Bundesinstitut für Risikobewertung

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Updated risk assessment on levels of 1,2-unsaturated pyrrolizidine alkaloids (PAs) in foods

BfR Opinion 026/2020 issued 17 June 2020[1]

The German Federal Institute for Risk Assessment (BfR) regularly addresses the question of food contamination resulting from 1,2-unsaturated pyrrolizidine alkaloids (PAs). PAs are secondary metabolites formed by plants as a defence against herbivores. In food, these are undesirable substances since they may damage the liver, and have shown mutagenic (genotoxic) and cancerous (carcinogenic) effects in animal studies.

When assessing the health risks of foods, that are either contaminated with pyrrolizidine alkaloids or contain these alkaloids naturally, the primary focus is on the genotoxic-carcinogenic effects of 1,2-unsaturated PA. No safe threshold value can be derived for the genotoxic-carcinogenic effects of 1,2-unsaturated pyrrolizidine alkaloids. Therefore, the assessment is made based on the margin of exposure (MOE) concept from the European Food Safety Authority (EFSA). Values calculated for the MOE according to this approach are no health-based guidance values, however, but serve to prioritise the need for risk management measures. An MOE value of 10,000 or higher is generally considered to be of low concern from a public health point of view and might therefore be considered as a low priority for risk management measures.

On the basis of new occurrence data from 2015 to 2019, the BfR has now estimated the current overall exposure of consumers in Germany to 1,2-unsaturated PAs across a wide range of relevant food groups (including honey, various (herbal) teas, milk and spinach). Observations indicate that the average levels of 1,2-unsaturated PAs as well as the levels in the 95th percentile have both been clearly reduced in recent years in most of the food groups considered. This decrease is especially pronounced in green tea, black tea and peppermint tea but also in camomile tea, herbal teas and rooibos tea. As a result, the intake of 1,2-unsaturated PAs via these foods has also declined in recent years.

The estimated chronic overall exposure across all food groups considered in this assessment leads in all scenarios considered to intake levels for both children and adults that result in MOE values of over 10,000, both for normal consumers and for high consumers. As a result, the occurrence of health impairments caused by the overall exposure to 1,2-unsaturated PAs calculated in this way can be considered to be of low probability. When interpreting these results, one must take into account the fact that the MOE values determined for high consumers lie only slightly above 10,000.

It should also be emphasised that people are also exposed to 1,2-unsaturated PAs by consuming other types of food. These foods could not be considered in the overall assessment due to a lack of data on consumption quantities and/or PA levels. Food groups that were not considered in the assessment include herbs/spices and food supplements, for example. While herbs/spices are consumed in relatively small quantities, for example, a preliminary estimate indicates that this food group could make a considerable contribution in terms of both long-term and short-term exposure to 1,2-unsaturated PAs. Model calculations made using practical assumptions show that a MOE for adult normal consumers would be clearly lower than 10,000 even if considering only the consumption of herbs/spices with high levels

[1] Replaces BfR Opinion 020/18 issued 14 June 2018

(3,000 µg/kg). The same applies for adult high consumers when considering herbs/spices with average concentrations (1,000 µg/kg).

In light of the above and the fact that even the consumption of small quantities of genotoxic carcinogens, especially if consumed on a regular basis, might be associated with an increased health risk, the recommendation remains the same as before, namely: to minimise the intake of these substances as far as it is reasonably possible (ALARA principle: "as low as reasonably achievable"). Accordingly, the BfR recommends to continue efforts to further reduce the levels of 1,2-unsaturated PAs across all foods groups by improving cultivation, harvesting and washing methods. This applies in particular to food groups such as herbs/spices as very high levels were found in some samples tested from this group.

An FAQ on PAs in foods is available from the BfR website: https://www.bfr.bund.de/en/frequently_asked_questions_on_pyrrolizidine_alkaloids_in_foods-187360.html

BfR risk profile:
1,2-unsaturated pyrrolizidine alkaloids (PAs) in foods
(Opinion no. 026/2020)

A	**Affected persons**	**General population [1]**				
B	**Probability of health impairment due to regular consumption of foods and food supplements containing pyrrolizidine alkaloids**	Practically impossible	Unlikely	**Possible**	Probable	Certain
C	**Severity of health impairment due to regular consumption of foods and food supplements containing pyrrolizidine alkaloids**	**The severity of the impairment may vary [2]**				
D	**Validity of available data**	High: The most important data are available and are internally consistent	Medium: Some important data are missing or inconsistent		Low: A large volume of important data is missing or inconsistent	
E	**Controllability by the consumer [3]**	Control not necessary	Controllable by precautionary measures	Controllable by avoidance	Not controllable	

Fields with a dark blue background indicate the properties of the risk assessed in this opinion
(for more details, see the text of opinion no. 026/2020 from the BfR dated 17 June 2020).

Explanations

The risk profile is intended to visualise the risk outlined in the BfR opinion. The profile is not intended to be used to compare risks. The risk profile should only be read in conjunction with the corresponding Opinion.
[1] Row A
The risks described primarily affect high consumers.
[2] Row C
A distinction must be made between acute and chronic effects. Chronic effects are always to be considered as more severe (cancer, irreversible).
[3] Row E
The BfR makes no recommendations for consumers in this Opinion. Recommendations are provided in the BfR's FAQ on PAs in foods. The BfR recommends adopting risk management measures to mitigate risk. The BfR takes the view that PA levels in relevant food groups should be kept as low as reasonably possible.

1 Subject of the assessment

In the past, the German Federal Institute for Risk Assessment (BfR) has regularly addressed the occurrence of 1,2-unsaturated pyrrolizidine alkaloids (PAs) in food as well as the resultant risks to human health. The updated overall assessment presented here has been prepared while taking into account new occurrence data for a range of relevant food groups. Food supplements, herbs and spices, rocket and flours have not been included in the estimate of overall exposure as a result of major uncertainties in terms of consumption data and in terms of occurrence data. These food groups were considered separately as a part of this opinion. In addition, new insights have also been taken into account in terms of the toxic properties and the analysis of 1,2-unsaturated PAs. The present opinion therefore replaces earlier opinions from the BfR on the risks to human health posed by exposure to 1,2-unsaturated PAs in foods.

2 Results

Regarding their potency as genotoxic carcinogens, 1,2-unsaturated PAs are considered as a group of equipotent substances whose effects are additive. Risk characterisation for this group of substances is therefore done by the *margin of exposure* (MOE) approach typically used in the European Union, with a $BMDL_{10}$ (*benchmark dose lower confidence limit 10%*) of 237 µg/kg body weight/day as a reference point.

On the basis of new occurrence data from 2015 to 2019, an updated estimate of the overall exposure to 1,2-unsaturated PAs across a wide range of relevant food groups (including honey, various (herbal) teas, milk and spinach) was conducted within this opinion. Observations indicate that the average levels of 1,2-unsaturated PAs as well as the levels in the 95th percentile have both been clearly reduced in recent years in most of the food groups considered. This decrease is especially pronounced in green tea, black tea and peppermint tea but also in camomile tea, herbal teas and rooibos tea. As a result, the intake of 1,2-unsaturated PAs via these foods has also declined in recent years.

The results of the risk characterisation can be summarised as follows.

- When assessing the health risks of 1,2-unsaturated PA, the primary focus is on the genotoxic and carcinogenic effects .

- The estimated chronic overall exposure across all food groups considered leads in all scenarios considered to intake levels for both children and adults that result in MOE values of over 10,000, both for normal consumers and for high consumers. As a result, the occurrence of health risks caused by the overall exposure to 1,2-unsaturated PAs calculated in this way can be considered to be of low probability.

  When interpreting these results, one must take into account the fact that the MOE values determined for high consumers lie only slightly above 10,000.

- Consumers are also exposed to 1,2-unsaturated PAs from other foods, which cannot yet be considered in the estimate of overall intake as presented here.

  Examples of such foods include herbs/spices and food supplements. A preliminary estimate for herbs/spices indicates that, although consumption quantities for this food

group are small, it could nonetheless make a considerable contribution to both long- and short-term exposure to 1,2-unsaturated PAs. As examples, model calculations made using practical assumptions show that a MOE could be clearly lower than 10,000, even if considering only the consumption of herbs/spices with high levels (3,000 µg/kg) for adult normal consumers or when considering the consumption of herbs/spices with average levels (1,000 µg/kg) for adult high consumers. In interpreting these findings, it should be noted that these MOE values result from the sole intake of 1,2-unsaturated PAs via the consumption of herbs/spices: in reality, a multitude of foodstuffs contribute to a consumer's overall exposure. Especially high levels were found in borage, oregano, lovage and mixed spices, and in one sample each taken of cumin and thyme. These herbs therefore constitute a relevant additional source of exposure. However, reliable data for PA levels and real-world consumption quantities for individual herbs are not available for these food groups. As a result, it has not been possible to include these in the estimate of overall exposure to 1,2-unsaturated PAs to date.

The general recommendation made in the European Union is to minimise exposure to substances that are both genotoxic and carcinogenic as far as it is reasonably possible (ALARA principle: as low as reasonably achievable). This is because even small quantities of these substances can be associated with an increase in health risks – especially if consumed on a regular basis.

The BfR therefore recommends to continue efforts to further reduce the levels of 1,2-unsaturated PAs to the lowest level technically possible across all foods groups by improving cultivation, harvesting and washing methods. This applies in particular to food groups such as herbs/spices, whose data still occasionally show abnormally high levels.

3 Rationale

3.1 Risk assessment

3.1.1 Agent

Pyrrolizidine alkaloids (PAs) are a large group of compounds that are formed primarily by plants, but also by fungi and bacteria (Robertson & Stevens 2017). To date, several hundred PAs and their *N*-oxides are known (Wiedenfeld *et al.* 2008). In plants, PAs have been identified in more than 350 species (Bunchorntavakul & Reddy 2013); based on chemotaxonomic considerations, however, the occurrence of these compounds is expected in over 6,000 species (Teuscher & Lindequist 2010). The ability to form PAs is found in the members of at least 13 plant families, especially in representatives of the composite family (Asteraceae), borage family (Boraginaceae), the legume family (Fabaceae/Leguminosae), the dogbane family (Apocynaceae), the buttercup family (Ranunculaceae) and the figwort family (Scophulariaceae) (Wiedenfeld *et al.* 2008). These compounds are presumably produced by plants as secondary metabolites as a protection against herbivores. The compounds also have the function of attracting certain insect species (Wink 2019).

Many structurally different PA occur in PA-forming plants.. In particular, compounds produced by some members of the borage family have very high structural diversity (These *et al.* 2013). Even within the same plant species, however, the exact composition of these PA profiles and levels may also be influenced by other factors such as growth conditions and the age of the plant. Concentrations may also differ from one part of a plant to another (Allgaier & Franz 2015).

Chemically speaking, PAs are esters that consist of a necine base (1-hydroxymethyl pyrrolizidine scaffold) and necic acids (aliphatic mono-/dicarboxylic acids). The necine base typically has another hydroxyl group at C7. PAs can occur as monoesters (esterification of the hydroxyl group at C9) or as diesters (esterification of the hydroxyl groups at C7 and C9). Cyclic diesters can also be formed by esterification of both hydroxyl groups with a dicarboxylic acid. Depending on the structure of the underlying necine base, PAs are essentially classified as belonging to the retronecine, heliotridine, otonecine or platynecine type (figure 1). While the necine base exhibits a double bond at the 1,2-position in the first three types, PAs of the platynecine type have a saturated necine base. PAs of the retronecine and heliotridine type differ only in terms of their diasteriomeric linking at C7 (Wiedenfeld *et al.* 2008).

