0.7685 | Yes | 0.157756 | 0.117057 |
| LogLogistic | Slope ≥1 | 3 | -44.2512 | 0.7381 | Yes | 0.134327 | 0.0779407 |
| LogProbit | Slope ≥1 | 2 | -45.0029 | 0.5299 | Yes | 0.25969 | 0.200231 |
| Multistage | Betas ≥1 | 2 | -44.5048 | 0.7685 | Yes | 0.157756 | 0.117057 |
| Logistic | – | 3 | -48.1729 | 0.0428 | No | – | – |
| Multistage-Cancer | – | 2 | -44.5048 | 0.7685 | Yes | 0.157756 | 0.117057 |
| Probit | – | 3 | -47.8633 | 0.0506 | No | – | – |
| Quantal-Linear | – | 2 | -44.5048 | 0.7685 | Yes | 0.157756 | 0.117057 |
| Gamma* | No restr. | – | – | – | – | – | – |
| Weibull | No restr. | 3 | -44.3373 | 0.6774 | Yes | 0.103333 | 0.0017579 |
| LogLogistic | No restr. | 3 | -44.2512 | 0.7381 | Yes | 0.134319 | 0.0048083 |
| LogProbit | No restr. | 3 | -44.2344 | 0.7505 | Yes | 0.151067 | 0.0073032 |
| Multistage | No restr. | 3 | -44.208 | 0.7742 | Yes | 0.120585 | 0.0703832 |
| Full model | – | 4 | -43.9486 | - | – | – | – |

*BMDS gave an error message when running the gamma model without restrictions.

**Females**

| Models | Restriction | N. parameters | Log-likelihood | P-value | Accepted | $BMD_{10}$ (mg/Kg b.w. per day) | $BMDL_{10}$ (mg/Kg b.w. per day) |
|---|---|---|---|---|---|---|---|
| Null model | – | 1 | -44.2314 | – | – | – | – |
| Gamma | Power ≥1 | 3 | -44.2314 | <0.0001 | No | – | – |
| Weibull | Power ≥1 | 2 | -44.2308 | 0.0002 | No | – | – |
| LogLogistic | Slope ≥1 | 2 | -44.2306 | 0.0002 | No | – | – |
| LogProbit | Slope ≥1 | 2 | -44.2314 | 0.0002 | No | – | – |
| Multistage | Betas ≥1 | 2 | -44.2308 | 0.0002 | No | – | – |
| Logistic | – | 2 | -44.2312 | 0.0002 | No | – | – |
| Multistage-Cancer | – | 2 | -44.2308 | 0.0002 | No | – | – |
| Probit | – | 2 | -44.2311 | 0.0002 | No | – | – |
| Quantal-Linear | – | 2 | -44.2308 | 0.0002 | No | – | – |
| Gamma* | No restr. | – | – | – | – | – | – |
| Weibull | No restr. | 1 | -38.524 | 0.1687 | Yes | –** | – |
| LogLogistic | No restr. | 1 | -38.524 | 0.1687 | Yes | –** | – |
| LogProbit | No restr. | 1 | -38.524 | 0.1687 | Yes | –** | – |
| Multistage | No restr. | 2 | -36.4543 | 0.4656 | Yes | 0.09524 | 0.06183 |
| Full model | – | 4 | -35.703 | – | – | – | – |

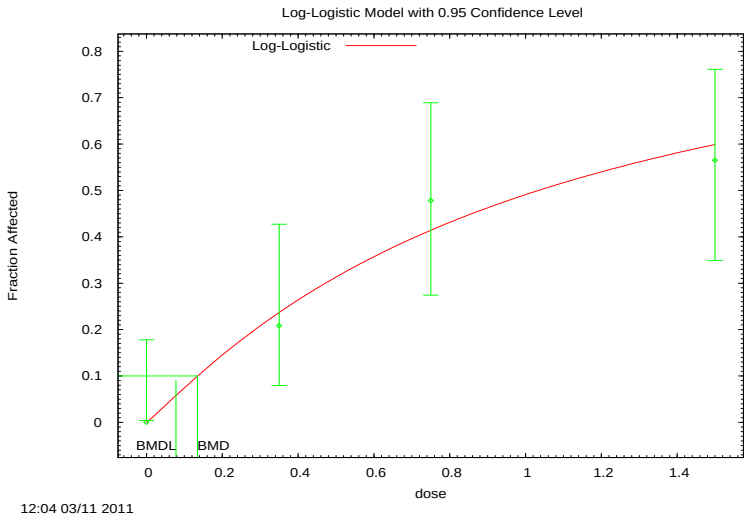
*BMDS gave an error message when running the gamma model without restrictions.
** BMD calculation failed

