Public statement on the use of herbal medicinal products containing toxic, unsaturated pyrrolizidine alkaloids (PAs) including recommendations regarding contamination of herbal medicinal products with pyrrolizidine alkaloids

Draft

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<td>Draft discussed by Working Party on Community monographs and Community list (MLWP)</td>
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1 In the context of this PS, the term "herbal medicinal products" (HMP) also includes "traditional herbal medicinal products" (THMP). Therefore only the term "herbal medicinal products" or "HMP" is used throughout.
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<td>Draft Revision 1 adopted by HMPC for release for consultation</td>
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- Herbal medicinal products
- Traditional herbal medicinal products
- HMPC
- pyrrolizidine alkaloids
Public statement on the use of herbal medicinal products containing toxic, unsaturated pyrrolizidine alkaloids (PAs) including recommendations regarding contamination of herbal medicinal products with PAs

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1. Introduction (Problem statement)

It became apparent during assessment of Symphytum officinale (monograph EMEA/HMPC/572844/2009) that the risk assessment of pyrrolizidine alkaloids (PAs) poses considerable difficulties, with several PAs being regarded as both hepatotoxic and carcinogenic. Considering that PAs are natural constituents of a number of plants used for medicinal purposes the HMPC decided to prepare a public statement on the use of herbal preparations containing PAs (EMA 2014).

Furthermore, it was increasingly reported that herbal teas including those used as medicines may contain variable amounts of PAs, although the plants used as ingredients are not known to produce PAs (BfR 2013). In the following, based on information from several Member States, it was recognised primarily as quality-related topic. Several national regulatory authorities addressed the issue of PA contamination in HMPs and also the HMPC prepared a statement to support harmonisation in this regard (EMA 2016). The Public statement on contamination of herbal medicinal products/traditional herbal medicinal products with pyrrolizidine alkaloids (EMA/HMPC/328782/2016) gave transitional recommendations for risk management and quality control.

After a 3-years-period, the HMPC decided to reconsider both Public statements (see HMPC meeting report January 2019 - EMA/HMPC/26549/2019) and published Calls for data before re-assessing and concluding on recommendations with respect to the risks associated with the use of herbal medicinal products containing PAs naturally or from contamination. Revision 1 is based on a review of newly available data and the improved evaluation methods. The specific contamination issue and subsequent recommendations for risk management and quality control are now included (see also section 1.4; 1.4; 3.2 and 4).

1.1. Occurrence of pyrrolizidine alkaloids (PAs)

Pyrrolizidine alkaloids are heterocyclic organic compounds derived from ornithine (Moreira et al. 2018). They occur in nature in more than 6,000 plants (in excess of 300 plant species of up to 13 families, mainly in the families of Boraginaceae (all genera), Asteraceae (tribes Senecioneae and Eupatorieae) and Fabaceae (genus Crotalaria)), representing about 3% of the world’s flowering plants (Prakash et al. 1999, Louisse et al. 2019, He et al. 2019). They are very effective insect-feeding deterrents and consequently have evolved independently on at least four occasions in a number of different plant families (Edgar et al. 2015). More than 350 different PAs, excluding the N-Oxides, were described up to now and it is assumed that about half of them are hepatotoxic (Fu et al. 2004; He et al. 2019).

Both, composition and concentration of PAs may fluctuate and depend on various factors such as species, age and part of the plant, variety (genotype/chemotype), season, location etc. (Hoogenboom et al. 2011; Bodi et al. 2014). Thus, all known PAs of a PA-containing plant are not necessarily present at the same time. The same species growing in different locations or in different seasons may contain different alkaloids (Mattocks 1986, Flade et al. 2019). 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% (up to 19% based on dry weight) in certain plant seeds. Reported concentrations vary from trace amounts up to 19% based on dry weight (EFSA 2011, Bodi et al. 2014).

1.2. Chemistry and types of PAs

Most PAs are esters of hydroxylated 1-methylpyrrolizidines. The basic components, called necines, are derived from bicyclic amino alcohols that, in turn, are derived from the polyamines putrescine and
spermidine via the cyclic pyrrolizidine-1-carbaldehyde. The acids with which the necines are esterified are called necic acids (EFSA 2011, Schramm et al. 2019).

Figure 1: General structure of PAs (Roeder 2000)

- **Necines**

In PAs of the retronecine- and heliotridine type, the necine base is made of two five-membered rings, inclined towards each other and sharing a common nitrogen at position 4. The necine can either be saturated or possess a double bond in the 1,2-position (ring (b), Fig. 2). In almost all cases the necine has a hydroxymethyl group at C-1 and usually a hydroxyl group at C-7 as well. Esterification can take place in these positions. In addition, the necine may have one or two hydroxy groups at C-2 or C-6 resulting in the formation of stereoisomers (Roeder 2000, Schramm et al. 2019).

Figure 2: Structure of necines (retronecin type) (Roeder 2000)

Otonecine-type PAs do not contain genuine bicyclic five-membered ring systems. They may act as a pyrrolizidine ring system due to transannular interactions of the keto group and the tertiary amine. The PAs derived from these structures constitute a subgroup of the otonecine alkaloids (OPAs) (Schramm et al. 2019). There are also several necine bases with unusual structures, e.g. 1-aminopyrrolizidine, ehretinine, 7β-angeloyloxy-1-methylene-8α-pyrrolizidine and tussilagine. These structures seem to be described only from few plants and may occur only in trace amounts (Schramm et al. 2019).

Figure 3: Otonecine: the binding between the N atom and the CO group is widened to such an extent that the indicated resonance structures result (Roeder 2000)

- **Necic acids**

Apart from acetic acid, the necic acids, possess 5 to 10 C atoms and differ from each other in their structure. They include mono- and dicarboxylic acids with branched carbon chains. Substituents may be hydroxy, methoxy, epoxy, carboxy, acetoxy or other alkoxy groups besides methoxy substituents. Thus, numerous structural, stereo- and diastereoisomers may be derived. Double esterification may
lead to 11- to 14-membered ring systems (macrocyclic diesters). The most widely known PAs are 11-
membered monocrotaline, 12-membered alkaloids senecionine and senkirkine, 13-membered
doronine, and 14-membered parsonsine (Roeder 2000).

Based on the combination of necine bases and necic acids and their linkage patterns the PAs have been
classified into five groups: senecionine-like PAs (>100 structures; mainly found in Senecioneae and
Fabaceae); triangularine-type PAs (>50 structures; mainly present in Senecioneae and Boraginaceae);
lycopsamine-like PAs (mainly found in Boraginaceae, Apocynaceae and Eupatorieae); monocrotaline
type (>30 structures; predominantly found in Fabaceae) and phalaenopsine and ipanguline-type PAs
(found in Orchidaceae, Convulvulaceae and in few representatives of other tribes including the
Boraginaceae). In addition to these five groups, there are also very simple PAs consisting only of the
necine base and a small acid residue and finally some PAs show unusual linkage patterns distinct from
that of the five main groups, such as madurensine, laburnamine and tussilagine (EFSA 2011, Schramm
et al. 2019).

- **Pyrrolizidine alkaloid N-Oxides (PANOs)**

Excluding otonecine alkaloids, which cannot form N-oxides (most likely due to the interactions of the
keto group and the tertiary amine,) together with the N-oxides of the other alkaloids more than 660
alkaloids are known (Roeder 2000, Schramm et al. 2019). Metabolised products (free bases) of N-
oxides are toxic.

Biosynthesis of PAs takes place in the roots where the alkaloids occur as N-oxides. The N-oxides are
very polar compounds which are readily soluble in water and insoluble in most organic solvents. Unlike
typical tertiary alkaloids, they are not able to non-specifically permeate biological membranes in their
unprotonated form. Due to their properties, N-oxidated PAs can easily be translocated to the target
organ(s) within the plant. They are taken up via membrane transporter molecules and stored in the
vacuoles (Hartmann & Toppel 1987). N-oxides can easily be reduced to the corresponding tertiary
alkaloids, not only in the alimentary tract or in experimental conditions but also within the plants (e.g.
by enzymatic reactions).

### Structural requirements for toxicity

The minimum structural requirements for toxicity of PAs are:

1. A double bond in 1,2 position of a pyrrolizidine moiety
2. A hydroxymethyl substituent (C-1 position) in the pyrrolizidine moiety, preferably with a second
   hydroxyl group in the C-7 position
3. Esterification of the primary hydroxymethyl group with a branched mono- or dicarboxylic acid
   containing at least 5 C-atoms (necic acid).


### 1.3. Human exposure to PAs via food

In Europe and most developed countries, levels of PA intake are mostly low. Beside the direct intake of
PAs via herbal medicinal products secondary contamination of food with PAs was observed: e.g. in
foods of animal origin (as milk, eggs, honey, pollen products), in grain and in packed lettuce boxes as
detected in Germany (Molyneux et al. 2011). Depending on individual preferences in food selection,
great variability of PA exposure in humans is likely.

Episodic and catastrophic, acute and chronic poisonings have been documented particularly in
developing countries. Thousands of people might be affected, as in India in 1972, Tajikistan in 1992 or

Globalisation of markets leads to situations where previously localised toxins are shipped around the world in contaminated products. During the past years it appears that, because of the lack of natural control factors, the expansion of certain invasive plants e.g. Senecio madagascariensis, Senecio jacobaea or Senecio inaequidens creates serious problems for animals and -via animal products- for humans, too (Molyneux et al. 2011, Tsutsumi 2011, Dormontt et al. 2014, CABI 2019).