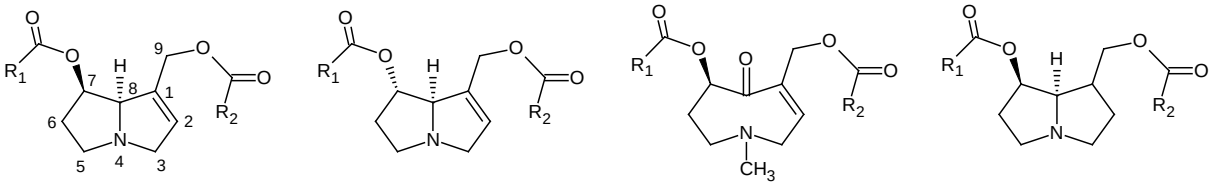

Figure 1: *Key structures of PAs.*

PAs can enter the food chain in a number of ways. One such way is when wild herbs containing PAs contaminate plant-based foodstuffs that are themselves free of PAs during harvesting. In addition, some plants used as foodstuffs actually form PAs themselves. This is the case with borage, for example. Some food supplements may also be produced using plants that form PAs or parts of these plants. In principle, another possibility is via the transfer from contaminated animal feed into foods of animal origin (meat, milk and eggs) (Mulder *et al.* 2016; de Nijs *et al.* 2017).

In general, 1,2-unsaturated PAs esterified with at least one branched carboxylic acid at C_5 are considered to have hepatotoxic, carcinogenic and mutagenic effects (BfArM 1992; Roeder 1992). These compounds include PAs of the retronecine, heliotridine and otonecine types but not those PAs of the platynecine type (figure 1). Accordingly, the present opinion considers only the 1,2-unsaturated PAs.

3.1.2 Assessments from national and international bodies

In recent years, the potential risks to health from 1,2-unsaturated PAs have been assessed by several scientific bodies. Both the assessment methods used and the conclusions drawn have changed over time. The section below provides a brief overview of key findings from earlier assessments.

In 1988, an expert committee from the International Programme on Chemical Safety (IPCS) of the World Health Organisation (WHO) concluded that various 1,2-unsaturated PAs were potentially toxic, whereby reactive pyrrole metabolites rather than the parent substances themselves were held responsible for the toxic effects. Following short- and medium-term intake of high doses, the pronounced liver-damaging effect is the primary concern. However, the toxicity of the compounds also has a cumulative character, which is why chronic exposure to low doses could also pose a health risk. The induction of liver cirrhosis and a possible carcinogenic effect were considered to be the main long-term effects in humans. At the time,

however, no reliable epidemiological studies were available that could have provided reliable information about the carcinogenic potential in humans. Based on a case report by Ridker *et al*. (Ridker *et al.* 1985), which described the occurrence of a liver damage following ingestion of 1,2-unsaturated PAs via comfrey leaves, the expert committee concluded that the medium-term daily intake of 15 µg/kg body weight (primary alkaloid: echimidine) could already cause liver damage in humans. Since the 1,2-unsaturated PAs from comfrey caused less pronounced effects in animal studies on acute toxicity, the ratio of LD_{50} values following intraperitoneal application was used to determine an equivalent dose of 10 µg heliotrine/kg body weight: this dose was also assumed to be capable of causing toxicity in humans (IPCS/INCHEM 1988). However, it should be noted that the cited case described by Ridker *et al*. exhibits major uncertainties concerning the dose-effect relationship.

The WHO's International Agency for Research on Cancer (IARC) has also assessed various 1,2-unsaturated PAs. While some of these compounds were classified as "possibly carcinogenic to humans", a classification of this kind was not possible for other compounds due to a lack of data (IARC 1983, 1987, 2002).

For medicines, maximum levels for the occurrence of 1,2-unsaturated PAs, including their *N*-oxides, were set in Germany as part of a so called "Stufenplanverfahren" in 1992. For medicines with recognised applications according to monographs pursuant to section 25(7) of the German Medicines Act (AMG), daily exposure following a maximum dose must not exceed the following intake quantities for 1,2-unsaturated PAs: 100 µg/person for external use, 1 µg/person for internal use, 10 µg/person for the use of coltsfoot leaves as a tea infusion. For these medicines, the duration of use has also to be limited to no more than 6 weeks a year. Medicines for internal use must also not be used during pregnancy and while breastfeeding. Aside from certain homeopathic medicinal products, medicines are exempted from these usage restrictions if their maximum dose results in a daily exposure to 1,2-unsaturated PAs not exceeding the amount of 0.1 µg/person for internal use and 10 µg/person for external use (BfArM 1992).

In 2001, the Australia New Zealand Food Authority (ANZFA, now: FSANZ, Food Standards Australia New Zealand) completed an assessment on 1,2-unsaturated PAs in food and concluded that no reliable epidemiological data were available that were capable of indicating a carcinogenic potential for these compounds in humans. The major toxicological endpoint in humans was considered to be PA-induced occurrence of hepatic veno-occlusive disease. On the basis of data from the case report from Ridker *et al*. (Ridker *et al.* 1985), the ANZFA – contrary to the WHO assessment published in 1988 – derived a "tentative no-observed-effect-level" for all PAs of 10 µg/kg body weight/day for the non-carcinogenic effects. After applying an uncertainty factor of 10 to account for intra-species differences in terms of susceptibility, a PTDI (provisional tolerable daily intake) of 1 µg/kg body weight/day was proposed (ANZFA 2001).

In 2002, the Permanent Senate Commission on Food Safety (SKLM) of the German Research Foundation (DFG) came to the following conclusion in an opinion called 'Stellungnahme zu Pyrrolizidinalkaloiden in Honigen, Imkereierzeugnissen und Pollenprodukten ': "*The available data on the concentrations of PAs in honeys obtained from plants that contain PAs (such as honey from Echium spp. or Senecio spp.) and the available data on PA exposure for consumers are to be considered inadequate. Since data on the toxicology of such PAs and on their metabolism in humans are also incomplete, no conclusive risk assessment can be completed at this time.*" (free translation into english) The SKLM recommended "*first focusing in particular on products that are manufactured by using pollen from plants that contain PAs. These products are distributed on the market as food supplements and are likely to*

be consumed in large quantities." (free translation into english) The Committee also recommended improving the analytical detection of 1,2-unsaturated PAs in honey and pollen (DFG 2002).

In 2005, the Dutch National Institute for Public Health and the Environment (RIVM) also assessed the risks posed by 1,2-unsaturated PAs. RIVM also included data taken from animal experiments. On the basis of an NOAEL (no observed adverse effect level) of 10 µg/kg body weight/day for non-carcinogenic changes from a chronic study in rats with riddelliine (NTP 2003) and taking into account an uncertainty factor of 100, a TDI (tolerable daily intake) of 0.1 µg/kg bodyweight/day was derived. In terms of the potentially carcinogenic effects of 1.2-unsaturated PAs, RIVM derived a VSD (virtually safe dose) from the same study of 0.00043 µg/kg body weight/day. This value specifies the dose that, following a linear extrapolation based on the animal data, is still associated with an increased cancer risk of 1 in 1,000,000 (RIVM 2014).

In its initial risk assessment of 1,2-unsaturated PAs in food that was published in 2007, the BfR had used an assessment approach that was harmonized with the medicines sector, which had been established in 1992 in the context of the above-mentioned "Stufenplanverfahren" for medicines containing PAs. As for certain medicines, it was also requested in the food sector that a maximum daily intake of 0.1 µg of 1,2-unsaturated PAs per person should not be exceeded wherever possible (BfR 2007).

Also in 2007, the European Food Safety Authority (EFSA) had identified gaps in knowledge concerning the exposure to PAs as part of an opinion on the assessment of PAs as undesirable substances in animal feed. In this assessment, EFSA noted that attention should be paid in particular to the occurrence of 1,2-unsaturated PAs in honey. In view of the risks to human health, PA-induced occurrence of veno-occlusive disease was also seen as the most sensitive endpoint (EFSA 2007).

In 2008, the risks resulting from exposure to 1,2-unsaturated PAs were assessed by the UK Committee on Toxicity of Chemicals in Food, Consumer Products and the Environment (COT). The case report by Ridker *et al*. (Ridker *et al.* 1985) was considered to be unsuitable for the assessment of non-carcinogenic effects. Instead, based on data from a chronic study in rats with riddelliine (NTP 2003), the COT identified a NOAEL of 10 µg/kg body weight/day and, by applying an uncertainty factor of 100, concluded that the occurrence of non-carcinogenic effects is not to be expected below an intake quantity of 0.1 µg riddelliine/kg body weight/day. COT also considered 1,2-unsaturated PAs to be genotoxic carcinogens with cumulative effects. The margin of exposure (MOE) calculation was based on data from the chronic study in rats with lasiocarpine (NCI 1978) and a $BMDL_{10}$ (benchmark dose lower confidence limit 10%) of 73 µg/kg body weight/day was derived as the underlying reference point. Based on guidelines published by the European Food Safety Authority (EFSA) (EFSA 2005), COT drew the following conclusions: "*MOEs of 10,000 and above, corresponding to doses of up to 0.007 µg/kg body weight/day, would be unlikely to be of concern*" (COT 2008).

In 2011, the BfR published an opinion on the risks to human health posed by 1,2-unsaturated PAs from honey. The increased incidence of hemangiosarcomas in rats was identified as the most sensitive endpoint. These were observed both in a long-term study with lasiocarpine (NCI 1978) and a long-term study with riddelliine (NTP 2003). Consequently, the MOE approach typically used in the European Union for substances having genotoxic-carcinogenic effects was used to assess the potential risks. Here, hemangiosarcoma data from both carcinogenicity studies were evaluated using dose-effect modelling based on the recommendations in the EFSA guidance "Use of the benchmark dose approach in risk assessment" as

published in 2009 (EFSA 2009). In agreement with the earlier COT assessment, a $BMDL_{10}$ of 73 µg/kg body weight/day was derived. This would result in values for the MOE of at least 10,000 provided that a daily intake of 0.007 µg of 1.2 unsaturated PA/kg bw and day is not exceeded.. For the risk assessment, the occurrence data of all 1,2-unsaturated PA were considered as a sum and an equivalence of the individual 1,2-unsaturated PA with regard to carcinogenic effect was assumed. To assess the effects on health following short-term intake, two case reports in humans (Stillman *et al.* 1977; Fox *et al.* 1978; Huxtable 1980) were provisionally used to identify an alternative dose range that was associated with pronounced detrimental effects on health following short-term intake. Such doses range from approximately 1 to 3 mg/kg body weight/day (BfR 2013b).