### Comments on results

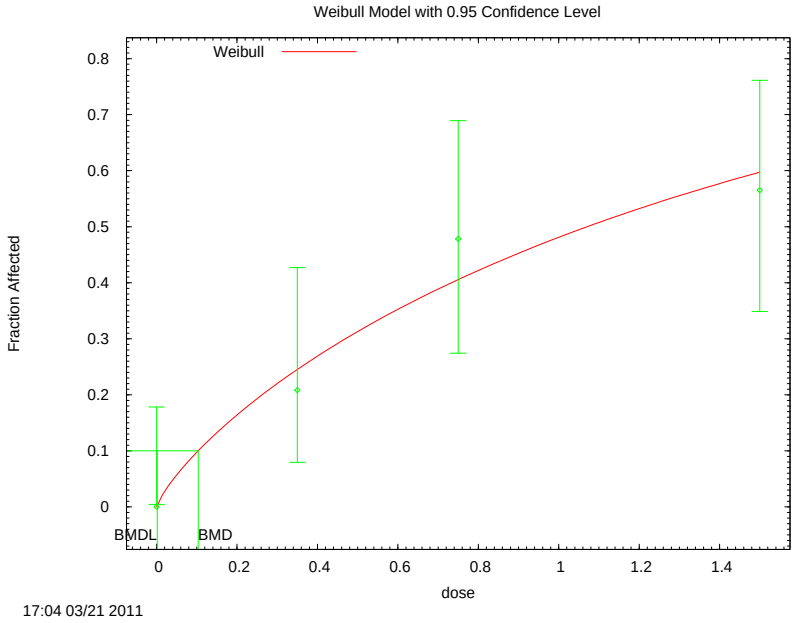
### Male rats

All dichotomous models satisfied the log-likelihood acceptability criterium when compared to the null model. However, the Logistic model did not pass the goodness-of-fit criterium, whereas the Probit model resulted to be borderline for the same criterium. Both models were considered as not acceptable for the $BMDL_{10}$ derivation. The two accepted models not allowing for restrictions (i.e. Quantal-Linear and Multistage-Cancer) gave a $BMDL_{10}$ of 0.12 mg/kg b.w. per day.

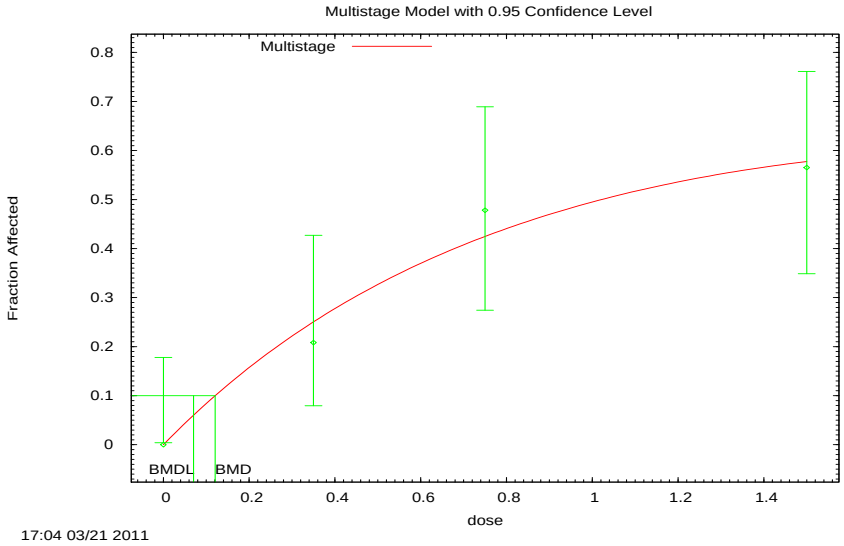
When default restrictions were applied to the models allowing for, $BMDL_{10}$ in the range 0.08 – 0.20 mg/kg b.w. per day were obtained. The lowest $BMDL_{10}$ of 0.08 mg/kg b.w. per day resulted from the LogLogistic model with the default restriction applied (see plot reported here below).