Many different studies on the determination of PA contamination in different food groups have been published. Most of them have been summarised by the EFSA (EFSA 2016).

Until now no limits for PAs in food exist within the EU, with the exception of refined echium oil for which a PA limit was given with 4 µg/kg (European Commission 2008). However, possible maximum levels for PAs in several food categories (e.g. tea, herbal infusions, food supplements and honey) are discussed (European Commission 2018). Within this discussion, limits for herbal infusions, such as 400 µg/kg for rooibos, anise, lemon balm, chamomile, thyme, peppermint, lemon verbena and 200 µg/kg for other herbal infusions are discussed (FSA 2019). With a standard single serving of 2 g herbal tea this would mean a maximum of 0.8 or 0.4 µg PA per serving.

- **Honey, pollen**

  The levels of toxic, unsaturated PAs and N-oxides found in many honeys could, according to published risk assessments, cause chronic diseases such as liver cirrhosis, pulmonary hypertension and cancer if these honeys are regularly consumed at the recommended serving sizes of 15–25 g (Edgar et al. 2011). PA levels up to 5600 µg/kg honey were found (Edgar et al. 2015). Investigations from Hong Kong and Australia also found PA contamination in a large proportion of honey samples examined (Chung & Lam 2017, Hungerford et al. 2019). A decrease of PA/PANO sum content caused by diminished PANO amounts was observed and systematically investigated. The observed decrease of PANO, on the one hand, was explained by a simple chemical derivatization or a dimerization and on the other hand as a result of enzymatic activity in honey caused by bee digestive enzymes. It could not be clarified whether the degradation of PANO involves a possible detoxification (Kaltner et al. 2018). Also for bee pollen products and, as well as products containing propolis and royal jelly a high number of the samples examined contained PA at such level, which in some cases led to recalls (Kempf et al. 2010a, Mulder et al. 2018, European Commission 2020).

- **Grain**

  There are many examples of acute poisonings in humans by PA contaminants in grain. All foreign seeds in grain, including those containing PAs, are removed normally prior to milling. These measures may be the reasons that large-scale, acute PA-poisoning incidents seen in some developing countries have not been seen in developed countries. However, chronic PA poisoning is still conceivable because it has been shown that complete removal of seeds containing PAs from heavily contaminated grain leaves readily detectable levels of PAs in the ‘cleaned’ grain. In addition, dust from PA-containing plants in the field during harvest or from their broken seeds is also a source of contamination that cannot be eliminated by measures concerning contaminating seeds (Molyneux et al. 2011).

  In a survey conducted in Hong Kong, PAs were detected in cereals and its products, in wheat, barley and rye flour as well as in plain bread up (Chung & Lam 2017). Also in Europe EFSA reports PA contaminations of cereal grains, their products and by-products and in other feed products, such as peas and carrots (EFSA 2017b).
• **Milk, eggs, meat**

Studies with single doses of different PAs or PA-containing plants in cows and goats showed that small amounts of PAs can pass into the milk (for most PAs rather low with approximately 0.1%, but for specific PAs up to 11%). This was also shown in rats (Coulombe 2003; COT 2008; Hoogenboom et al. 2011). In several surveys only small amounts of PAs could be detected in milk and dairy products. No positive findings were recorded for yoghurt, cheese or infant formula (Chung & Lam 2017, De Nijs et al. 2017, Mulder et al. 2018).

When different PA-containing plants were given to laying hens in the feed very different results were found in eggs: very high contents (Coulombe et al. 2003, Mulder et al. 2016), but also very low (Chung & Lam 2017; Mulder et al. 2018) or no detectable contents (Eröksüz et al. 2003; Mulder et al. 2016) at all.

Oral dosing of animals with radiolabelled PAs results in most of the radiolabel being eliminated within 24 hours, however small amounts of radiolabelled dihydropyrrolizine adducts remain detectable for many months in edible tissues, particularly in the liver (Edgar et al. 2011). Also in feeding studies in laying hens PA concentrations found in muscle tissue were lower than in liver tissue (Mulder et al. 2016). In two other studies none of the analysed bovine, porcine, or poultry meat and liver samples contained measurable amounts of PAs (Chung & Lam 2017, Mulder et al. 2018).

• **Other food such as salads, teas, spices, liquors**

Some leafy PA-producing plants, e.g., species of *Borago* and *Symphytum* are recommended as salads. The leaves of the common weed *Senecio vulgaris* accidently co-occurred with salad leaves of similar appearance being sold in supermarkets in Germany (BfR 2007a). However, from different herbal products purchased from supermarkets and farmer markets across Germany, only 1 product showed (Cramer et al. 2013).

PA-producing plants are also recommended for making teas, e.g., *Symphytum* spp. and sauces, e.g., traditional "Fränkische Grüne Sosse" contains borage (*Borago officinalis*) (BfR 2018).

Several studies examining tea samples were published. Apart from minor differences, the following similarities were found: PAs were detected in the majority of all tea samples; while in many cases the contents were below the LoQ, tea samples with very high contents were also found. Rooibos tea contained the highest concentrations of PAs through all studies (Bodi et al 2014, Mulder et al. 2015, Shimshoni et al. 2015, Chung & Lam 2017, Mulder et al. 2018), while also huge differences were seen between these studies for single types of tea, such as melissa tea, peppermint tea, chamomile tea.

Additionally, it should be added that in 2017 a single report of 73 000 µg PAs/kg in chamomile tea was published (Test 2017). It should be taken into account that the leaching of the PAs from dry tea material into infusions in the process of the brewing process might be incomplete (Picron et al. 2018).

It has been reported that a woman who consumed 20–30 µg of PAs per day via cooking spices during her pregnancy gave birth to a child suffering fatal liver damage (Rasenack et al. 2003). A number of publications have investigated the occurrence of PAs in spices (frozen and dried spices and herbs). Especially in borage, lovage, oregano, majoram and cumin high contents of PAs were detected (Chung & Lam 2017; BfR 2019; Kaltner et al. 2020). In addition, ginger root samples (dried and milled powder) showed high PA contents, possibly indicating a cross-contamination during processing or a horizontal transfer of PAs between living plants via the soil (Kaltner et al. 2020).

In 9 out of 38 liqueurs on plant base (bitters, digestives) PAs could be detected (Chmit et al. 2019).
- **Food supplements**

Investigation of food supplements focused on supplements that explicitly contained material of PA-producing plants or on supplements with no labelling concerning ingredients being PA producers. In the majority of all investigated samples, PAs were detected; but the concentrations were highly variable. The highest PA levels were found in herbal food supplements made from plant material of known PA producers. Supplements containing oil-based extracts of PA-producing plants were free of PAs, indicating that the hydrophilic PAs will not be co-extracted in the lipophilic oil fraction, or are effectively removed during oil refinement.

Furthermore, 60% of the (herbal) food supplements contained measurable amounts of PAs, even though that no PA-containing plant was labelled. Relatively high mean concentration were detected in products (13 out of 15) containing St John’s wort (Hypericum perforatum) (Mulder et al. 2018).

### 1.4. Contamination of herbal medicinal products

Beside the long known PA content in some plants traditionally used as medicines such as from the genera *Symphytum*, *Borago*, *Petasites* and *Tussilago* (CPMP 1992), an apparently rather wide-spread contamination of herbal products including medicinal products from plants not containing PAs (and therefore not usually tested for PAs) was later reported.

In Germany, the BfR conducted a research project "Determination of Pyrrolizidine Alkaloids in Food and Feed". In the project 221 different commercially available herbal tea and tea samples as well as herbal drugs were analysed for their PA content. Total PA contents from 0 to 3430 μg/kg dry matter were measured in the herbal tea and tea samples, including fennel tea, chamomile tea, peppermint tea, nettle tea and melissa tea. Considerable variations in PA contents, also for the same tea variety were found (BfR 2013).

Different publications exist, which report on detection of PAs in products used as medicine, independent from the regulatory status in different regions of the world for such products (e.g. Letsyo et al. 2017a, Letsyo et al. 2017b, Chmit et al. 2019, Steinhoff 2019, Suparmi et al. 2020). It has been shown that PA-containing weeds contaminate plant-derived raw materials used for the production of food, food supplements and herbal medicinal products (HMPs). The herbal raw materials generally appear to be contaminated by (very) low levels of PAs, but due to analytical methods (LC-MS/MS) even trace amounts of PAs can now be detected and quantified (EFSA 2011, Steinhoff 2019).

### 2. Discussion

The relevant literature on toxic, unsaturated PAs and PA-containing preparations was searched principally via PubMed. The cut-off date was June 2020. Information provided by Interested Parties upon the Calls for data ending August 2019 was also assessed.

#### 2.1. Regulatory status and assessment of PAs or PA-containing products

Some regulatory guidance documents concerning limits of intake of toxic, unsaturated PAs exist either in the field of medicinal products or in the field of food/food supplements.

**Medicines**

In Germany in 1992, a graduated plan concerning medicinal products containing PAs with a necine system unsaturated in 1,2 position came into force. The maximum daily dose of such PAs for internal use is set at 1 μg for a duration of maximum 6 weeks per year and 0.1 μg without any limitation in the duration. The maximal daily dose of PAs in case of cutaneous application is 100 μg for a duration of
maximum 6 weeks per year and 10 µg without any limitation in the duration of use (Bundesanzeiger 1992).

In Belgium medicinal products for internal use containing PAs are not allowed to be marketed (Albert 2000) and in Austria it has to be proven that the medicinal product which contains herbal preparations from PA-containing plants has no PAs in the final product (Bundesanzeigerblatt 1994). Several other countries refer to the CPMP document "Herbal drugs with serious risks-Listing of herbs and herbal derivatives withdrawn for safety reasons" (CPMP 1992).