An opinion on the risk assessment of PAs in food and animal feed was also published by EFSA in 2011. Within this evaluation a detailed characterisation of the hazard potential of 1,2-unsaturated PAs was carried out. EFSA concluded that 1,2-unsaturated PAs are genotoxic carcinogens. The increased incidence of hemangiosarcomas in rats was identified by EFSA as the most sensitive endpoint. In its assessment, EFSA also used the MOE approach and derived a $BMDL_{10}$ of 70 µg/kg body weight/day as a reference point. EFSA also considered the occurrence data of all 1,2-unsaturated PAs as a sum and assumed an equivalent potency in terms of their carcinogenic effects. On the basis of MOE values below 10,000, EFSA concluded that a risk to health in terms of carcinogenic effects could be possible for infants and children who consume large quantities of honey. An assessment of other food groups was not possible, since no reliable occurrence data were available at this time. Alongside the assessment of potential risks due to the chronic intake of 1,2-unsaturated PAs, potential acute toxic effects were also considered in the EFSA opinion. Based on the two case reports in humans described above (Stillman *et al.* 1977; Fox *et al.* 1978; Huxtable 1980), PA doses were identified that were associated with pronounced impairments to human health after short-term intake. Such doses range from approximately 1 to 3 mg/kg body weight/day (EFSA 2011).

In another opinion published in 2013, the BfR assessed risks to health posed by 1,2-unsaturated PAs in teas and herbal teas. In this opinion, the BfR concluded that the MOE values for persons who frequently consume large quantities of tea and herbal tea are considerably lower than 10,000 (BfR 2013a).

For the medicines sector, the Committee on Herbal Medicinal Products (HMPC) of the European Medicines Agency (EMA) published an opinion in 2014 entitled ‘Public statement on the use of herbal medicinal products containing toxic, unsaturated pyrrolizidine alkaloids (PAs)’. For medicines taken orally, the HMPC drew the following conclusions: *“… Risk assessment by various scientific organisations […] deduced a permitted daily intake of 0.007 µg PA/kg body weight. Assuming a 50 kg person this would mean a daily intake of 0.35 µg per day (from all sources: food and herbal medicinal products) for adults. The potential daily intake of toxic, unsaturated PAs via food cannot be ignored especially as consumers/patients are not able to avoid them. On the basis of the available kinetic data, it seems clear that ingested PAs will be absorbed and metabolised. HMPC concludes that the short-term (maximum 14 days) daily intake of 0.35 µg of toxic unsaturated PAs/day from herbal medicinal products might be acceptable. …”* (EMA 2014).

In June 2015, 1,2-unsaturated PAs were evaluated for the first time by the *Joint FAO/WHO Expert Committee on Food Additives* (JECFA). So far, only a short summary of the preliminary results from the assessment has been published. The assessment is primarily based on the chronic study in rats with riddelliine (NTP 2003) conducted as part of the NTP programme. On this basis, the occurrence of hemangiosarcomas in the liver in female rats was

identified as the most sensitive endpoint, and a $BMDL_{10}$ of 182 µg/kg body weight/day was derived and used for MOE calculations. Based on the consumption of honey and tea, exposure was estimated for children and adults, and the following conclusion was drawn: *"…The calculated MOEs for adult frequent consumers of tea and honey, and for average tea consumption by children indicated a concern…*" (JECFA 2015).

A comprehensive opinion on 1,2-unsaturated PAs in various foods was published by the BfR in 2016. Here, a $BMDL_{10}$ of 73 µg/kg body weight/day was used as a reference point for the calculation of MOE values. On the basis of new data on the occurrence of 1,2-unsaturated PAs, it was possible to take additional food groups into account for the risk assessment in this opinion. Especially herbal tea, black tea and honey accounted for a considerable proportion of the overall intake of 1,2-unsaturated PAs in children. For adults, herbal tea and black tea were particularly relevant for the intake of 1,2-unsaturated PAs. In addition, food supplements – and especially those supplements based on plants capable of forming PAs – were identified as an important source of exposure. On the basis of MOE values considerably lower than 10,000, both for children and for adults, this opinion highlighted a potential risk concerning carcinogenic effects following high levels of consumption of foods with high concentrations of PAs. To assess potential acute toxic effects for certain food supplements with high concentrations of 1,2-unsaturated PAs, results from case reports and animal experiments on non-carcinogenic (non-neoplastic) effects were used as a provisional source of data. In addition, the BfR had derived a health-based guideline value (HBGV) of 0.1 µg PA/kg bw and day as an orientation value for the assessment of the risk of non-carcinogenic damage based on a NOAEL of 10 µg/kg bw and day for the occurrence of non-carcinogenic liver damage in a rat study and by applying a suitable extrapolation factor of 100. For both children and adults, the orientation value was exceeded when considering high consumers of highly contaminated products (BfR 2016b).

Following the recent publication of new data on the occurrence of 1,2-unsaturated PAs and on the exposure to 1,2-unsaturated PAs from several food groups by EFSA in 2016, the risk assessment for 1,2-unsaturated PAs in foods was subsequently updated by EFSA in 2017. This assessment took honey, teas, herbal teas and food supplements into account. EFSA did not consider it necessary to provide an extensive update of the characterisation of the hazard potential posed by 1,2-unsaturated PAs. However, due to the update of the EFSA guidance on BMD modelling published in spring 2017 ("Update: use of the benchmark dose approach in risk assessment") (EFSA 2017a), a remodelling of the dose-response data in accordance with the current recommendations was considered necessary (EFSA 2017b). Taking into account this new guidance, EFSA conducted a remodelling of the findings from the previously considered studies with lasiocarpine and riddelliin. The occurrence of hemangiosarcomas was confirmed as the most sensitive endpoint. However, EFSA concluded that the data from the riddelliin study provide a more reliable basis for modelling the dose-response relationship, in line with the new recommendations on BMD modelling. A new $BMDL_{10}$ of 237 µg/kg body weight/day was derived. The differences compared to the previous $BMDL_{10}$ of 70 µg/kg body weight/day are probably the primary result of the limited quality of the available data. For example, a pronounced incidence of early substance-related mortality was observed, which presumably had a relevant impact on the dose-effect relationship (EFSA 2017b). Despite the higher reference point of 273 µg/kg body weight/day compared with the one from older opinions of 70 µg/kg body weight/day, the MOE values calculated in this opinion for high consumers of highly contaminated products were clearly below 10,000. The EFSA therefore considered a risk of carcinogenic effects to be possible under certain consumption conditions. In addition, certain food supplements were considered in this opinion as a relevant source of exposure and it was concluded that even the occurrence of acute toxic effects

is possible following consumption of certain food supplements based on PA-forming plants (EFSA 2017b).

In 2018, the BfR published an updated risk assessment on 1,2-unsaturated PAs in food. Taking into account the new $BMDL_{10}$ of 237 µg/kg body weight/day, this risk assessment concluded that the consumption of certain foods can result in a level of exposure corresponding to MOE values that are clearly below 10,000. A risk of carcinogenic effects from the intake of food contaminated with PAs was therefore considered possible (BfR 2018).

In 2019, an assessment of 1,2-unsaturated PAs in food, with a focus on culinary herbs, was published. Due to the very limited data available on both short- and long-term consumption of various herbs, a conclusive assessment of the associated risks was not possible. On the basis of a number of model calculations, however, the BfR came to the conclusion that, while the absolute quantities consumed are probably low, culinary herbs can make a major contribution to both long- and short-term exposure to 1,2-unsaturated PAs (BfR 2019).

3.1.3 Hazard characterisation

3.1.3.1 Toxic properties of 1,2-unsaturated PAs

The primary target organ for toxic effects is the liver. However, other organs such as the lungs can also be affected by PA-induced damage. The cause of this organotropism is probably the conversion of the 1,2-unsaturated PAs to reactive metabolites (pyrrol esters) that occurs primarily in the liver.

The formation of these reactive pyrrol metabolites is considered to be the primary cause for both the non-carcinogenic damage to sinusoidal endothelia in the liver and of the genotoxic-carcinogenic effects (Fu *et al.* 2004; Wiedenfeld *et al.* 2008; Allgaier & Franz 2015; Fu 2017; Ma *et al.* 2018). The precise mechanism of the damage caused by the pyrrol compounds is not yet fully understood, however. At higher doses, reprotoxic effects have also been observed in animal experiments with 1,2-unsaturated PAs (Fu *et al.* 2004; Chen *et al.* 2010; Edgar *et al.* 2014; Allgaier & Franz 2015).

3.1.3.1.1 Non-carcinogenic effects of 1,2-unsaturated PAs

In livestock, severe cases of poisoning caused by the consumption of wild herbs rich in PAs are regularly occurring (Fu *et al.* 2017; Panziera *et al.* 2018). In cattle, for example, the occurrence of liver cirrhosis has been observed following intake of alpine ragwort from hay and silage. In horses, the intake of PA-rich *Senecio* species while grazing is also known to lead to seneciosis that are marked by degenerative effects on the liver. In livestock, these types of poisoning symptoms due are known by various names, including ‘walking disease’ (USA), ‘dunziekte’ (South Africa), ‘Winton disease’ (New Zealand) or ‘Schweinsberg disease’ (Germany) (Petzinger 2011b, a).

Especially inn livestock and rodents, the occurrence of enlarged hepatocytes, with the formation of large, hyperchromatic cell nuclei, has also been observed following chronic exposure, which has been ascribed to the antimitotic effects of 1,2-unsaturated PAs (NTP 2003; Fu *et al.* 2004; Wiedenfeld *et al.* 2008; Fu *et al.* 2017). In the chronic toxicity study in rats with riddelliine described in greater detail in section 3.1.3.1.2, the increased incidence of

hepatocytomegalia was also the most sensitive non-carcinogenic effect. This was observed from a dose of 33 µg/kg body weight and treatment day upwards (NTP 2003).

Also in humans, severe and occasionally fatal poisoning cases have been regularly observed following the intake of high doses of 1,2-unsaturated PAs. In Afghanistan, for example, several thousand cases of endemic poisoning have been documented over the last few decades, with 2010 being the most recent year with a multitude of cases. The poisonings were attributed to the consumption of cereal crops that had been contaminated with *Heliotropium* species capable of forming PAs (Molyneux *et al.* 1991; Kakar *et al.* 2010). In Asia, poisonings have also been associated with the consumption of certain herbs used as part of traditional Chinese medicine, which either contain 1,2-unsaturated PAs themselves or are confused with or contaminated with PA-rich wild herbs (Dai *et al.* 2007; Ma *et al.* 2018; Zhuge *et al.* 2019).

In humans, the non-carcinogenic effects of 1,2-unsaturated PAs manifest in particular in the liver, in the form of veno-occlusive changes (HSOS, *hepatic sinusoidal obstruction syndrome*; synonym: HVOD, *hepatic veno-occlusive disease*). In a process presumably mediated by the reactive metabolites, this causes damage to the sinusoidal endothelial cells, which can then swell up or become detached as a result. This, in turn, leads to a characteristic change in and stenosis of sinusoidal vessels. Following the (sub)acute intake of higher quantities of 1,2-unsaturated PAs, clinical symptoms such as severe stomach pain, pain centred on the liver, anorexia, fatigue, ascites, jaundice and hepatomegalia can be observed (Allgaier & Franz 2015; Yang *et al.* 2019b; Zhuge *et al.* 2019). Such symptoms can occur following the short-term intake of very high doses or following medium-term exposure to average doses (BfR 2013a). While severe poisonings are often fatal, complete remission is possible in cases of milder poisoning (Allgaier & Franz 2015; Zhuge *et al.* 2019).