However, when the same models were run without restrictions, different results were obtained. Namely, $BMDL_{10}$ were in the range 0.002 – 0.070 mg/kg b.w. per day, with the lowest $BMDL_{10}$ obtained from the Weibull model (plot reported below). The CONTAM Panel noted that a great difference exists between the $BMDL_{10}$ and the $BMD_{10}$ in the unrestricted Weibull model calculation, as well as for calculations with the unrestricted Log Logistic and Log Probit models ($BMD_{10}/BMDL_{10}$ ratios are 59, 28 and 21 for the unrestricted Weibull, Log Logistic and Log Probit models, respectively), indicating a low confidence of the analysis.



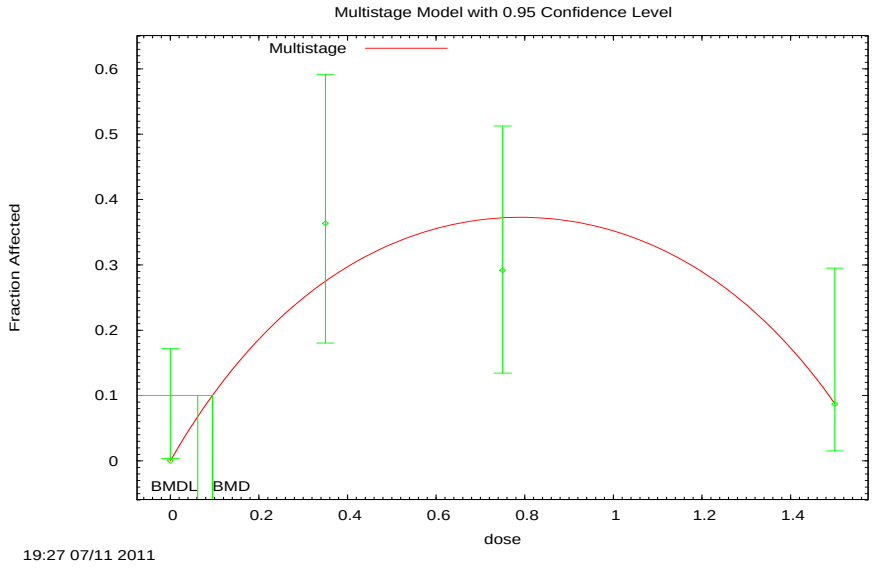

On the other hand, a low difference between $BMDL_{10}$ and $BMD_{10}$ was noted for the unrestricted Multistage model ($BMDL_{10}$ = 0.07 mg/kg b.w. per day, $BMD_{10}/BMDL_{10}$ ratio of 1.7. See plot reported below). The CONTAM Panel selected this $BMDL_{10}$ for the dose-response analysis




## Female rats

Although no clear dose-response relationship can be observed in the incidence of liver angiosarcomas in female rats, an attempt was made to derive the $BMDL_{10}$ for this set of data. The unrestricted, Weibull, Log Logistic, Log Probit and Multistage models satisfied the log-likelihood acceptability the goodness-of-fit criteria. However, BMD/BMDL calculation failed for the former three models, whereas a $BMDL_{10}$ of 0.06 mg/kg b.w. per day was calculated with the Multistage model (plot reported below). The CONTAM Panel noted that the calculated $BMDL_{10}$ supports the $BMDL_{10}$ value selected from males. However, the high variability of the BMD calculations observed with the restricted and unrestricted quantal dose response models indicated a low confidence of the dose response analysis of the incidence of haemangiosarcoma in female rats. The CONTAM Panel

concluded that the high mortality rate observed in the mid and high dose female groups impaired the dose-response analysis. The calculated $BMDL_{10}$ of 0.06 mg/kg b.w. per day was therefore disregarded from the dose response analysis.