In 2016, several EU regulatory authorities addressed the issue of PA contamination in HMPs. In May 2016, following a review of the available data, the EMA (HMPC) issued a Public statement to support Member States considering a harmonised approach in implementing appropriate controls for their markets. A contamination level of HMPs leading to a daily intake of maximum 1.0 µg PAs per day during a transitional period of 3 years was considered acceptable from a public health point of view. During this period, producers of HMP should be required to take the necessary measures to reduce the contamination to a level resulting in a daily intake not exceeding 0.35 µg PAs per day (EMA 2016).

In January 2019, the HMPC agreed to recommend an extended transitional period for a further 2 years due to ongoing discussions and efforts for harmonisation. Manufacturers should continue to take appropriate actions including implementation of enhanced GACP to ensure that the daily intake does not exceed 1.0 µg PAs per day.

Table 1: Examples of proposed reference values for unsaturated PAs and their N-Oxides

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<tr>
<td>Bundesgesundheitsamt (BGA) (1992)</td>
<td>1 µg per day (maximum 6 weeks per year)</td>
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<tr>
<td>BfArM (2016)</td>
<td>0.1 µg per day (no restriction) (for medicinal products only) maximum 1 µg per day</td>
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<tr>
<td>EFSA (2011)</td>
<td>70 µg/kg per day</td>
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<tr>
<td>EFSA (2017)</td>
<td>237 µg/kg per day</td>
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<tr>
<td>Food Standards Australia New Zealand (FSANZ) (2001)</td>
<td>1 µg/kg bw per day (safe level, provisional) (tolerable daily intake - based on avoidance of veno-occlusive disease –; cancer risk considered not proven)</td>
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<tr>
<td>Rijksinstituut voor Volksgezondheid en Milieu (RIVM) (2007) (Kempf et al. 2010b)</td>
<td>0.1 µg/kg bw per day (based on virtual safe dose of 0.43 ng/kg bw per day)</td>
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<tr>
<td>Rijksinstituut voor Volksgezondheid en Milieu (RIVM) (2015)</td>
<td>1 µg/kg of herbal teas and other food products and beverages containing herbs or herbal extracts</td>
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<tr>
<td>Committee on Toxicity (COT) (2008)</td>
<td>0.1 µg/kg bw per day (non-cancer unlikely) 0.007 µg/kg bw per day (cancer unlikely)</td>
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Foodstuffs

Some regulatory data were also available for foodstuffs, even though uniform regulations were missing in this field as well (IPSC 1988; EFSA 2007; COT 2008; Mulder et al. 2010).
In 2011 EFSA published an opinion on toxic, unsaturated PAs in food (EFSA 2011) which focus mainly
on the occurrence of PAs in honey. EFSA pointed out that on the basis of the genotoxic and
carcinogenic properties of 1,2-unsaturated PAs, it was not appropriate to establish a Tolerable Daily
Intake (TDI) and decided to apply the Margin of Exposure (MOE) approach instead. A Benchmark Dose
(giving 10% response) (BMDL10) for excess cancer risk of 70 µg/kg bw per day was calculated for
induction of liver haemangiosarcomas by lasiocarpine in male rats and used as the reference point for
comparison with the estimated dietary exposure. Whilst the MOEs for adults (calculated on
consumption data) were seen to be of low concern (MOE of 10,000 or higher), it was concluded that
there is a risk for those juveniles who are high consumers of honey.

In 2017, EFSA published a renewed opinion concerning toxic unsaturated PAs (EFSA 2017b). This was
preceded by a revision of the “Use of benchmark dose approach in risk assessment” (EFSA 2017a). In
that, model averaging is recommended as the preferred method for calculating the BMD confidence
interval, while acknowledging that the respective tools are still under development and may not be
easily accessible to all. The set of default models to be used for BMD analysis has been reviewed, and
the Akaike information criterion (AIC) has been introduced instead of the log-likelihood to characterise
the goodness of fit of different mathematical models to a dose–response data set. This BMD model
averaging approach was applied on the data sets on the incidence of liver haemangiosarcoma in male
and female rats exposed to lasiocarpine (NTP 1978) and riddelliine (NTP 2008). The BMD modelling for
riddelliine using model averaging resulted in a narrower BMDL10–BMDU10 interval, fully included within
the two higher tested doses (equivalent to 237-714 µg/kg bw per day), despite the relatively high
uncertainty related to the poor information on the dose response relationship of the study. Therefore,
EFSA selected the BMDL10 of 237 µg/kg bw per day, derived for the incidence of liver
haemangiosarcoma in female rats exposed to riddelliine as RP for the chronic risk assessment of PAs
(EFSA 2017).

2.2. Pharmacokinetics of PAs

Bio-activation occurs primarily in the liver by the action of several different mixed function oxidases to
dehydropyrrolizidine alkaloids (dehydro-PAs, pyrrolic esters). These dehydro-PAs possess an allylic
structure which makes them increasingly reactive. Once formed, the pyrrolic esters can rapidly bind
with DNA, protein, amino acids and glutathione even in the presence of adequate amounts of GSH
(Stegelmeier et al. 1999, Kempf et al. 2010b). It assumed that even chronic exposure to low dosages
of toxic PAs may lead to the accumulation of pyrrole–protein adducts and ultimately result in liver
damage (Ruan et al. 2014, Ma et al. 2018). Metabolism steps which either lead to activation or
detoxification are described in the literature. Although conjugation with GSH should be a step-in
detoxification, there is evidence that, among others, the 7-GSH-DHP conjugate may be a potential
reactive metabolite of PAs leading to DNA adduct formation (Geburek et al. 2020). The non-toxic
metabolites are quickly excreted.
N-Oxides cannot be directly converted into pyrroles. However, on oral ingestion they are reduced either by the gut enzymes or the liver microsomes and NADP or NADPH to the free bases which are toxic (Wiedenfeld 2011).

**Absorption**

Different PAs are transferred across the ileum and jejunum, but not the stomach, as measured by Swick et al. (1982) in rabbits.

Studies with different PAs were performed in rats (i.v., oral, cutaneous). Generally, it could be shown that resorption rates per plasma concentrations were significant lower for PA N-oxides than for PAs (Brauchli et al. 1982, Wang et al. 2011, Yang et al. 2020) regardless of the way of administration. Riddelliine was completely absorbed from the gavage dose within 30 minutes in all rats and mice (Williams et al. 2002).

Also in Caco-2 monolayer model, PAs showed absorption with apparent permeability coefficient values higher than those of corresponding N-oxides. Except for only few N-oxides all PAs and PA N-oxides investigated were absorbed via passive diffusion. While, for the few N-oxides, in addition to passive diffusion as their primary transportation, efflux transporter-mediated active transportation was also involved but to a less extent. Furthermore, a good correlation between lipophilicity and permeability of retronecine-type PAs and their N-oxides with absorption via passive diffusion was observed (Yang et al. 2020).

Diffusion and penetration of lycopsamine from an ointment containing Symphytum officinale extract varied from 0.11% and 0.72% (within 24 hours) through a synthetic membrane and 0.04-0.22% through human skin (Jedlinski et al. 2017).

**Metabolism to toxic metabolites**

The metabolic pattern and DNA adduct profiles produced by human liver microsomes are similar to those formed in rat liver in vitro and in vivo, indicating that the results of mechanistic studies with experimental rodents are highly relevant to humans (Yan et al. 2008). One metabolite, identified as a demethylation product, was the main metabolite when lasiocarpine was exposed to liver microsomes from human, pig, rat, mouse, rabbit, and sheep even though human liver microsomes displayed some distinctive features (indicating that humans may be more prone to lasiocarpine-induced acute toxicity than many other species). Liver microsomes from resistant species (i.e., rabbits and sheep) produced lower levels of the reactive metabolites (Fashe et al. 2015). When the in vitro degradation rate of frequently occurring PAs by liver enzymes present in S9 fractions from human, pig, cow, horse, rat,
rabbit, goat, and sheep liver were investigated, almost no metabolic degradation of any PA was observed for susceptible species such as human, pig, horse, or cow. It was assumed that the observed high biotransformation rate of non-susceptible species mainly represented a detoxification and the potential of toxic metabolites that might be formed in low concentration is that high that they are able to bind to proteins and possibly inhibit S9 enzymes effectively, so that the species-specific balance between activation and inactivating pathways decides on the degree of toxicity (Kolrep et al. 2018).

The levels of secondary pyrrolic metabolites formed from senecionine in different liver microsomes were found to be formed in the order: mouse>human>rat (Xia et al. 2020).

Conversion of PAs to reactive pyrrolic metabolites occurs by C- and N-oxidation catalysed by cytochrome P450 monoxygenases (Prakash et al. 1999, Fu et al. 2004) while flavin-containing monoxygenases and carboxylesterases are considered to be involved in detoxification pathways (Fu et al. 2004). The most commonly identified isoforms catalysing bio-activations are isoforms of the CYP3A subfamily (CYP3A4 and CYP3A5), but CYP2B and CYP2D isoforms also have this activity (Prakash et al. 1999, Huan et al. 1998, Fu et al. 2004, Ruan et al. 2014, Fu 2017). The abundance of this enzyme in liver varies over a 30-fold range between individuals, which suggests an inter-individual variation in toxification of PAs. It was reported that the panel of CYPs capable of mediating metabolic activation of retronecine-type PAs is more diverse than that for otonecine-type PAs, which might contribute to the differences in hepatotoxic potency between these two types of toxic PAs (Ruan et al. 2014). However, all of the dehydro-PAs contain an identical pyrrolic moiety regardless of the structures of their parent PAs (Ma et al. 2018b). Because of their extreme instability, the dehydro-PAs have not yet been identified either in vivo or in vitro (FSANZ 2001, Edgar et al. 2011, Fashe et al. 2015, Xia et al. 2020).