Alongside effects on the liver, (some) 1,2-unsaturated PAs or rather their metabolites can also cause damage to pulmonary endothelial cells. This can then cause pulmonary hypertension and, as a consequence, right ventricular enlargement (pulmonary heart disease, *cor pulmonale*) (Wiedenfeld *et al.* 2008; Edgar *et al.* 2014; Allgaier & Franz 2015).

The available information from reports of poisoning cases in humans allow only for a limited range of conclusions to be drawn about the dose-effect relationship of the non-carcinogenic damage in humans:

(a) Toxic effects following short-term intake of high doses

The literature provides two relatively well-documented case reports in which poisonings are described after 4 and 14 days of exposure, respectively. One case concerned a six-month-old female infant (body weight: 6 kg), while the second case involved a two-month-old male infant (body weight: unknown). Both babies had been given a herbal tea that had been prepared from *Senecio longilobus* (Stillman *et al.* 1977; Fox *et al.* 1978; Huxtable 1980). The female infant's clinical symptoms included ascites and pleural effusion, with sinusoidal liver fibrosis presenting after two months and cirrhosis of the liver six months later. The male infant presented with hematemesis, developed jaundice with pronounced hepatomegalia, and exhibited convulsions of the CNS, bradycardia and periods of apnoea. He died six days later.

In the case of the female infant, the herbal tea contained (as percentages of dry weight) 1,2-unsaturated PAs as free alkaloids (primarily riddelliine) at a concentration of 0.3% as well as *N*-oxides (primarily of retrorsine, with smaller proportions of senecionine and senecionine) at 1%. In the case of the male infant, the concentrations in

the tea were 0.5% for free alkaloids and 1% for *N*-oxides, respectively. Based on the occurrence data and the dosage scheme, the female infant was estimated to have received between 70 and 147 mg (equivalent to 12–25 mg/kg body weight) of 1,2-unsaturated PAs in total over a two-week period. In the male infant's case (assumed body weight: 5.5 kg), the estimated overall intake of 1,2-unsaturated PAs was approximately 66 mg over a period of four days (equivalent to 17 mg/kg body weight).

The estimated daily dose was therefore 0.8–1.7 mg/kg body weight for the female infant and 3 mg/kg body weight for the male, for a mixture of various 1,2-unsaturated PAs, with the primary components being riddelliine and retrorsine-*N*-oxide. In both cases, the overall doses consumed led to severe health impairments of the patients.

(b) Toxic effects following medium- to long-term exposure

The literature supplies one case of poisoning with the clinical symptoms of HSOS, which occurred following four months of consumption of a preparation made from comfrey leaves (the exact *Symphytum* species is not named). The leaves contained up to 0.27 g alkaloid/kg. In addition, a herbal tea had also been consumed over a prolonged period that also contained 1,2-unsaturated PAs. The authors estimate that a daily dose of 15 µg alkaloid/kg body weight had been consumed (primary alkaloid: echimidine) over a period of six months. Since exposure was from multiple sources, however, this estimate involves several relevant uncertainties (Ridker *et al.* 1985; IPCS/INCHEM 1988; COT 2008).

The occurrence of HSOS, which in one case proved fatal, was also diagnosed in the case of four Chinese women who had consumed a herbal tea prepared from *Heliotropium lasiocarpum* over a period ranging from 19 to 46 days. According to estimates, daily amounts of 0.59, 0.49 (fatal case), 0.60 and 0.71 mg of 1,2-unsaturated PAs (heliotrine)/kg body weight had been consumed over 45, 46, 19 and 21 days, respectively (Kumana *et al.* 1983, 1985; Culvenor *et al.* 1986; IPCS/INCHEM 1988).

In two cases occurring in India, which presented with HSOS following the intake of *Heliotropium eichwaldii* for medicinal purposes over a period of 20 to 50 days, exposure was estimated at 3.3 mg of 1,2-unsaturated PAs (heliotrine)/kg body weight/day (Datta *et al.* 1978; IPCS/INCHEM 1988).

Following a cluster in HSOS cases in Afghanistan and India after the consumption of cereal crops contaminated with PAs, it was estimated that a daily quantity of 0.033 and 0.66 mg/kg body weight had been consumed over a period of six and two months, respectively. The primary alkaloids were assumed to be heliotrine and crotananine/crotaburmine (Mohabbat *et al.* 1976; Tandon *et al.* 1976; Krishnamachari *et al.* 1977; IPCS/INCHEM 1988).

The literature also documents a few more recent case reports of poisonings after the intake of 1,2-unsaturated PAs (Ruan *et al.* 2015; Rollason *et al.* 2016; Sun *et al.* 2018). However, these cannot be used to derive robust data about the quantities consumed by the patients concerned.

3.1.3.1.2 Genotoxic/carcinogenic effects of 1,2-unsaturated PAs

In animal experiments in which rats were given 1,2-unsaturated PAs via their feed or gavage, an increased incidence of tumours was observed. A corresponding cancer risk for humans is assumed (IARC 1976; Danninger *et al.* 1983; IARC 1983; IPCS/INCHEM 1988; IARC 2002).

Accordingly, lasiocarpine, monocrotaline and riddelliine (for example) have been classified to date by the WHO (IARC, *International Agency for Research on Cancer*) as “potentially carcinogenic in humans” (IARC 2019). For most of the 1,2-unsaturated PAs identified, however, data that would allow for a robust classification in this sense has not been forthcoming to date. Nonetheless, also for other 1,2-unsaturated PAs animal studies have indicated that these PAs also exhibit a carcinogenic potential. In terms of carcinogenic effects, the liver is once again the primary target organ, although PA-induced tumours have also been found in other organs, including the lungs, kidneys, skin, bladder, brain, spinal medulla, pancreas and adrenal glands (Chen *et al.* 2010).

The most reliable data available in terms of carcinogenic effects – especially regarding the dose-effect relationship following oral intake – are provided by two chronic toxicity studies in which the carcinogenic potential of lasiocarpine and riddelliine were investigated following lifelong oral consumption (NCI 1978; NTP 2003). The key findings of these studies are presented in brief in the following:

In a study performed by the National Cancer Institute (NCI) with lasiocarpine, F344 rats received the substance as a lifelong dose with feed over a max. period of 104 weeks (0, 7, 15, 30 mg of lasiocarpine/kg feed ≙ 0, 0.35, 0.75, 1.5 mg/kg body weight/day). A total of 24 animals were investigated per dose group and sex. In the group receiving the highest dose, pronounced early substance-related mortality was observed in both sexes: all male rats had died by the 88th week and all female rats had died by the 69th week. An increased incidence of hemangiosarcomas of the liver was identified as the most sensitive endpoint (males: 0/23 (controls), 5/24 (low-dose group), 11/23 (medium-dose group), 13/23 (high-dose group); female rats: 0/24 (controls), 8/22 (low-dose group), 7/24 (medium-dose group), 2/23 (high-dose group)) (NCI 1978). The early substance-related mortality presumably prohibits the adequate detection of carcinogenic effects, especially in the female rats.

In a study performed by the *National Toxicology Program* (NTP) with riddelliine, both B6C3F1 mice and F344 rats were given riddelliine via gavage on five days a week as a lifelong dose over a max. period of 105 weeks (male mice: 0, 0.1, 0.3, 1, 3 mg/kg body weight and treatment day; female mice: 0, 3 mg/kg body weight and treatment day; male rats: 0, 1 mg/kg body weight and treatment day; female rats: 0, 0.01, 0.033, 0.1, 0.33, 1 mg/kg body weight and treatment day). Rats were the more sensitive species in terms of the toxic effects of riddelliine. In the high-dose group, all male rats had died by the 70th week and all female rats by the 97th week. Treatment-related tumour formation was considered to be causative for the high mortality in the high-dose group. Treatment was therefore discontinued for the male rats after 72 weeks. An increased incidence of hemangiosarcomas of the liver was identified as the most sensitive endpoint (males: 0/50 (control), 43/50 (1 mg/kg body weight and treatment day); females: 0/50 (control), 0/50 (0.01 mg/kg body weight and treatment day), 0/50 (0.033 mg/kg body weight and treatment day), 0/50 (0.1 mg/kg body weight and treatment day), 3/50 (0.33 mg/kg body weight and treatment day), 38/50 (1 mg/kg body weight and treatment day)) (NTP 2003).

In addition, it has been shown for various derivatives of the 1,2 unsaturated PA that they have a genotoxic potential.. Following metabolic activation, these compounds are capable of damaging genetic material both *in vitro* and *in vivo*. Investigations discovered PA-induced DNA adducts, DNA cross-links, spontaneous DNA repair, micronuclei, chromosomal aberrations, sister chromatid exchanges and point mutations. The last type, point mutations, have been detected in bacteria and *Drosophila* as well as in transgenic rats (Chen *et al.* 2010). The studies in rats also demonstrated that, following treatment with riddelliine, a correlation exists between the PA-specific DNA adducts, the mutation frequency and the

formation of hemangiosarcomas in the hepatic endothelial cells that constitute the starting tissue for hemangiosarcoma formation (Chen *et al.* 2010). Increased expression of the tumour suppressor p53 was also observed in endothelial cells after malignant transformation by riddelliine, along with an increased rate of mutation in the K-*ras* proto-oncogene in riddelliine-induced hemangiosarcomas (Hong *et al.* 2003). In light of these findings, it is assumed that carcinogenicity is a direct result of these genotoxic effects. The characteristic DNA adducts are generally considered to be an initial step in the process of chemical carcinogenesis (Fu 2017); the underlying mechanism of the carcinogenic effects observed in the animal experiments, however, is not yet understood in its entirety.

3.1.3.2 Factors that influence toxicity *in vivo*

3.1.3.2.1 Key principles of chemical structure

The characteristic toxic effects are primarily mediated by those PAs that exhibit certain structural characteristics (Mattocks 1986; IPCS/INCHEM 1988; Roeder 1992; NTP 2003; Fu *et al.* 2004; Teuscher *et al.* 2004; Li *et al.* 2011; Petzinger 2011b). These include:

- Double bound at the 1,2-position of the necine base
- Esterification of the hydroxyl group at C9 and/or the hydroxyl group at C7
- Branching of the alkyl side chain in at least one of the necic acids

3.1.3.2.2 Bioavailability

Investigations with riddelliine, senecionine, and adonifoline in rodents have shown that these 1,2-unsaturated PAs can reach the systemic circulation quickly after oral intake (Williams *et al.* 2002; Wang *et al.* 2011). The degree of systemic bioavailability for individual PAs can vary significantly, however, depending on their respective structures (Wang *et al.* 2011). Experiments involving human CaCo-2 cells indicate that even gastrointestinal uptake via the cells in the gut can vary considerably, depending on the respective structure of individual 1,2-unsaturated PAs (Hessel *et al.* 2014). The extent of resorption via the intestinal mucosal barrier is therefore probably a key factor that influences the toxic potential of individual 1,2-unsaturated PAs. On the other hand, the systemic bioavailability of the parent substance or *N*-oxides is probably of less relevance when assessing potential toxic effects: this is because 1,2-unsaturated PAs are protoxins whose metabolism (together with the toxification that results from this process) is already completed in the liver (Fu *et al.* 2004; Allgaier & Franz 2015). Accordingly, the proportion of the substance reaching the liver after resorption in the gut – where it is then converted into reactive metabolites – is probably the decisive factor.