## ABBREVIATIONS

| | |
|---|---|
| ANZFA | The Australian New Zealand Food Authority |
| APCI | Atmospheric-pressure chemical ionization |
| AUC | Area under the curve |
| BfR | The Federal Institute of Risk Assessment/(Bundesinstitut für Risikobewertung, Germany |
| BH | Bulk honey |
| BMDL | Benchmark dose lower confidence limit |
| BMR | Benchmark response |
| BSA | bovine serum albumin |
| b.w. | Body weight |
| CA | Chromosomal aberration |
| CID | Collision-induced dissociation |
| Comprehensive Database | EFSA Comprehensive European Food Consumption Database |
| CONTAM Panel | EFSA Scientific Panel on Contaminants in the Food Chain |
| COC | UK Committee on Carcinogenicity of Chemicals in Food, Consumer Products and the Environment |
| COT | UK Committee on Toxicity of Chemicals in Food, Consumer Products and the Environment |
| CYP | Cytochrome P450 |
| DAD | Diode array detection |
| DHP | 6,7-dihydro-7-hydroxy-1-hydroxymethyl-5H-pyrrolizine |
| DM | Dry matter |
| DW | Dry weight |
| EFSA | The European Food Safety Authority |
| EI | Electron ionization |
| ELISA | Enzyme-linked immunosorbent assay |
| ESI | Electrospray ionization |
| EU | European Union |
| FAB-MS | Fast-atom bombardment mass-spectrometry |
| FID | Flame ionization detector |
| FMO | Flavin-containing monooxygenases |
| GC | Gas chromatography |
| GC-MS | Gas chromatography-mass spectrometry |
| GD | Gestational day |
| GI | Gastrointestinal |
| GSH | Glutathione |
| GST | Glutathione-*S*-transferase |
| HC group | *Heliotropium circinatum* group |
| HD group | *Heliotropium dolosum* group |
| HepG2 | Human hepatoma cells |
| HPLC | High performance liquid chromatography |
| HR-GC | High resolution capillary gas chromatography |
| HVOD | Hepatic veno-occlusive disease |
| IARC | International Agency for Research on Cancer |
| i.p. | Intraperitoneal |
| i.v. | Intravenous |
| JECFA | Joint FAO/WHO Expert Committee on Food Additives |
| LB | Lower bound |
| LC | Liquid chromatography |
| LOD | Limit of detection |
| LOQ | Limit of quantification |
| ML | Maximum level |

| | |
|---|---|
| MN | Micronuclei |
| MOE | Margin of exposure |
| MRM | Multiple reaction monitoring |
| MS | Mass spectrometry |
| MS/MS | Tandem mass spectrometry |
| NAD | Nicotinamide adenine dinucleotide |
| NMR | Nuclear magnetic resonance |
| NOAEL | No-observed-adverse-effect level |
| NOEL | No-observed-effect level |
| NPD | Nitrogen-phosphorus detector |
| NTP | National Toxicology Program |
| OVA | Egg ovalbumin |
| PA | Pyrrolizidine alkaloid |
| PANO | PA-*N*-oxide |
| PND | Post-natal day |
| PIS | Precursor ion scan |
| PTDI | Provisional tolerable daily intake |
| QuEChERS | Quick, Easy, Cheap, Effective, Rugged and Safe |
| RH | Retail honey |
| RIVM | The Dutch National Institute for Public Health and the Environment/ Rijksinstituut voor Volksgezondheid en Milieu |
| s.c. | Subcutaneous |
| SCE | Sister chromatid exchange |
| SCX | Strong cation exchange |
| SD | Standard deviation |
| SIM | Single ion monitoring |
| SPE | Solid phase extraction |
| SRM | Single reaction monitoring |
| SV group | *Senecio vernalis* group |
| TDI | Tolerable Daily Intake |
| TLC | Thin layer chromatography |
| TOF | Time-of-flight |
| UB | Upper bound |
| UDS | Unscheduled DNA synthesis |
| UHPLC | Ultra high performance liquid chromatography |
| UV | Ultraviolet |
| VEGF | Vascular endothelial growth factor |
| VSD | Virtually safe dose |
| WHO-IPCS | World Health Organization - International Programme on Chemical Safety |