A rapid and extensive conversion of riddelliine to the N-oxide was shown, with the exception that female rats produced lower serum concentrations of the N-oxide. All rodents produced small amounts of retronecine. The elimination half-times increased in the following order: riddelliine<retronecine<N-oxide consistent with metabolism of parent compound. Internal exposures (AUC_{0-\infty}) increased in the order: retronecine<riddelliine<N-oxide, with male rats as the exception (Williams et al. 2002). Distribution

Heliotrine (i.p.) was present in the liver after 2 minutes (3.7% of total dose), the level peaking at 5 minutes (6.3%), and dropping to 2.2% at 1 hour and 0.5% at 2.5 hours. In adult rats, the level in the liver at 5 hours was 0.07% of the total dose. Five minutes after i.p. dosing, 30-40% of the initial dose remained in the peritoneal cavity, and the blood level of heliotrine was 60 mg/l, dropping to 3 mg/l at 1 hour. Blood levels of senecionine in rats (i.p.) were 0.38, 0.32, and 0.14 mg/l at 0.5, 1, and 2 hours after injection, respectively (IPCS 1988).

Concerning distribution of radioactivity from a triturated PA analogue (i.v.); in rats the highest concentrations of radioactivity were seen in the liver, lungs, kidneys, and spleen (respectively, 3.9%, 0.19%, 0.18%, and 0.27% of the dose given). Radioactivity in the expired air was negligible. The binding of radioactivity in the liver, and especially the lungs, was more persistent than in other organs (Mattocks 1977). When tritium-labelled indicine N-oxide was given i.v. to mice or monkeys, at 2 hours the highest concentrations of radioactivity were in the kidneys, liver, and intestines (El Dareer et al. 1982).

Studying the distribution of the uniformly $^{14}$C-labelled senecionine in lactating mice, after 16 hours, 0.04% of the radioactivity had been recovered in the milk; the liver contained 1.92% (IPCS 1988).
Excretion

The urinary excretion of monocrotaline in rats was 50-70% within the first day (IPCS 1988). Similar results were reported by Mattocks (1977) and White (1977). Despite minor differences between alkaloids, about 80% of ingested PAs were excreted unchanged in the urine and feces in rats (Stegelmeier et al. 2016). Indicine N-oxide given i.v. to mice, monkeys, or rabbits disappeared from the serum with initial half-lives ranging from 3 to 20 minutes. Over 80% of tritium-labelled indicine N-oxide given i.v. was excreted in the urine of mice or monkeys within 24 hours. Urinary excretion of indicine N-oxide was also rapid in rabbits, but somewhat slower in human beings (Powis et al. 1979, El Dareer et al. 1982).

Excretion of pyrroles continued for a little longer. In rats given retrorsine, the urine in the first 24 hours contained 10.6% unchanged alkaloid, 13.3% N-oxide, and 13.4% pyrrolic metabolites.

During the second day, only 0.1% alkaloid, 0.2% N-oxide, and 1.8% pyrroles were excreted. Biliary excretion also occurred. About one-quarter of an i.v. dose of retrorsine in rats was excreted in the bile as pyrrolic metabolites, and 4% as unchanged alkaloid; most of this excretion occurred during the first hour after the injection (White 1977). The proportion of urinary excretion of unchanged base increases with the hydrophilicity of the alkaloid, e.g. being 62% for heliotrine N-oxide, 30% for heliotrine, and only 1-1.5% for lasiocarpine (IPCS 1988). After small doses of tritiated senecionine or seneciphylline (0.3-3.3 mg/kg) given to rats, most radioactivity was eliminated in the urine and faeces within 4 days. Giving uniformly 14C-labelled senecionine in lactating mice, after 16 hours, 75% of the radioactivity had been recovered in the urine and 14% in the faeces.

Newly weaned mice are more susceptible to retrorsine-induced hepatotoxicity than adult mice, along with generation of more of the corresponding reactive metabolite, intensified liver GSH depletion, and formation of more protein modifications. The observed higher susceptibility of newly weaned mice to retrorsine liver injury resulted from greater internal exposure to retrorsine, due to slower elimination of the parent compound (Yang et al. 2018).

To summarise, the available evidence suggests that ingested PAs are rapidly metabolised and that the excretion of unchanged alkaloid and of most metabolites is rapid as well. Thus, within a few hours, only a relatively small proportion of the dose remains in the body, much of it in the form of metabolites bound to tissue constituents. It is unlikely that a significant amount of unchanged alkaloid will remain in the body after the first day.

2.3. Mechanism of toxic action of PAs

PA exposure over longer periods of time is mainly known to damage the liver (due to the liver being the main production site), lung or the blood vessels. Kidney, GI tract, pancreas and bone marrow are damaged to a lesser extent. Venous occlusions in the liver and lung, megalocystosis, inhibition of cell division (mitosis) and liver cirrhosis are all signs of PA toxicity. Genotoxic effects are seen as well (Mattocks 1986, Fu et al. 2004).

PAs themselves are chemically non-reactive. As ester alkaloids, they may be partially saponified by nonspecific hydrolases to the corresponding necines and necic acids both in the intestinal tract and during transit to the liver. Like the parent alkaloids, the fission products are non-toxic and are excreted via the renal system (Roeder 2000). Bio-activation (similar to aflatoxins) is necessary for toxic actions of PAs (Coulombe 2003, Ma et al. 2018b).

The cyclic diesters are thought to be the most toxic alkaloids and the noncyclic diesters are of intermediate toxicity, whilst the monoesters are the least toxic (Stegelmeier et al. 2016, Moreira et al. 2018). Saturated PAs are non-toxic according to the literature.
The extent of toxicity depends on the structure and the resulting metabolic pathways and
detoxification rates. Furthermore, many other factors such as species, age, sex or biochemical,
physiologic and nutrition status might influence bio-activation (Stegelmeier et al. 2016).

The activated PA metabolites, are mono- or, more commonly, bifunctional biological alkylating agents,
which undergo facile release of their ester groups to form positively charged, dihydropyrrolizine
carbonium ions that rapidly react with negatively charged nucleophilic functional groups (SH, OH and
NH) on proteins, nucleotides and other substances they encounter, e.g. glutathione (GSH), to form
dihydropyrrolizinadducts. Highly reactive electrophilic pyrroles are short lived. They quickly bind with
and damage nearby hepatic molecules as the endothelial cells lining in the sinusoids of the liver, close
to where the pyrroles are produced (Edgar et al. 2011). A number of publications highlight different
aspects of the underlying biochemical and pathophysiological mechanisms of toxicity (e.g. Liu et al.

Some PAs or their metabolites are more stable. So, they may circulate and transported to the lungs
where they cause similar effects in the arteries and alveolar capillaries. The ensuing thickening of
vessel walls in both the liver and lungs leads to their occlusion and consequently to restriction of blood
flow. The resulting conditions, hepatic veno-occlusive disease (VOD) (also known as sinusoidal
obstruction syndrome) and pulmonary arterial hypertension, lead to liver cirrhosis and right heart
congestive failure respectively (Edgar et al. 2011). Some pyrrole-tissue adducts may persist for
months and years as well. Adducts in tissues and dehydro-PA-induced neoplasms still have been
identified years after exposure. With time nucleic acids, proteins, and glycolipids containing dehydro-
PA-derived adducts are metabolized and repaired. Consequently dehydro-PA-derived adducts are
cleared at a low rate. As the adducted “pyrroles” are removed from cellular proteins or nucleic acids it
may be that they retain their electrophilicity and again react with cellular components (Stegelmeier et
al. 2016).

2.3.1. Single and repeat dose toxicity in animals

An in-depth description of the older literature concerning acute or chronic toxicity of PAs or their
metabolites is presented in some general documents, e.g. IPCS 1988, EFSA 2011. PAs are noted
mainly for the poisoning of livestock and large-scale outbreaks have been recorded from most parts of

The relative toxicity of PAs varies between mammalian species. The differences probably arise from
different toxicokinetics (bio-availability and bio-activation) and the stability and relative reactivity of
the resulting pyrroles (Coulombe 2003, Stegelmeier et al. 2016, Dalefield et al. 2016), but also from
other factors, such as differences in ruminal microflora, which might degrade PAs and decrease so the
amount entering hepatic portal circulation (Wiedenfeld & Edgar 2011). Nevertheless, the fundamental
metabolic and cytotoxic processes are common to all species (Molyneux et al. 2011). Pigs and poultry
are most susceptible, while horses, cattle and rats are less so and mice, sheep and goats are relatively
resistant to PA toxicity (Prakash et al. 1999, Stegelmeier et al. 2016). The toxicity of N-oxides is
similar of that of the parent alkaloid (IPCS 1988).