3.1.3.2.3 Detoxification

The metabolism of 1,2-unsaturated PAs and their *N*-oxides takes place primarily in the liver, and includes metabolic pathways that involve toxification as well as detoxification (Mattocks 1982; Wiedenfeld *et al.* 2008). The extent of toxicity for individual 1,2-unsaturated PAs is therefore likely to be strongly dependent on the (quantitative) dominance of the toxification or detoxification pathway. One key detoxification reaction is the hydrolysis of the esters at positions C7 and C9, as mediated by the carboxylesterases (figure 2). The metabolites released by this cleavage, namely necine bases and necic acids, are highly soluble in water and are rapidly excreted; they therefore appear to have no high toxicological relevance (Fu *et al.*

2004; Allgaier & Franz 2015). The susceptibility of the esters to hydrolysis is therefore particularly relevant for the toxic potential of various 1,2-unsaturated PAs. Due to steric hindrance, esters with complex branched necic acids are hydrolysed more slowly: as a result, these compounds can in principle exhibit greater levels of toxicity (Mattocks 1982; Wiedenfeld *et al.* 2008). In principle, the *N*-oxidation mediated by cytochrome P450 monooxygenases (CYPs) and/or flavin-dependent monooxygenases (FMOs) is another metabolic pathway leading to detoxification. Due to structural requirements, however, this is possible only for the 1,2-unsaturated PAs of the retronecine and heliotridine types (figure 2). Just as with the metabolites mentioned above, the *N*-oxides are readily soluble in water and are typically excreted rapidly. It should be noted, however, that *N*-oxides ingested orally can again be converted into the reduced – and therefore toxic – form of the alkaloids by reductases in the gut and/or the liver (Allgaier & Franz 2015; Yang *et al.* 2017; Yang *et al.* 2019a). Alongside these metabolic pathways, other potential routes of detoxification are also conceivable. The glucuronidation of 1,2-unsaturated PAs has also been observed *in vitro*, for example (He *et al.* 2010).

3.1.3.2.4 Formation of reactive metabolites

The 1,2-unsaturated PAs are protoxins: the toxic effects are not mediated by the parent substances themselves but by reactive metabolites that can be formed during metabolism.

The toxification of 1,2-unsaturated PAs is also catalysed by enzymes of the cytochrome P450 system. Ebmeyer et al. observed for example that the 1,2-unsaturated PA lasiocarpine caused a rise in the micronuclei rate in a V79 cell line expressing human CYP3A4, whereas this genotoxic effect had not been observed in the CYP-deficient parent cell line (Ebmeyer *et al.* 2019). On the basis of *in vitro* investigations with human Supersomes™, it can be deduced that CYP3A4, CYP3A5 and CYP2A6 are the enzymes primarily involved in humans, among other CYP enzymes involved (Ruan *et al.* 2014). With PAs of the retronecine and heliotridine types, these CYP enzymes mediate a hydroxylation of the necine base at position C3 or C8. Since the hemiaminal structure formed is relatively unstable, the spontaneous cleavage of water may occur, resulting in the formation of an aromatic pyrrol system. In the otonecine type PA, toxification results from spontaneous rearrangement reactions following CYP-mediated cleavage of the methyl group at C4. The alpha carbon atoms in the aromatic pyrrol system become ‘activated’ by the aromatic system: as a result, the spontaneous cleavage of the necic acids at C7 or C9 can lead to the formation of highly reactive carbocations at this position. These carbocations are strong alkylating agents that can covalently bind to nucleophilic structures of various biomolecules. The formation of protein and DNA adducts may therefore occur. As a result of the bifunctionality of many 1,2-unsaturated PAs, the cross-linking of these structures (protein-DNA cross-links, DNA-DNA cross-links) is also possible (figure 2) (Fu 2017).

Alternatively, the reactive pyrrol esters can also be hydrolysed into racemic 6,7-dihydro-7-hydroxy-1-hydroxymethyl-5H-pyrrolizine (DHP) by spontaneous reaction with water (figure 2). While this is less reactive compared with the esters, it still possesses alkylating properties (Fu *et al.* 2004; Xia *et al.* 2018). In addition, the reactive metabolites can also bind to reduced glutathione (GSH) (figure 2) (Xia *et al.* 2015; Chen *et al.* 2016). The reaction with GSH was long considered to be a detoxifying mechanism. However, recent research indicates that the GSH derivatives formed, together with other potential pyrrol metabolites, also exhibit a certain degree of reactivity. Using the human cell line HepG2 as a model, Xia et al. were able to demonstrate that characteristic DNA adducts could also be generated by various pyrrol metabolites, for example (including 7,9-di-valine-DHP, 7-valine-DHP, 7-GSH-DHP, 7-cysteine-DHP, dehydroretronecine) (Xia *et al.* 2018).

A recent cell culture experiment by He et al. has also shown that, in the presence of the potential metabolites 7-GSH-DHP, 7-cysteine-DHP and 7,9-cysteine-DHP, and in the presence of dehydromonocrotaline and dehydroriddelliine, the formation of characteristic DNA adducts occurs in the human bronchoalveolar cell line A549: due to the lack of metabolic capacity in this cell line these DNA adducts were not formed by the parent substances riddelliine and monocrotaline (He *et al.* 2019). Such metabolites, which have higher stability and improved solubility in water compared with the pyrrol esters primarily formed, could therefore represent a kind of transport form and accordingly (at least partially) explain the toxicity observed for 1,2-unsaturated PAs in extra-hepatic tissues (Xia *et al.* 2018). Alternatively, it also appears possible that the metabolic activation of 1,2-unsaturated PAs also takes place to a certain degree in certain kinds of extra-hepatic tissues, which would also contribute to the toxicity in these tissues.

The saturated PAs of the platynecine type, however, for which no toxic potential or at most a low toxic potential is postulated, exhibit a quite different kind of metabolism. Ruan et al. have observed *in vitro* for example that, following the incubation of platynecine-type PAs in the presence of S9 from rat liver as a metabolic activation system, no reactive pyrrol esters are formed. While a pronounced CYP-catalysed conversion occur, this instead led to the formation of stable metabolites (Ruan *et al.* 2013).

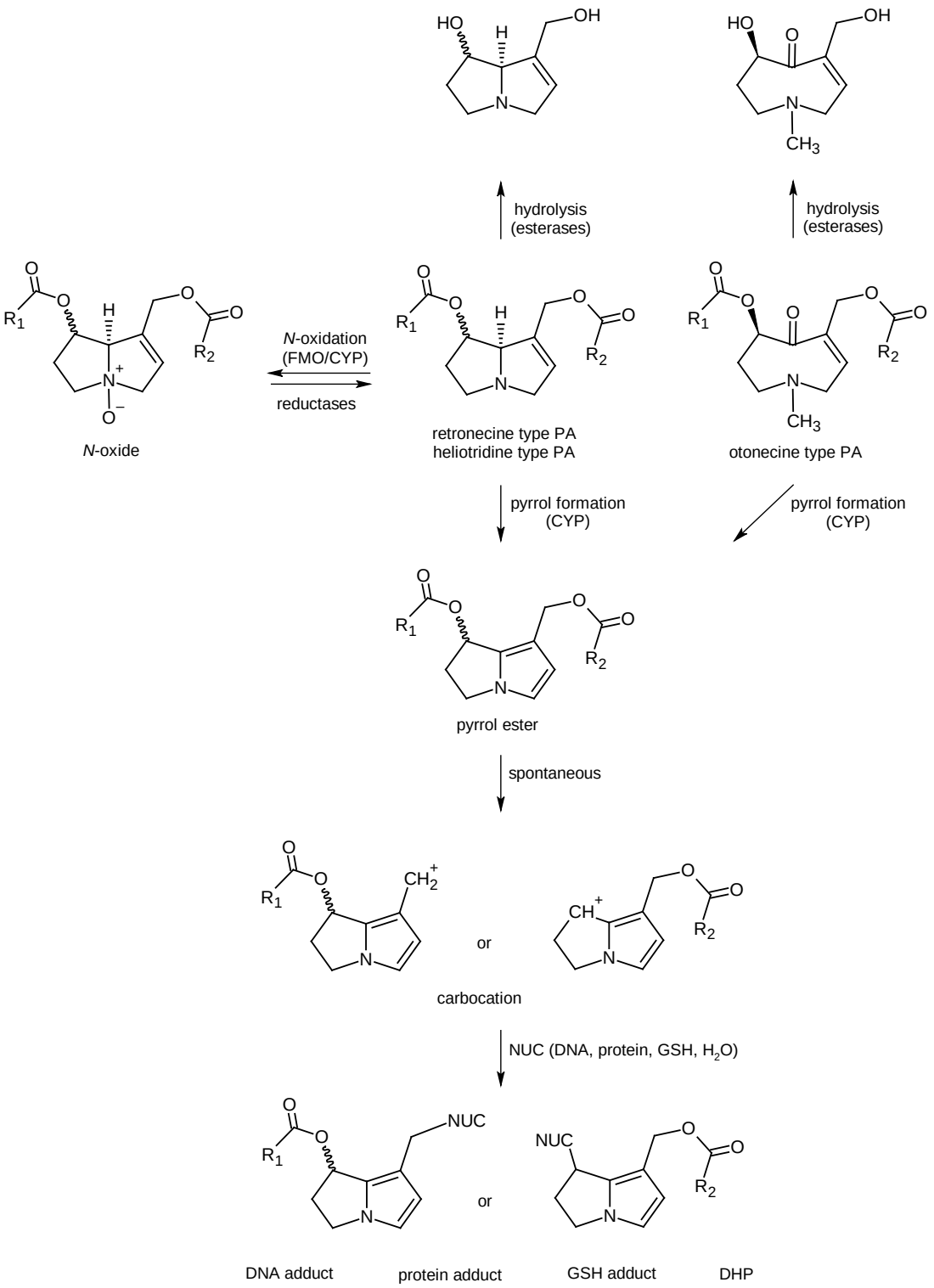

Figure 2: Important metabolic pathways for 1,2-unsaturated PAs. R_1 and R_2: necic acids; FMO: flavin-dependent monooxygenases; GSH: glutathione; DHP: (±)-6,7-dihydro-7-hydroxy-1-hydroxymethyl-5H-pyrrolizine, NUC: nucleophilic structures, such as DNA or proteins.

3.1.3.3 Transferability of findings from animal experiments to humans

3.1.3.3.1 Interspecies differences in terms of susceptibility

Differences between various species in terms of their sensitivity to the toxic effects of 1,2-unsaturated PAs are well known – especially with respect to the non-carcinogenic effects. Chickens, ducks, horses, cattle, pigs, rats and mice are assumed to be much more susceptible than sheep, goats, rabbits and guinea pigs, for example. Differences in terms of sensitivity may also exist between various animal strains and even between the sexes within the same strain (Fu *et al.* 2004).

Species-dependent susceptibility is considered to result from differences in the balance of detoxifying and toxifying metabolic pathways (Fu *et al.* 2004). As a result of the complexities found in the metabolism of 1,2-unsaturated PAs, differences of paramount importance for toxicity can occur in various metabolic steps, including:

- Hydrolysis of (pyrrol) esters by esterases
- *N*-oxidation by CYP and FMO
- Formation of the reactive pyrrol system by CYP
- Binding of pyrrol metabolites to GSH by glutathione *S*-transferases (GSTs)

In addition, gastrointestinal metabolism could also be a co-determinant of toxicity, especially in ruminants (Fu *et al.* 2004).