Acutely intoxicated animals show signs of liver failure, including anorexia, depression, icterus, visceral
oedema, and ascites and clinical pathological changes include massive elevations in activity of serum
enzymes (AST, SDH, ALK, and GGT) and increased amounts of serum bilirubin and bile acids. Gross
and histologic changes often includes pan lobular hepatocellular necrosis accompanied by haemorrhage
with minimal inflammation but also other findings such as increased sinusoidal platelet aggregation in
the damaged tissue regions (FSANZ 2001, Stegelmeier et al. 2016, Prelasco et al. 2017, Hessel-Pras
et al. 2020). There is conclusive evidence from studies on experimental animals that the effects of a
single exposure to PAs may progress relentlessly to advanced chronic liver disease and cirrhosis,
following a long interval of apparent well-being, and without any other latent or provocative factor.
(IPCS 1988). Results concerning the late onset of changes in the lung after a single exposure to
monocrotaline were described in animals (Huxtable 1990). Chronic poisoning may not be immediately
apparent, clinically, since animals may only develop transient elevations in serum enzyme activities
and mild elevations in serum bilirubin and bile acids. It was postulated that hepatocellular damage
might be progressive as damage continues with focal hepatocyte necrosis and subsequent
inflammation, fibrosis, and ultimately cirrhosis. With the resultant loss of hepatic function, animals
develop liver failure when they are unable to compensate when stressed with seasonal poor nutrition,
pregnancy, or lactation. Such failure may present as photosensitivity, icterus, or increased
susceptibility to other hepatic diseases (Stegelmeier et al. 2016).

In Big Blue transgenic rats receiving riddelliine for 12 weeks a number of genes involved in liver injury
and abnormalities were altered. Significant changes were seen in genes, which are linked to cell death,
cellular growth and proliferation, oxidative stress and liver morphology. Liver endothelial cells were
more involved than liver parenchymal cells (Mei et al. 2007).

Heliotrine at doses of 50 mg/kg body weight or more, administered to rats during the second week of
gestation, has been shown to induce several abnormalities in the fetus. Doses of 200 mg/kg bw
resulted in intrauterine deaths or resorption of fetuses. Dehydroheliotridine, the metabolic pyrrole
derivative of heliotrine, was 2.5 times more effective on a molar basis than its parent PA in inducing
teratogenic effects. The ability of PAs to cross the placental barrier in the rat and to induce premature
delivery or death of litters has been demonstrated. The embryo in utero appears to be more resistant
to the toxic effects of PAs than the neonate (IPCS 1988). Prenatal PAs exposure in rats induced fetal
hepatic and pulmonary toxicities (observed only in foetuses) through the generation of pyrrole
metabolites and oxidative injury. Furthermore, fetal serum transaminase activities were reduced (Guo
et al. 2019).

Alkaloids/toxic metabolites have been shown to be secreted in the milk of lactating dairy cattle and
rats, and both male and female young have been shown to suffer toxic damage, even when suckled by
retrorsine-treated mothers, who apparently are not affected themselves. Such suckling animals may
also be in apparent good health while the livers show toxic effects (Schoental 1959). Furthermore for
dehydroheliotridine and monocrotaline immunosuppressant activity could be shown in young mice and
rats showed of (FAO/WHO 2011).

In experimental animals, protein-rich and sucrose-only diets have given some measure of protection
against the effects of the alkaloids, as has pre-treatment with thiols, anti-oxidants, or zinc chloride. On
the other hand, PAs have been shown to act synergistically with aflatoxin in causing cirrhosis and
hepatoma in primates and to up-regulate EtOH-induced hepatocytotoxicity by inducing the
inflammatory cytokines and enhancing the apoptotic effects of ethanol in vitro (Lin et al. 1974,
Neumann et al. 2017).

**Toxic Actions of Dehydro-PA and DHP**

Pyrrolic derivatives prepared chemically from PAs, as well as some analogous compounds, have been
tested in experimental animals and in vitro systems, and showed a variety of toxic actions.

- **Dehydro-PA derivatives (DHP esters, pyrrolic esters)**

When given orally to rats, DHP esters are destroyed almost immediately in the aqueous acid of the
stomach and show no toxic action. When given i.p., they cause severe local irritation and peritonitis;
s.c. injection leads to skin lesions. After i.v. injection of pyrroles into the tail veins of rats, toxic injuries
appear principally in the lungs. Depending on the dose, these include vascular lesions and pulmonary
oedema; a progressive alveolar proliferation similar to that produced by very much larger doses of the parent alkaloid. Injections of DHP esters or synthetic analogues into mesenteric veins of rats lead to liver damage after smaller doses than the alkaloids themselves (IPCS 1988).

- **DHP (pyrrolic alcohols)**

These alcohols are much less reactive than the pyrrolic esters but far more persistent. They are seen as secondary toxic metabolites but also as the ultimate and common toxic metabolites of all dehydro-PAs which are not acute toxicants but can cause extensive extrahepatic injury, involving almost all rapidly developing tissues, especially in young animals. They have been shown to be immunotoxic, cytotoxic, genotoxic, pneumotoxic and carcinogenic (FSANZ 2001, Edgar et al. 2011).

The effects of dehydroheliotridine on 14-day-old rats were studied. All rats given i.p. doses of 0.4 mmol/kg bw survived, but a dose of 0.6 mmol/kg killed most animals within 10 days. Toxic effects were mainly found in rapidly developing tissues. In young rats, it caused fur loss, tooth defects, and atrophy of hair follicles, gut mucosa, spleen, thymus, testis, and bone marrow. The lungs were not affected. Pathological effects in the liver were confined to necrosis of isolated cells and antimitotic action, which was manifested as a mild megalocytosis in rats surviving 4 weeks or more (IPCS 1988).

The persistent antimitotic action on the liver that leads to the formation of giant hepatocytes can be produced both by pyrrolic esters (Hsu et al. 1973a, b), and by pyrrolic alcohols (Peterson et al. 1972, IPCS 1988). Both kinds of metabolites can lead to similar alkylation products. The antimitotic action must be accompanied or followed by a stimulus of cell division to be sufficient. Such a stimulus may be provided by the acute necrotic effect of primary pyrrolic metabolites or by any other cause of acute liver injury that leads to tissue regeneration. In very young animals, the stimulus can be the enhanced rate of replication that already exists in them.

Dehydroheliotridine was found to be teratogenic when given i.p. to female hooded rats on gestation day 14 (IPCS 1988).

### 2.3.2. Acute and chronic toxicity in humans

To date, over fifteen thousand acute human PA-poisoning cases have been documented (Yang et al. 2020). In man, PA poisoning is usually manifested as acute veno-occlusive disease (VOD) characterised by a dull dragging ache in the right upper abdomen, rapidly filling ascites resulting in marked distension of the abdomen and sometimes associated with oliguria, swelling feet and massive pleural effusion. There might be vomiting of blood in advanced stages of the disease. Acute liver damage includes centrilobular haemorrhagic necrosis and hepatomegaly with accompanying ascites. It can also manifest as subacute disease with vague symptoms and persistent hepatomegaly, in which the small hepatic veins become occluded by endothelial proliferation and medial hypertrophy leading to restricted blood flow, necrosis of surrounding tissue, fibrosis, nodular regeneration and in many cases, cirrhosis (Prakash et al. 1999). In some cases, a single episode of acute disease has been described to progress to cirrhosis (even in a period as short as 3 months from the acute phase), in spite of the fact that the patient has been removed from the source of toxic exposure and has been given symptomatic treatment (Tandon et al. 1977, Stuart & Bras 1957). Tissue-bound DHP adducts are considered to be a source of ongoing alkylation either by releasing 6,7-dihydropyrrrolizine carbonium ions capable of forming new adducts directly, or via the hydrolytic release of dihydropyrrrolizine alcohols (Mattocks 1986). In literature it was postulated that, following dietary exposure to PAs, in vivo alkylation continues until the reservoir of labile tissue-bound adducts is eliminated, mainly as soluble conjugates (e.g. with GSH) in urine and bile. This may take many months so that even a single dietary exposure...
to PAs continues to produce silently progressing chronic diseases, which are unlikely to be attributed to PAs in food (Edgar et al. 2011).

Mortality to PAs can be high with death due to hepatic failure in the acute phase or due to haematemesis resulting from ruptured oesophageal varices caused by cirrhosis. Less severely affected cases may show clinical, or even apparently complete, recovery. It was reported that after acute poisoning in man with significant acute toxicity, approx. 20% will die rapidly and 50% of patients will recover completely. Of the survivors, about 20% appear to recover clinically but may go on to develop cirrhosis and liver failure years later. Others may develop subacute liver pathological changes, which will either eventually resolve or go on to cirrhosis and liver failure (FSANZ 2001). In several publications the mortality of VOD is given with approx. 50% (Stickel & Seitz 2000).

**Figure 5**: Clinical natural history of VOD of the liver. B and C may be present with no clinical history of preceding illness (Stuart & Bras 1957)

Furthermore, the possibility of the development of toxic pulmonary disease in man cannot be ruled out. It is possible that the greater capacity of the liver to repair damage would lead to the situation where at some low levels and rates of exposure to PAs, liver damage may be minimal while lung damage continues to develop. In this scenario, sporadic small doses of PAs over an extended period, expected from current levels of dietary exposure, may produce cancer and pulmonary hypertension rather than liver damage (Edgar et al. 2011). There is a report of an outbreak of *Trichodesma* poisoning in the former USSR in which the symptoms were mainly neurological (IPCS 1988).

In the 1970s and 1980s, studies from Hong Kong, the United Kingdom and the USA reported instances of human disease that have been caused by the use of medicinal products containing PAs, resulting in fatality or the development of cirrhosis (IPCS 1988, Ridker et al. 1985) and also more recent cases of such PA poisoning via medicinal used herbs are reported (Gao et al. 2012).