As a result, the impact of metabolism on the species-dependent toxicity of 1,2-unsaturated PAs is difficult to investigate experimentally. In a recent *in vitro* experiment, for example, Kolrep et al. observed that various 1,2-unsaturated PAs were particularly well converted in the presence of S9 liver preparations of species considered to be less sensitive (Kolrep *et al.* 2018). Since this study only investigated the decrease in the parent substance but not the formation of the metabolites relevant for toxicity, however, these findings do not allow for a comparable statements to be made about the relevant formation of the reactive pyrrol esters in various species. This becomes clear by the findings from a study conducted by Geburek et al. In this study, metabolic experiments with liver microsomes were used to demonstrate that a decrease in the parent substance does not necessarily correlate with the formation of reactive metabolites, which were indirectly determined in this study by measuring the formation of glutathione adducts (Geburek *et al.* 2020).

In order to obtain information on the sensitivity of different species, it therefore seems more favourable to determine the formation of the reactive pyrrole metabolites, if necessary indirectly.. Thus, Lin et al. were able to determine differences in species sensitivity by measuring the formation of reactive pyrrole metabolites. It was shown that the level of tissue-bound pyrrol derivatives was considerably higher in the presence of microsomes from rat liver than in the presence of liver microsomes from guinea pigs, which are considered less sensitive (Lin *et al.* 2002). These results are consistent with those from White et al., who showed that the administration of retrorsine in rats also led to a significantly higher hepatic pyrrol protein adduct level when compared with guinea pigs (White *et al.* 1973). These interspecies differences presumably result from the high esterase-mediated hydrolysis rate of 1,2-unsaturated PAs in guinea pigs. *In vitro* experiments have shown that, in the presence of

guinea pig liver microsomes, the 1,2-unsaturated PAs monocrotaline and clivorine are primarily hydrolysed via esterases and therefore detoxified. In contrast, the same studies showed that these compounds were primarily converted into reactive pyrrol metabolites in the presence of rat liver microsomes (Dueker *et al.* 1992; Lin *et al.* 2002). A recent *in vitro* study from Fashe et al. has also shown that greater amounts of reactive pyrrol metabolites ((3*H*-pyrrolizidine-7-yl)methanol and mono-GSH pyrrol adducts) were formed in the presence of liver microsomes from species known to be susceptible than in the presence of liver microsomes from less sensitive species (Fashe *et al.* 2015).

3.1.3.3.2 Transferability to humans

Against the background of the species differences described above, the question of the transferability of data from animal studies to humans arises. While the occurrence of non-carcinogenic effects following short- or medium-term intake of higher doses of 1,2-unsaturated PAs is also well documented in humans by numerous case reports (Dai *et al.* 2007; Kakar *et al.* 2010; Molyneux *et al.* 2011; Ma *et al.* 2018; Zhuge *et al.* 2019), epidemiological studies that could provide clarification about the potential carcinogenic potential in humans are missing. Nonetheless, available data indicate that the genotoxic and carcinogenic potential of 1,2-unsaturated PAs observed in animal studies is in principle also relevant for humans.

Since both the non-carcinogenic and genotoxic-carcinogenic effects of 1,2-unsaturated PAs can be attributed to the formation of the reactive pyrrol esters (Fu 2017; Ma *et al.* 2018), the occurrence of non-carcinogenic damage that has been observed in humans is strong evidence for concluding that the conversion of 1,2-unsaturated PAs into the toxic pyrrol esters takes place in humans – at least on a qualitative basis. These conclusions are further supported by the fact that multiple studies have now shown that characteristic pyrrol protein adducts are also detectable in the blood of patients with severe PA-induced liver damage (Lin *et al.* 2011; Gao *et al.* 2015; Ruan *et al.* 2015; Ma *et al.* 2018). These can be recognised as biomarkers for systemic exposure to toxic pyrrol metabolites. A study in rats has also demonstrated that the level of PA-induced pyrrol protein adducts and the level of DNA adducts thought to be responsible for the genotoxic-carcinogenic effects are generally correlated with one another (Xia *et al.* 2016). Findings from an *in vitro* study performed by Xia et al. also showed that the conversion of riddelliine into DHP and riddelliine-*N*-oxide occurred to a similar extent in the presence of liver microsomes from humans and rats. When bovine thymus DNA was added as a nucleophile to the preparations, formation of the characteristic DNA adducts also occurred to a comparable degree (Xia *et al.* 2003). Data from another recently published study by Geburek et al., which investigated the metabolic activation of reactive metabolites using liver microsomes from humans and rats, also indicate that bioactivation occurs to a similar extent (Geburek *et al.* 2020).

Overall, the findings indicate that the reactive pyrrol esters are also formed in humans in relevant quantities, and that the results both for hepatotoxic effects and for genotoxic-carcinogenic effects are transferable from rat studies to humans.

A study recently performed by Ning et al., which is based on PBK modelling (physiologically-based kinetic modelling), further indicates that, when compared with rats, humans could actually be more susceptible to the hepatotoxic effects of 1,2-unsaturated PAs (Ning *et al.* 2019). However, a number of assumptions are made in this study that could not be conclusively evaluated so far.

3.1.3.4 Carcinogenic potency of various 1,2-unsaturated PAs

As already discussed, the formation of reactive pyrrol esters – more specifically, the damage caused by these to cellular structures – is considered responsible for the toxicity of 1,2-unsaturated PAs (Fu 2017; Ma *et al.* 2018). In general, conversion into reactive pyrrol metabolites appears possible for all 1,2-unsaturated PAs; for some derivatives it has already been experimentally proven (Xia *et al.* 2013). Since the toxicokinetics of individual compounds can differ depending on their respective structures, however, it can be assumed that this may have an impact on the potency of different derivatives of the 1,2-unsaturated PA and that different 1,2-unsaturated PA therefore vary in their toxic potency.. Such differences have already been demonstrated experimentally for different derivatives with regard to different endpoints.

Nevertheless, risk assessments conducted to date have considered the individual 1,2-unsaturated PAs in terms of their toxic potency as a group of equipotent substances with cumulative effects. Recently, however, discussions have started to focus on approaches more capable of accounting for the differing potency of individual 1,2-unsaturated PAs and their *N*-oxides in the future. A comprehensive review of this topic has been published by Merz and Schrenk, for example (Merz & Schrenk 2016).

To date, various sets of data are available that supply information about the varying toxic potency of various 1,2-unsaturated PAs. The available data comprise the following endpoints in particular:

- LD_{50} values in rodents (i.p.-application, i.v.-application)
- Primary DNA damage, e.g. DNA adducts, DNA cross-links, DNA strand-breaks (*in vitro* and some *in vivo*)
- Chromosome damage, e.g. micronuclei formation, chromosomal aberrations (*in vitro*)
- Mutagenicity, e.g. in bacteria and in *Drosophila*
- Cytotoxicity in cell culture (*in vitro*)

Based on this, for example Merz and Schrenk propose so-called "interim relative potency" factors for 1.2-unsaturated PA and its *N*-oxides based on data on acute toxicity in rodents, cytotoxicity in cell culture and genotoxicity in the fruit fly *Drosophila* (1.0 for cyclic and open-chain diesters with 7S configuration, 0.3 for monoesters with 7S-configuration, 0.1 for open-chain diesters with 7R-configuration and 0.01 for monoesters with 7R-configuration, *N*-oxides are treated as the parent substance), which could be considered in the risk assessment (Merz & Schrenk 2016). In contrast, Chen et al. have derived interim relative potency factors based on carcinogenicity data for only a few 1,2-unsaturated PAs (Chen *et al.* 2017). In addition, Allemang et al. investigated the genotoxicity of various 1,2-unsaturated PAs in a micronuclei test using the human HepaRG cell line and have proposed these findings as a potential basis for comparing potency (Allemang *et al.* 2018). In contrast, Louisse et al. investigated the intrinsic genotoxic potential of 37 individual PAswith HepaRG cells using the γH2AX test (Louisse *et al.* 2019), while Lester et al. studied the intrinsic potency of various 1,2-unsaturated PAs by determining DNA adducts formation *in vitro* in a metabolically competent rat liver cell sandwich model (Lester *et al.* 2019). An investigation of the cytotoxic potential of various 1,2-unsaturated PAs on primary rat hepatocytes *in vitro* has also been published recently (Gao *et al.* 2020).

Research findings have also shown, for example, that *N*-oxides typically exhibit lower potency when compared with the parent compounds. However, the differences observed

varied in relation to the specific test system used (Xia *et al.* 2013; Field *et al.* 2015; Merz & Schrenk 2016; He *et al.* 2017; Yang *et al.* 2017). In addition, *N*-oxides could also be converted by bacterial reductases in the gut or by hepatic reductases into their reduced and therefore more toxic forms (Allgaier & Franz 2015; Yang *et al.* 2017; Yang *et al.* 2019a). For conservative considerations, the same toxic potency is usually still considered for the *N*-oxides as for the parent compounds.

The BfR comes to the conclusion that the potency factors derived on the basis of the currently available data cannot yet be usefully applied for the assessment of possible health risks from exposure to 1,2-unsaturated PA in food for various scientific reasons. In particular, the potency factors proposed to date do not permit any reliable conclusions to be drawn about the genotoxic-carcinogenic potency of various derivatives after oral ingestion *in vivo*. This conclusion is in line with the conclusions drawn by EFSA (EFSA 2017b). Overall, against the background of the various uncertainties in the different areas (toxicology, analytics, exposure assessment), the consideration of potency factors derived from the current state of knowledge would imply a level of precision that has in fact not been archived, yet.

3.1.4 Analytical determination of levels of 1,2-unsaturated PAs in food

The European Commission and member states are currently discussing the introduction of maximum levels for the occurrence of 1,2-unsaturated PAs in certain foods, such as tea and herbal tea products, food supplements, pollen/pollen products and certain spices. Depending on the food group concerned, these maximum levels under discussion correspond to the 80th to 98th percentile of current concentrations occurring in food (EFSA 2017b) and are not directly derived using toxicological methods. The maximum level must refer to a clearly defined spectrum of individual analytes and has to be determined as a so called lower bound-level. Accordingly, individual analytes not determined in the sample – i.e. whose concentrations are below the limit of quantification – are included in the sum calculation with the numerical value of "zero".

The biodiversity of plants that are capable of forming PAs is very high. To date, these compounds have been detected in over 600 different plant species, most of which are members of the Asteraceae, Boraginaceae and Fabaceae families. This high degree of biological variability is expressed in a large number of biosynthesised structures, with the resulting PA compounds identified numbering in the hundreds (Wiedenfeld *et al.* 2008). When estimating the exposure of consumers to 1,2-unsaturated PAs, their analytical determination should ideally be quantitative in nature. To this end, two separate methodological approaches have been pursued in recent years:

- In **sum parameter analysis**, chemical modifications are used to convert the individual PAs into their common core structure, which is then quantified as a final step.

- In **targeted analysis**, the concentrations of individual toxins are quantified and these concentrations are then summed up. This is generally possible with restriction to marker substances for which reference standards are available.

3.1.4.1 Sum parameter analysis

Sum parameter analysis for the quantification of 1,2-unsaturated PAs aims at converting the many naturally-occurring individual compounds into their few common necine core structures (see figure 1). The number of compounds to be analysed and the required reference substances is thus drastically reduced and the total content of 1,2-unsaturated PA is quantified in form of the core structure equivalent. The accuracy of the levels determined with this method is affected by the fact that all PA structures must be quantitatively converted into the core structure without reaction-mediated decomposition taking place. Chemical conversion is not possible for the 1,2-unsaturated PAs of the otonecine type. Accordingly, derivatives of this structural type cannot be determined with this method.

In sum parameter analysis, any individual PAs that are present in the *N*-oxide form must first be reduced to their corresponding tertiary bases by means of reduction with zinc dust. After enrichment and purification using solid-phase extraction, all of the PAs present in the sample are converted in a second reduction step to the necine base core structure that is the underlying form for all PAs. $LiAlH_4$ in tetrahydrofuran is frequently used for the reduction of the ester compound (Kempf *et al.* 2008). Instead of the two-stage reduction, optimised methods also use a direct conversion of the *N*-oxides into the core structure (Cramer *et al.* 2013). Following derivatisation, the retronecine or heliotridine core structures are generally measured using mass spectrometry, which can be performed equally well in combination with a liquid chromatography separation system or with a gas chromatography separation system (Kempf

et al. 2008; Cramer *et al.* 2013). One key challenge for the routine suitability of this method is the necessity of an internal standard for the quantification and correction of the various losses taking place during processing. An isotope-labelled retronecine derivative is currently used, which must be synthesised internally in the lab.

3.1.4.2 Targeted analysis

During targeted analysis, the concentrations of individual compounds are quantified and subsequently summed up. These compounds are structurally well-known and are generally also available as reference standards. The most widespread method currently used for the analysis of undesirable substances in food is liquid chromatography in combination with mass spectrometry (LC-MS/MS). For this method, tandem mass spectrometers (also known as triple quadrupoles) are primarily used in selected/multiple reaction monitoring (SRM, MRM) mode. This type of detection offers high sensitivity combined with very high specificity. This is achieved by a double focusing of the mass, whereby a precursor ion of a certain mass is selectively filtered in the first quadrupole of the tandem mass spectrometer. In the case of 1,2-unsaturated PAs, this is typically the protonated molecule ion formed in the positive ionisation mode. Only this precursor ion can pass through the first quadrupole. This ion is then fragmented to generate substance-specific product ions that are detected in the second mass spectrometer. In general, an analyte is considered to be uniquely identified in a sample if the chromatographic retention time and at least two mass conversions of the precursor ion into product ions are detected within a defined intensity ratio and also comply with the reference ('target') substance (SANTE 2017). Mass spectrometers whose selectivity is achieved by their high resolution are also increasingly used for the analysis of 1,2-unsaturated PAs at the moment. The high-resolution full-scan data thereby produced offer in particular the option of identifying an effectively unlimited number of analytes in one measurement. Identification criteria are described in documents such as the 'Guidance document on analytical quality control and method validation procedures for pesticide residues and analysis in food and feed' (SANTE 2017). Accordingly, an analyte is considered identified if, in addition to retention time, the accurate masses of at least two ions match up with a mass accuracy of at least 5 ppm.

3.1.4.3 Analyte selection

The targeted analysis method is typically used in the context of current food monitoring work. With this method, it is not possible to determine the sum level of 1,2-unsaturated PAs as a sum of all naturally-occurring individual compounds. Rather, only those compounds are included in the quantification for which targeted analysis is carried out. The specific analytes included in the quantification must be specified precisely for the future monitoring of maximum levels, to ensure uniform enforcement of these levels during monitoring. The analytes summarised in table 1 represent the current consensus for the methodological spectrum of analytes within Europe. At the point in time when the methods were established, this selection of analytes was primarily based on the availability of reference standards. When in 2013 the responsible monitoring authorities became aware of the problem of PA-forming plants as a source of contamination in the production of plant-based foods, 17 analytes were in the analyte spectrum of the methods. In the following year, the methods were extended to 28 1,2-unsaturated PAs. This analyte selection was used as the basis for determining the levels of 1,2-unsaturated PAs in many foods, both in Germany and other EU member states. To enable comparability between the older and more recent occurrence data, established methods were no extended by reference standards that became available at a later date. The choice not to extend the methods' analyte spectrum, although a much higher number of 1,2-unsaturated PAs are formed in the environment, can also be understood by considering the fact that

the sum level in PA-forming plant species is represented by just a few primary compounds ('marker substances') (Mädge 2020). This is also reflected in the contamination profile for foods. An evaluation of occurrence data from many samples has shown that certain 1,2-unsaturated PAs do not occur in amounts with quantitative relevance. In practical terms, this means that extending the methods by additional compounds would increase analytical effort without leading to any relevant increase in the quantified 'sum level' of 1,2-unsaturated PAs.

Against this background, the BfR recommended reducing the analyte spectrum used in the methods from 28 to 21 analytes in 2015 (BfR 2015). This is now in line with the current EFSA recommendation that initially recommended the analysis of 17 compounds (figure 3).

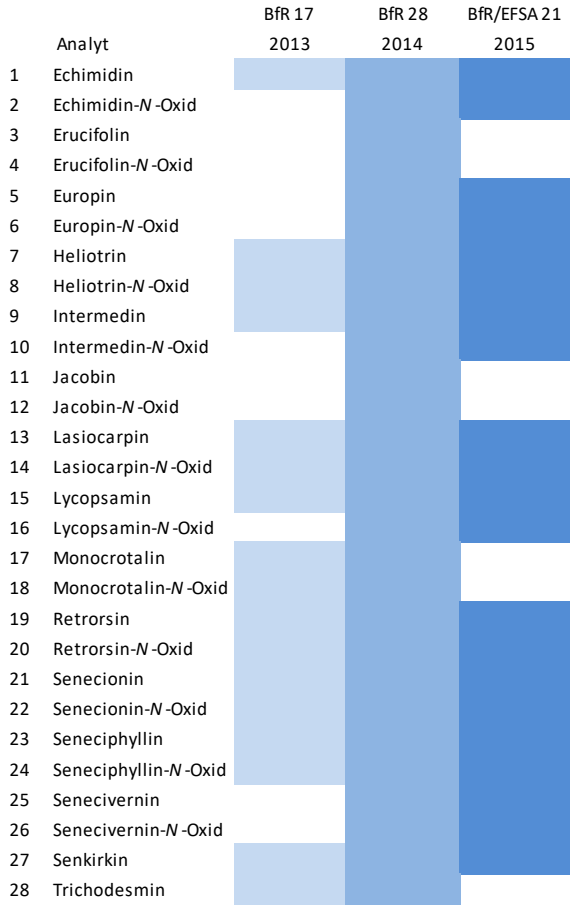

Figure 3: Developments over time in the specification of the analyte spectrum for determining the total level of 1,2-unsaturated PAs.

Table 1: Analyte selection currently recommended by the BfR for monitoring the maximum levels discussed for the occurrence of 1,2-unsaturated PAs in food as a sum of individual levels (BfR 2016a).

PA [abbreviation]	Type of ester form	Necine base	Naturally-occurring isomers
Echimidine [Em]	Open-chain diester	Retronecine	Heliosupine [Hs]
Echimidine *N*-oxide [EmN]	Open-chain diester	Retronecine	Heliosupine-NO [HsN]
Europine [Eu]	Monoester	Heliotridine	
Europine *N*-oxide [EuN]	Monoester	Heliotridine	
Heliotrine [Ht]	Monoester	Heliotridine	
Heliotrine *N*-oxide [HtN]	Monoester	Heliotridine	
Intermedine [Im]	Monoester	Retronecine	Lycopsamine [Ly], indicine [Id], echinatine [En], rinderine [Rn]
Intermedine *N*-oxide [ImN]	Monoester	Retronecine	Lycopsamine-NO [LyN], indicine-NO [IdN], echinatine-NO [EnN], rinderine-NO [RnN]
Lycopsamine [Ly]	Monoester	Retronecine	Intermedine [Im], indicine [Id], echinatine [En], rinderine [Rn]
Lycopsamine *N*-oxide [LyN]	Monoester	Retronecine	Intermedine-NO [ImN], indicine-NO [IdN], echinatine-NO [EnN], rinderine-NO [RnN]
Lasiocarpine [Lc]	Open-chain diester	Heliotridine	
Lasiocarpine *N*-oxide [LcN]	Open-chain diester	Heliotridine	
Retrorsine [Re]	Cyclic diester	Retronecine	Usaramine [Us]
Retrorsine *N*-oxide [ReN]	Cyclic diester	Retronecine	Usaramine-NO [UsN]
Senecionine [Sc]	Cyclic diester	Retronecine	Senecivernine [Sv], integerrimine [Ig]
Senecionine *N*-oxide [ScN]	Cyclic diester	Retronecine	Senecivernine-NO [SvN], integerrimine-NO [IgN]
Senecivernine [Sv]	Cyclic diester	Retronecine	Senecionine [Sc], integerrimine [Ig]
Senecivernine *N*-oxide [SvN]	Cyclic diester	Retronecine	Senecionine-NO [ScN], integerrimine-NO [IgN]
Seneciphylline [Sp]	Cyclic diester	Retronecine	Spartioidine [St]
Seneciphylline *N*-oxide [SpN]	Cyclic diester	Retronecine	Spartioidine-NO [StN]
Senkirkine [Sk]	Cyclic diester	Otonecine	

A special situation arises for PAs that occur as isomers. These isomers are virtually impossible to distinguish using LC-MS/MS since they have similar chromatographic retention times (co-elution) and in mass spectrometry form identical precursor and product ions, whose intensity distribution is also often very similar. Isomers are very hard to distinguish with the methods currently being used and conclusions about the exact pattern of isomers in a sample cannot be made with absolute clarity. Table 1 presents the most important naturally-occurring isomers for the respective 1,2-unsaturated PAs. As an example, the structure of 1,2-unsaturated PAs in the intermedine group has 4 carbon atoms at which stereocentres could be present: theoretically, the occurrence of 16 separate stereoisomers would therefore be possible (see figure 4). Five isomers have been described in the literature to date. Both the Asteraceae (predominantly the genus *Eupatorium*) and Boraginaceae plant families (almost all genera) are capable of forming these isomers: accordingly, all five of these isomers are relevant for the monitoring of contamination in food. The analyte spectrum currently recommended is based primarily on the reference standards available at the date the methods were established. In relation to the compounds presented in figure 4, this includes lycopsamine and intermedine. Since the established methods were not further extended to include indicine, echinatine or rinderine, and the co-eluting isomers are not clearly distinguishable by analysis, echinatine and rinderine levels in samples contaminated with *Eupatorium* have been reported as lycopsamine and intermedine. As regards the perception and assessment of the frequency of individual PA compounds, this implies that lycopsamine and intermedine are frequently reported while their isomers are not.