Liver damaging agents, e.g. viruses, bacterial endotoxins, aflatoxins and environmental copper, can act synergistically and increase liver damage and cancer caused by PAs (Yee et al. 2000, IPCS 1988).

Although all age groups might be affected by PA poisoning, children are particularly vulnerable to the effects of PAs. One of the explanations therefore might be, that in neonates and foetuses, liver copper levels are naturally high (Riordan & Richards 1980, Edgar et al. 2011) which could potentiate the effects of PAs.

In 2011, the first identification of pyrrolo-protein adducts in the blood of a patient who was diagnosed as HSOS and confirmed to intake a PA-containing herb was reported. Blood pyrrolo-protein adducts in further patients were identified, each of whom consumed PA-containing plants (intake of the herb ranging from 5 to 200 days via self-medication), but not in healthy subjects (Ma et al. 2018b).

Furthermore, based on PBK modelling it was hypothesised, that liver toxicity shows inter-species and inter-ethnic human differences with the average Caucasian being more sensitive than the average Chinese, mainly due to more efficient reactive metabolite formation. In addition, humans are reported
being more susceptible to lasiocarpine and riddelliine-induced liver toxicity than rat (Ning et al. 2019b).

### 2.3.3. Genotoxicity and Carcinogenicity of PAs

#### Genotoxicity

Several PAs, PA derivatives, and related compounds have been shown to produce genotoxic effects (mutations, sister chromatid exchanges, chromosomal aberrations) in plants and several cell culture systems after metabolic activation (Kraus et al. 1985, Fu et al. 2004, Mei et al. 2010). Some PAs induce micronuclei formation in erythrocytes in the bone marrow and foetal liver in mice (IPCS 1988). Chromosomal aberrations have been demonstrated in rats and humans with VOD. In humans, this is believed to have been caused by fulvine (Martin et al. 1972).

DNA-adduct formation may play a role in the genotoxicity of riddelliine. Riddelliine induced a higher frequency of mutations in non-neoplastic endothelial cells (but not in parenchymal cells) in the cII gene mutation assay in transgenic Big Blue rats. The predominant mutations observed were G:C to T:A transversions, which are consistent with riddelliine-induced formation of DNA adducts involving G:C base pairs (Mei et al. 2007).

- **DHPs (pyrrolic alcohols)**

  Several DHPs were shown to have an inhibitory action in cultures of human KB cells, cultured rat liver cells and to cause chromosome breaks and sister chromatid exchange. Cell death was preceded, first by the swelling and disruption of organelles, including mitochondria, and then by the rupture of plasma membranes with the release of cell components (IPCS 1988).

#### Carcinogenicity

In the early 1970, a series of PAs were found to induce tumours, mainly liver tumours, in rats and other experimental rodents. To date, more than 20 purified plant PAs, a PA N-oxide, dehydro-PAs, and plant extracts have been demonstrated to induce tumors in rodents (Fu 2017). The carcinogenic activity of PAs appears to parallel their mutagenic behaviour, but not their hepatotoxicity. In rats, appropriately low repeated doses of several alkaloids have been shown to induce tumours. In one study, a single dose has been carcinogenic (Culvenor 1983). In the study of Schoental & Magee (1957) a single dose of lasiocarpine provoked after approximately 13 months changes in the liver which were described as being very similar to those observed in the earlier stages of hepatic carcinogenesis due to several pyrrolizidine alkaloids after multiple dosing.

It is notable that dose rates that have been effective in inducing tumours in rats are mostly equivalent to 0.2–6 mg/kg bw per day for the initial period and 0.2-3 mg/kg bw per day for the 12-month period. These dosages are roughly similar in magnitude to estimated intake rates (0.01-10 mg/kg bw per day) in several episodes of human toxicity. Comparison of the total intakes resulting in human toxicity with the total doses to death observed in the chronic toxicity studies on rats indicates that human beings are more susceptible and suggests that human beings may survive for sufficient time to develop cancer after only a brief exposure at this level or a longer exposure at a markedly lower level (Culvenor 1983, IPCS 1988).

From a 2-year study on lasiocarpine (24 rats per sex in each treatment group) it was concluded that under the conditions of this bioassay, lasiocarpine was carcinogenic in Fischer 344 rats producing hepatocellular tumors and angiosarcomas of the liver in both sexes and hematopoietic tumours in female animals (NTP 1978).
A 2-year study carried out as part of the National Toxicology Program showed that riddelliine induced liver hemangiosarcomas in both male and female rats and male mice, hepatocellular adenomas and carcinomas in male and female rats, and lung alveolar adenomas in female mice. Riddelliine was classified as “reasonably anticipated to be a human carcinogen” (NTP 2008).

The proposed mechanism for the induction of liver hemangiosarcoma suggests that the active metabolite of riddelliine interacts with endothelial DNA, causing damage, including karyomegaly, cytomegaly, and apoptosis, to endothelial cells of the liver. The enlarged endothelial cells obstruct the blood vessels causing local hypoxia. Hepatic hypoxia was shown to induce VEGF (Vascular Endothelial Growth Factor) production by hepatocytes. Increases in VEGF then induce increases in endothelial cell replication. The increased replication enhances the probability that DNA damage, either spontaneous or drug-induced, will escape repair and become fixed as mutations that eventually lead to hemangiosarcomas. It was suggested that hypoxia also triggers replication in the endothelial cells. (Nyska et al. 2002, Smith et al. 2004).

Figure 6: Proposed mechanism for the induction of liver hemangiosarcoma by riddelliine in rats (Nyska et al. 2002)

Carcinogenesis related gene expression patterns resulting from the treatment of comfrey and riddelliine are found to be very similar, even though the number of genes altered by comfrey was much higher, possible due to the fact that comfrey is a complex mixture compared to the isolated substance (Guo et al. 2007).

All potentially carcinogenic PAs studied for DNA adduct formation so far are reported to generate the same 4 adducts in vivo and in vitro in cell systems. These 4 DNA adducts have been proposed to be biological biomarkers of PA-induced liver tumour formation (Allemang et al. 2018).

No information is available on the long-term follow-up of the human population, to ascertain whether the exposure to PAs could have resulted in an increased incidence of liver cancer or other types of cancer. However, various PAs have been shown to be carcinogenic for experimental animals, which imply that a potential cancer risk for human beings should be seriously considered.

- **DHP (pyrrolic alcohols)**

Dehydroheliotridine was described as being carcinogenic. It could be shown that rats given 9 i.p. injections of this compound over 23 weeks had a shorter life span and suffered a significantly higher incidence of tumours than control rats (IPCS 1988).
Mechanistic studies with retrorsine, monocrotaline, clivorine, lasiocarpine, riddelliine N-oxide, retrorsine N-oxide and monocrotaline N-oxide generated the same set of DHP derived DNA adducts described as being responsible for liver tumour induction (Yan et al. 2008).

**Further considerations on carcinogenicity risk in humans**

For riddelliine, NTP concluded that the predominance of hemangiosarcoma was likely due to the greater genotoxicity and toxicity in the endothelial cell than in the hepatocyte. (NTP 2008) and also for other 1,2-unsaturated PAs, the carcinogenic potency is likely to be related to a combination of the genotoxic potential and the toxicity (EFSA 2011). In the NTP-reports on both lasiocarpine and riddelliine, a proliferative effect was observed also on hepatocytes, but this effect was not clearly dose-related, and resulted in malignancy only in the high-dose groups, in a few individuals. In contrast, liver hemangiosarcoma occurred at all dose levels in the rat lasiocarpine study. From a risk perspective, liver hemangiosarcoma is therefore considered the key effect.

The relevance of PA-induced hemangiosarcoma in rodents requires careful consideration when assessing human carcinogenic potential of PAs. The human intake of PAs through food and herbal medicinal products has presumably been fairly constant over the last decades (or longer), yet the incidence of liver hemangiosarcoma in humans is very low. Exact data on the occurrence of liver hemangiosarcoma in the population is difficult to obtain, but all information points to the fact that this is a very rare diagnosis.

Angiosarcoma is a malignant neoplasm of endothelial cells of blood vessels or lymphatic vessels and as such included in the overarching term of soft tissue sarcomas (STS), which in turn is a heterogeneous group of neoplasms of mesenchymal origin that comprise more than 50 histology subtypes, many of them very rare. STS constitutes less than 1% of all malignancies in adults. In the literature it has recently been estimated that angiosarcoma accounts for approximately 2-3% of all STS and primary hepatic angiosarcomas in turn accounts for <5% of all angiosarcomas (Zheng et al. 2014). Hepatic angiosarcoma account for 0.1%-2% of all primary hepatic malignancies, and therefore it is considered to be the third most common primary hepatic malignancy (Kumar et al. 2019).

In a review all available epidemiological information on the incidence of liver hemangiosarcoma based on studies in Sweden, UK, USA and Norway were summarized. The conclusion was that the incidence of liver hemangiosarcoma was approximately 0.5-2.5 cases per 10.000.000 individuals per year (Zocchetti 2001). Furthermore, it has been estimated that about 20-25% of the cases are associated with known etiologic factors such as vinyl chloride monomer exposure, use of Thorotrast (thoriumdioxid) in angiography, exposure to inorganic arsenic and treatment with androgenic-anabolic steroids (Zocchetti 2001, Falk et al. 1981, Rademaker et al. 2000). However, a much more common association that is often overlooked is hepatic fibrosis and cirrhosis, which is reportedly present in 40% of biopsy specimens at the time of diagnosis (Pickhardt et al. 2015). In the majority of cases the aetiology however remains unknown (Wilson et al. 2019).

Another risk that cannot be excluded at present is that intake of PAs would result in other forms of neoplasms in humans than in rodents. It is of course difficult to assess this risk, but the MOE framework, used by EFSA and HMPC to arrive at an acceptable daily intake of PAs, has been devised to accommodate such species differences.

**Relative toxicity of different PAs**

Investigation concerning toxicity suggest that structural differences of the various PAs have an influence on the toxicity. Among the same type of PAs, variations in the number of ester substitutions, lipophilicity, and steric hindrance of the necine acid groups could significantly affect the rate of
metabolic activation. It could be shown that retronecine-type PAs are much more susceptible than that of otonecine-type PAs. It was also shown that pyrrole−protein adducts formed in-vitro by otonecine-type PAs were significantly lower than those by retronecine-type PAs having similar necine acids.

Furthermore, among the nine retronecine-type PAs tested, the open-ring diester showed the highest efficiency for pyrrole−protein adduct formation, followed by the 12-membered macrocyclic diester and then by the 11-membered macrocyclic diester, while the monoester showed the lowest efficiencies (Ruan et al. 2014).

It was seen worthwhile to find out whether it would be possible to identify potency factors for the different 1,2-unsaturated PAs and their N-oxides, in order to evaluate the possible effects of combined exposure. However, since the preferred data for comparing potency would be carcinogenicity, the available data so far did not appear to be sufficient to distinguish between the potency of the PAs tested (FAO/WHO 2016).

Different approaches were published, which take into account the different structures and/or the different metabolism of the different PAs.

Merz & Schrenk proposed provisional potency factors for a series of 1,2-unsaturated PAs, based on available data on i.p. and i.v. acute LD50s in rat and mouse, genotoxic potency in Drosophila melanogaster, and in vitro cytotoxicity data in a model of chicken hepatocytes (Merz & Schrenk 2016). Chen et al. proposed to derive relative potency factors (RPFs) for a series of PAs for which information on tumour incidence following exposure in rats is available (Chen et al. 2017). However, EFSA concluded in 2017 that, due to the limitations in the analysed data set and the provisional nature of the semi-quantitative approach proposed by Merz & Schrenk, it is not adequate to use the derived RPFs for the cumulative risk assessment of PAs in food. Similarly, the approach proposed by Chen et al. has also important limitations and its use is not considered adequate for the risk assessment of PAs (EFSA 2017b).

Benchmark Dose (BMD) analysis was used to calculate the critical effect dose for 15 PAs representing 6 structural classes for micronuclei formation in HepaRG cells which express metabolising enzymes at levels similar to primary human hepatocytes. When BMD confidence intervals were used to rank PAs, lasiocarpine was the most potent PA and plotted distinctly from all other PAs examined (Allemang et al. 2018). When comparing 37 PAs representing different chemical classes in different potency classes according to the results of the concentration-dependent genotoxicity in the γH2AX in cell western assay in HepaRG human liver cells, the group with the highest potency consists particularly of open diester PAs and cyclic diester PAs (including riddelliine). The group of the least potent or non-active PAs includes the monoester PAs, non-esterified necine bases, PA N-oxides, and the unsaturated PA trachelanthamine (Louisse et al. 2019). While lasiocarpine was 3.5-fold more active than riddelliine in the in vitro H2AX-test, the predicted in vivo genotoxicity of riddelliine appeared to be 2.6-fold higher than that of lasiocarpine. This was explained by the differences in kinetics with a slower clearance of riddelliine compared to lasiocarpine (Chen et al. 2019).

The relative potencies of a series of structurally diverse PAs were explored by measuring DNA adduct formation in vitro in a rat sandwich culture hepatocyte (SCH) cell system. The adducts generated are consistent with those identified in vivo as biomarkers of PA exposure and potential liver-tumour formation and affirmed that PA toxicity varies considerably with chemical structure (Lester et al. 2019).

In a comprehensive study incubating a set of PAs (22) belonging to different structural types with rat or human liver microsomes together with GSH revealed differences in the degree of GSH conjugate formation. Because of the probable toxic potency of the GSH-DHP conjugates the formation could be used to estimate the potency of PAs. The highest amounts of GSH conjugates were detected for the
The development of models and the subsequent prediction of in vivo toxicity using such models requires evaluation of the models and predictions made. The lack of in vivo carcinogenicity data for other PAs than lasiocarpine and riddelliine may turn out a serious bottleneck for further development of an alternative testing strategy for prediction of PA toxicity. Furthermore, especially considerations on toxicogenetics/biokinetics issues will be needed to develop a robust understanding of relative potencies for a realistic risk assessment of PA-mixtures (Allemang et al. 2018, Lester et al. 2019, Ning et al. 2019a).

3. Conclusions and recommendations

3.1. Intake limits

Hepatotoxicity following the intake of toxic, unsaturated PAs is established. However, the dose-effect relationship remains unclear and inter-individual differences in susceptibility are large. Furthermore, hepatotoxicity caused by PAs may easily be misinterpreted as the result of other aetiologic factors, such as alcohol abuse for example (Stickel & Seitz 2000).

However, there are no substantial, long-term follow-up data to assess whether exposure to toxic, unsaturated PAs results in increased incidence of chronic liver disease or cancer in man. Toxic, unsaturated PAs could also be possible carcinogens in man, since a number of them have been demonstrated to induce cancer in experimental animals. In addition, in several instances of human toxicity, the reported daily rates of intake of PAs were in close range of those known to induce tumours in rats. Estimates of intakes causing toxic effects in human beings indicate that they are more sensitive than rats and domestic animals. The lowest intake rate causing VOD in a human being was estimated to be 0.015 mg/kg bw per day. It was a result of a self-medication with a comfrey preparation (Symphytum officinale).

The International Agency for Research on Cancer (IARC) evaluated several PAs for carcinogenicity in 1976 and 1983. It was concluded that there was in experimental animals "sufficient or limited evidence" for the carcinogenicity of monocrotaline, retrorsine, isatidine, lasiocarpine, petasitenine, senkirkine, and of extracts of the PA-containing plants Petasites japonicum, Tussilago farfara, Symphytum officinale, Senecio longilobus, Senecio numorensis, Farfugium japonicum and Senecio cannabifolius. The main target organ is the liver, where liver cell tumours and haemangioendothelial sarcomas were observed. In some instances, tumours in extra-hepatic tissues (lung, pancreas, intestine) were also observed, namely with monocrotaline, retrorsine, and lasiocarpine. Some PAs, for example, retrorsine, have been shown to be carcinogenic after a single dose. The pyrrolic metabolites have also been shown to be carcinogenic for rats. However, IARC concluded that the compounds are not classifiable as carcinogenic for humans. Due to the NTP data on riddelliine carcinogenicity, IARC changed the classification into “possibly carcinogenic to humans”, while NTP itself concluded that riddelliine is “reasonably anticipated to be a human carcinogen” (IARC 2002, NTP 2008).

Low level, intermittent dietary exposure to toxic, unsaturated PAs can be expected, so that slowly progressing chronic diseases such as cancer, cirrhosis and pulmonary hypertension are possible outcomes from eating foods sometimes containing relatively low levels of PAs. Hepatotoxicity may not always be the most prominent effect. P450 enzymes are also subject to induction by many (herbal) medicinal products and their use could significantly enhance the toxicity of PAs in the diet. The extended time period of progressive chronic disease development adds to the difficulty in identifying...
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It has to be considered that honey-containing products as mead, candy etc. may also contain toxic, unsaturated PAs. Familial susceptibility to PAs toxicity can also be expected. It should not be forgotten that anti-mutagenic compounds will also be ingested from food plants so that the impact of both mutagenic and anti-mutagenic compounds will be modulated by polymorphisms in genes associated with nutrient or xenobiotic uptake, distribution and metabolism (Ferguson & Philpott 2008).

Because of their known involvement in human poisoning and their possible carcinogenicity, exposure to toxic, unsaturated PAs should be kept as low as practically achievable (IPCS 1988, EFSA 2007, BfR 2007b). According to the published literature, it is possible that the average dietary daily intake might already be more than the amounts of toxic, unsaturated PAs which are seen to be safe.

### 3.2. Recommendations

In the evaluation of HMPs containing toxic, unsaturated PAs Member States should take steps to ensure that the public are protected from exposure and the following thresholds should be applied.

Even though that the HMPC allows the TTC concept for the risk evaluation of herbal preparations containing identifiable genotoxic compounds this applies only to preparations/compounds where an established safety assessment method cannot be applied by the lack of data (EMA 2007; Bucholzer et al. 2014). The existing data on toxic, unsaturated PAs were seen by different bodies sufficient to allow a specific safety assessment (EFSA 2011).

The CHMP concluded in 2019 that the BMDL10 approach as used by EFSA still lacks an international harmonised calculation methodology (EMA 2019) and the TDS0 approach should be in place according to ICH M7 (EMA 2013). Within the Carcinogenic Potency Database oral TDS0 values are listed for clivorine, monocrotaline, lasiocarpine, riddelline, senkirkine and retrorsine. Given the quality of the underlying studies, it would be most appropriate to take the TDS0 value of lasiocarpine (0.39 µg/kg per day). Applying the factor of 50,000 and based on a body weight of 50 kg for adults this would result in a limit of 0.39 µg PAs per day for adults, close to the limit given in the former Public statement on PAs (EMA 2014).

However, also the TDS0/NOAEL approach bears a variety of weaknesses, such as dependence of values on dose-selection during study design or uncertainties based on expert decisions relating to statistical significance of findings. These limitations have led to the development of the BMD approach in the first place. Taking into account that the rationale of benchmark dose modelling applied by EFSA (EFSA 2017b) covers better the underlying biological processes for PAs and applying the rationale used by HMPC before, the HMPC decided to follow the BMDL10 approach used by EFSA.

**Oral use**

Risk assessment by EFSA (EFSA 2017b) deduced a BMDL10 of 237 µg/kg. According to ICH this BMDL10 has to be divided by 10,000 to achieve the acceptable intake=0.0237 µg/kg body weight. Assuming a 50 kg person this would mean a daily intake of 1.0 µg per day for adults.2

**Sensitive groups**

Children:

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2 For ~18% (average) of the European population the body weight is given with less than 60 kg (EUROPEAN COMMISSION 2006). This number would increase to up to 30%, if only taking into account woman. Therefore, the calculation is linked to a body weight of 50 kg. This is in accordance with ICH M7.
If children are included in the usage of certain products the daily amount of toxic, unsaturated PAs has to be adjusted to the body weight of the age group: e.g. body weight of 20 kg would lead to an acceptable daily intake of 0.5 µg toxic, unsaturated PAs per day.

Pregnant and breast-feeding woman:

Sensitive groups such as pregnant and breast-feeding woman are also covered by the limit calculated above. If these limits are complied with, the chapter 4.6 of the SmPC of the products concerned should be phrased according to the "Guideline on risk assessment of medicinal products on human reproduction and lactation: from data to labelling" (EMEA/CHMP/203927/2005) (EMA 2008).

Cutaneous use

Until now only rudimentary data concerning absorption of PAs through the skin exist. The study by Brauchli et al. (1982) suggests that at least in rats, the dermal absorption could be 20-50 times less than absorption via the intestinal route. The used test model (rat) is not sufficient for the risk assessment in humans. For lycopsamine a diffusion of not more than 0.3% through human skin (in vitro) reported (Jedlinski et al. 2017). The limitation of the study is that penetration was analysed only in case of one PA.

It is to ensure that the amount of toxic, unsaturated PAs within the daily dose is <1.0 µg for adults. The use is restricted to intact skin.

Higher contents of toxic, unsaturated PAs within the products would be possible if for the relevant product (means the relevant matrix, because absorption might be greatly influenced by the excipients, for instance essential oils as enhancers) low absorption rates (generated with modern analytical techniques; in animal species which are more comparable to human beings in relation to the skin or in vitro human skin preparations) can be shown, not exceeding the daily intake of 1.0 µg toxic, unsaturated PAs for adults.

Sensitive groups

Children:

If children are included in the usage of certain products the daily amount of toxic, unsaturated PAs has to be adjusted to the body weight of the age group: e.g. body weight of 20 kg would lead to an acceptable daily intake (herbal medicinal products) of 0.5 µg toxic, unsaturated PAs per day.

Pregnant and breast-feeding woman:

Sensitive groups such as pregnant and breast-feeding woman are also covered by the limit calculated above. If these limits are complied with, the chapter 4.6 of the SmPC of the products concerned should be phrased according to the "Guideline on risk assessment of medicinal products on human reproduction and lactation: from data to labelling" (EMEA/CHMP/203927/2005).

Contamination of medicinal products with PAs

Also for contaminations of medicinal products (either contamination of the active ingredient or excipients) with PAs the same limit of 1.0 µg per day for adults applies. For children and adolescents the maximum intake should be calculated according to the body weight. For HMP with PA-containing herbal substances/preparations as active ingredient, the sum of PAs from the active ingredient and possible contaminations with PAs should not exceed the given limits.
3.3. Quality measures to reduce contamination with PAs

**Recommended strategy for risk management**

The main approach for risk management of the PA contamination of HMP should be according to the concept of ALARA, i.e. as low as reasonably achievable. In principle, contamination of herbal substances with PA containing weeds should not occur at all for reasons of requirements on pharmaceutical product quality and compliance with GACP/GMP.

**Quality aspects: control of PAs due to contamination in Herbal Medicinal Products**

With regard to actions to be undertaken by Member States arising from the concerns relating to the quality of HMPs, two main aspects needs to be addressed:

1. Implementation of suitable testing procedures to ensure PA levels are controlled in line with limits agreed.
2. Implementation of measures to avoid or reduce PA contamination in HMPs.

**4. Implementation of suitable testing procedures to control PA levels**

**4.1. Analytical methods**

Highly sensitive analytical methods are required to provide the level of quantification needed to control PAs. There have been no official test methods available for PAs in HMPs. The HMPC has therefore requested that the European Pharmacopoeia (Ph. Eur.) consider development of an appropriate analytical method validation for PAs in HMPs as a matter of priority. An expert group was founded at the European Directorate for the Quality of Medicines (EDQM) in September 2017 and a draft was adopted in September 2019, which is published in Pharmeuropa for consultation with interested parties.

This analytical procedure (SPE-LC-MS/MS method) described there as an example is very close to the one published by BfR (BfR 2014). The same 28 PAs are assayed, but 43 alkaloids are eluted. Numerous validation criteria are described. No chemical reference substance CRS will be provided by the Ph. Eur. and the sampling procedure is not described.

Until an official analytical method is available Marketing Authorisation Holders (MAHs) are advised to use the SPE-LC-MS/MS method as published by BfR (BfR-PA-Tea-2.0/2014). Other suitable validated methods may be acceptable (Ma et al. 2018a, Picron et al. 2018).

The test method should allow quantification of at least the following toxic PAs:

| 5. Europine | 15. Lycopsamine | 25. Seneciverine |
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10. Intermedine-N-oxide 20. Retrorsine-N-oxide

4.2. Specifications for herbal substances, herbal preparations, HMPs

The most appropriate stage for testing to take place should be considered; i.e. whether at the level of the herbal substance, the herbal preparation or the herbal product. Regulatory specifications should be created to reflect the controls introduced on PAs. In any event, the controls to be applied on PAs should take account of the final posology of the HMPs.

An appropriate sampling plan should be developed depending whether the herbal substance (spot contamination) or the herbal preparation/finished product (homogenous sample) is tested. Sampling should be in accordance with Commission Regulation 401/2006/EC (European Commission 2006).

4.3. Implementation of measures to avoid or reduce PA contamination in HMPs

Due to worldwide cultivation/collection and season-dependent sourcing processes, a complete elimination of PA contamination at all sourcing sites seems to be impossible. The findings of widespread contamination by PAs in HMPs has confirmed that the situation with PA contamination is serious and on an unprecedented scale. A detailed Code of Practice (CoP) has been developed by FAO and WHO (Codex Alimentarius 2014). The CoP focuses on weed control and provides guidance on good management practices to prevent and reduce PA contamination by control measures for the management of PA-containing plants as well as measures for control of plant release and spread.

In addition, from 2013 onwards, the German HMPs industry has initiated measures, which were intended to avoid and/or reduce PA contamination. Such measures consisted e.g. in causal research, in analytical testing in order to minimise the content of PAs in HMPs and in the establishment of a CoP that was elaborated together with herb growers (BAH 2016, Dittrich et al. 2016). The German CoP provides a framework for the implementation of individual measures in pharmaceutical companies as well as for the agricultural production steps. The main principle of the CoP is the identification of potential risks for each process step along the entire process chain comprising e.g. cultivation, harvesting, incoming goods inspection, drug processing up to the release of the final medicinal product.

The results obtained by collection of data and annual evaluations, confirm the efficiency of the performed measures according to the German CoP. Over the past few years, a clear reduction of the total PA burden of HMPs can be seen that follows an asymptotic function (Steinhoff 2019). However, the data provided by industry must be interpreted with caution due to various restrictions concerning the collection of data and they do not allow the authorities to draw any regulatory relevant conclusions (Wiesner et al. 2020).

The challenge to GACP is considerable as already small numbers of PA-containing weeds may lead to contaminations exceeding the threshold recommended above. Available agricultural measures to reduce PA weeds by way of selective herbicides, manual weeding/sorting, seed cleaning, inspection of fields before harvesting etc., need to be put in place to achieve the reduction of PA contamination.
5. Abbreviations

- ALARA: As Low As Reasonably Achievable
- BfR: German Federal Institute for Risk Assessment (Bundesinstitut für Risikobewertung)
- BMC: Bench Mark Concentration
- BMC10: Bench Mark Concentration (giving 10% response)
- BMC50: Bench Mark Concentration (giving 50% response)
- BMD: Bench Mark Dose
- BMD10: Bench Mark Dose (giving 10% response)
- BMDL10: Bench Mark Dose Lower Confidence Limit
- CoP: Code of Practice
- CRS: Chemical Reference Substance
- CYP: Cytochrome P450
- DHP(s): pyrrolic alcohols
- GSH: Glutathione
- HSOS: Hepatic Sinusoidal Obstruction Syndrome
- IARC: International Agency for Research on Cancer
- LC-MS/MS: Liquid chromatography tandem mass spectrometry
- MOE: Margin of exposure
- MS: Mass Spectrometry
- MS/MS: Tandem mass spectrometry
- NTP: National Toxicology Program (USA)
- NOAEL: No Observed Adverse Effect Level
- PA(s): Pyrrolizidine alkaloid(s)
- PAN0: PA-N-oxide
- SPE-LC-MS/MS: Solid Phase Extraction (SPE) in combination with Liquid Chromatography tandem mass spectrometry (LC-MS/MS)
- STS: Soft Tissue Sarcomas
- TD50: dose giving a 50% tumour incidence
- TDI: tolerable daily intake
- VOD: veno-occlusive disease
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