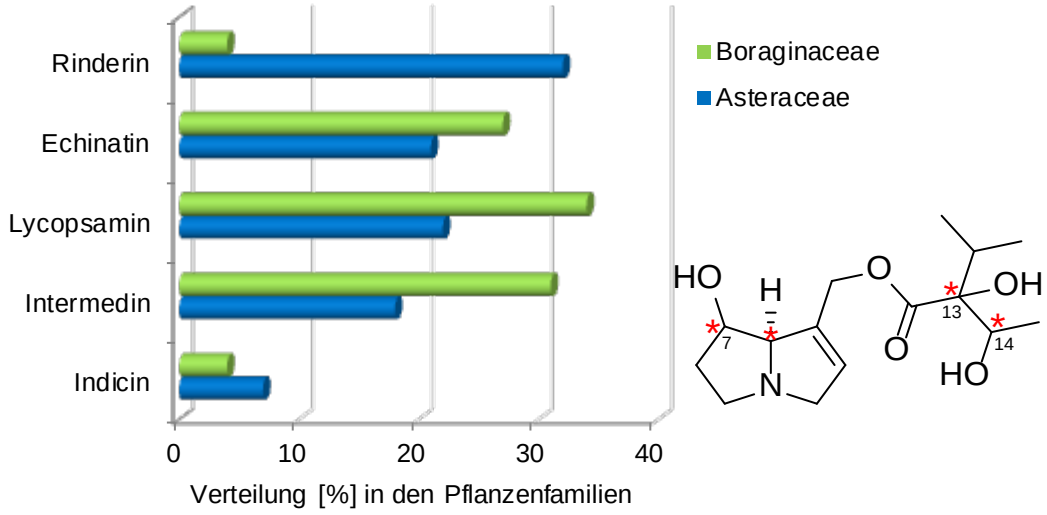

Figure 4: Naturally-occurring isomers in the intermedine group and their distribution in the Asteraceae (top) and Boraginaceae (bottom) families (Hartmann & Witte 1995; Dubecke et al. 2011).

For the reasons explained here, a pragmatic approach to specifying the analyte spectrum for monitoring the levels of 1,2-unsaturated PAs would be to retain the 21 analytes from table 1 previously selected while including the isomers in the analyte spectrum. This extended analyte spectrum is in line with analytical practice as applied to date and would ensure comparability between occurrence data already obtained. From an analytical perspective, this would be the most practicable way of ensuring uniform enforcement within monitoring: if maximum levels were exceeded, the laboratories would not have to prove the absence of isomers not named in the analyte spectrum with a great effort and possibly with legally contestable results.

3.1.5 Exposure assessment

3.1.5.1 Data on the occurrence of 1,2-unsaturated PAs in food

For an overall assessment of the intake of 1,2-unsaturated PAs in food, the BfR has used occurrence data provided by the German Federal Office of Consumer Protection and Food Safety (BVL). These data originate from the food monitoring programmes of the German states and it is assumed that these data therefore represent the levels in food as available on the market. Since the BVL has not supplied data for milk, however, occurrence data from EFSA were used here.

In an earlier BfR opinion published in 2016, entitled 'Pyrrolizidine alkaloids: Levels in foods should continue to be kept as low as possible' (BfR opinion no. 030/2016, dated 28 September 2016), occurrence data from the period 2011 to 2015 were used. The present overall assessment is an update to this document. The occurrence data considered by this opinion therefore follow on directly from the earlier data set and cover the period from 2015 to mid-2019. For the following exposure assessment, the samples drawn as samples due to complaints, samples from the national residue control plan (NRP) or import samples, suspect or follow-up samples, and other samples subject to other sampling and reporting reasons were excluded from the submitted data set. When considering the analytical results for individual analytes, measured values below the limits of detection/quantification (LOD/LOQ) were adjusted using the modified lower-bound approach. For this purpose, values below the LOD were set to zero and those below the LOQ were set to the LOD.

The sum of 21 analytes was calculated (table 1) for each individual sample. It should be noted that some of the individual analytes already reflect the sum of multiple individual isomers. In the subsequent evaluation, only those samples were included from the BVL data for which analytical results were available for all 21 analytes. For the rationale here, see also section 3.1.4.3.

Occurrence data were available for the following foods, which were grouped together appropriately: honey, various types of tea, mixtures of tea-like products, herbs/spices, flour, flower pollen, food supplements, rocket and spinach; note that the concentrations for tea and tea-like products do not relate to the infusion but to the tea as dry matter. For milk, occurrence data from EFSA (Mulder et al. 2015) was used, as in the earlier opinion published in 2016. This includes data on 182 samples, for which 35 individual analytes had been measured between January 2014 and April 2015. Of these, sum concentrations in 171 samples were below the LOD. Table 2 lists the data on levels of 1,2-unsaturated PAs (sum of 21 analytes, including co-eluting isomers) for these various food groups, together with the corresponding statistical key figures.

Table 2: Data used for the exposure assessment on the levels of 1,2-unsaturated PAs (sum of 21 analytes, including co-eluting isomers) using the modified lower-bound approach.

Food	N[1]	<LOD [2] [%]	Mean [µg/kg]	Median [µg/kg]	P95[3] [µg/kg]
Honey	244	56	3.0	0	17.6
Nettle tea	6	17	57.0	23.7	230.5
Fennel tea	101	57	6.6	0	22.2
Fruit tea	24	75	1.5	0	4.9
Green tea	98	64	8.0	0	37.1
Camomile tea	84	32	29.3	8.7	119.8
Herbal tea (nfs[4])	229	27	97.4	15.0	409.7
Peppermint tea	122	53	33.5	0	105.3
Rooibos tea	175	11	107.1	38.0	493.5
Black tea	111	45	11.9	1.0	65.1
Iced tea	13	92	0.1	0	1.0
Mixtures of tea-like products	69	38	29.3	2.5	133.9
Tea products for babies and infants	28	43	37.0	0.9	91.7
Spices	327	34	2,905.7	53.3	10,871.1
Flour	19	95	0.04	0	0.74
Milk[5]	182	94	0.01	0	0.04
Flower pollen	18	6	720.7	124.5	5,083.1
Food supplements	61	44	39.3	0.3	124.7
Rocket	17	76	9,790.7	0	166,384.0
Spinach	30	50	2.4	0.1	9.7

[1]N = number of samples (occurrence data from German state food monitoring programmes, 2015 to mid-2019); [2] LOD = limit of detection;; [3]P95 = 95th percentile; [4]nfs = not further specified; [5]EFSA concentration data (Mulder *et al.* 2015)

The food groups of rocket, herbs/spices, flower pollen, rooibos tea and herbal tea (nfs[2]) have the highest average levels. For nettle tea, iced tea, flour, flower pollen and rocket, less than 20 samples are available. As a result, the 95th percentile is the same as the maximum and is therefore subject to high resultant uncertainties.

The comparison of the occurrence data from 2015 to 2019 used in the present opinion with the data gathered from 2011 to 2015 used in opinion no. 030/2016 of 28 September 2016, as shown in Table 3, shows that both the average levels and the levels in the 95th percentile have been reduced, in some cases considerably. Particularly noteworthy is the reduction of the average levels by a factor of more than ten for green tea, peppermint tea and black tea. In chamomile tea, herbal tea, and rooibos tea a decrease by more than a factor of two can be observed. One exception here is herbs/spices, which have considerably higher average levels, although the 95th percentile is also higher for herbs/spices in comparison with the other food groups considered. A comparison of both data sets shows that the number of samples for herbs/spices has increased from 40 to 327, whereby borage was not represented at all in the previous dataset and the number of samples for oregano, lovage, and

[2]nfs: not further specified, i.e. herbal tea with no details of the types of herbs used

spice mixtures as herbs/spices with a large proportion of high levels has increased considerably. Due to the major differences between the data sets it is not possible to conclude from the currently higher contents observed how the contents in the herbs/spices group have developed over the last few years. As regards tea products for babies and infants, it should also be noted that other products were sampled for the current data set than for the earlier opinion: the data sources are therefore essentially different. For this reason, the currently lower levels offer no means of determining the actual direction of the trend for this group in recent years.

The methodological differences in selecting the samples being included in the data analysis should be noted, however. In opinion no. 030/2016, all samples were included in which at least 17 of the 1,2-unsaturated PAs had been investigated; the analytes did not necessarily need to be the same ones. For the current opinion, samples were included only if they had analytical results for all 21 of the individual analytes listed in table 1.

Table 3: Comparative overview of the current levels of 1,2-unsaturated PAs (sum of 21 analytes including co-eluting isomers) with the levels of those PAs included in opinion no. 030/2016, dated 28 Sep 2016 (sum of at least 17 arbitrary 1,2-unsaturated PAs), in µg/kg.

Food group	Current levels			Levels in 030/2016		
	N[1]	Mean	95th percentile	N[2]	Mean	95th percentile
Honey	244	3.0	17.6	129	11.0	40.2
Nettle tea	6	57.0	230.5	27	272.3	857.1
Fennel tea	101	6.6	22.2	44	53.4	222.0
Fruit tea	24	1.5	4.9	14	1.8	7.6
Green tea	98	8.0	37.1	26	423.0	1,471.5
Camomile tea	84	29.3	119.8	35	272.4	1,192.4
Herbal tea (nfs[3])	229	97.4	409.7	20	437.1	1,800.4
Peppermint tea	122	33.5	105.3	30	494.3	2,990.0
Rooibos tea	175	107.1	493.5	22	596.8	1,672.0
Black tea	111	11.9	65.1	33	571.2	3,620.4
Tea products for babies and infants	28	37.0	91.7	15	0.3	3.1
Spices	327	2,905.7	10,871.1	40	265.2	1,857.5

[1]N = number of samples (occurrence data from German state food monitoring programmes, 2015 to mid-2019);
[2]N = number of samples (occurrence data from BfR opinion no. 030/2016); [3]nfs = not further specified

3.1.5.2 Consumption data

As a data basis for the consumption of children under 5 years of age, consumption data from the VELS study was used (Heseker *et al.* 2003; Banasiak *et al.* 2005). This nationwide study was carried out from 2001 to 2002 in Germany, covering 816 infants and young children aged from 6 months to under 5 years old. The parents logged the food consumed by each child in two nutritional records kept over 3 consecutive days. Due to the presence of consumption data for individual days, the two 3-day nutritional protocols are suitable for use in exposure assessments considering both acute and chronic health risks. It should be noted, however, that the use of a few single day measurements for the calculation of a lifelong intake is associated with uncertainties that must be taken into account, especially when mak-

ing statements on detailed food groups or estimates with a high percentage of non-consumers. In the evaluation, short-term and long-term consumption data was considered for children no longer being breastfed. The exposure assessment was made based on occurrence data available for honey, fennel tea, fruit tea, green tea, camomile tea, herbal tea (nfs), peppermint tea, rooibos tea, iced tea, tea with juice[3], milk and spinach. As there were few consumers of nettle tea, these were considered collectively with consumers of herbal tea (nfs) and linked with the occurrence data for herbal tea (nfs) in the exposure assessment.

The data set for consumption by adolescents and adults was taken from the National Food Consumption Study II (NVS II) published by the Max Rubner Institute (MRI). NVS II is the current representative study for food consumption in the German population. The study, which surveyed about 20,000 individuals aged between 14 and 80 on their eating habits using three separate survey methods (*dietary history*, *24-hour recall* and *weighing protocol*), was conducted between 2005 and 2006 throughout Germany (Krems *et al.* 2006; MRI 2008). The analyses of consumption are based on the data from the two independent *24h-recalls* from NVS II, which were surveyed in a computer-aided interview using 'EPIC-SOFT'. Data was evaluated from 13,926 people for whom both interviews were available. Due to the presence of consumption data for individual days, the *24h-recall method* is suitable for use in exposure assessments considering both acute and chronic health risks. Intake estimates were based on the individual body weight of the respondents. The evaluation considered both the short-term (acute) and long-term (chronic) consumption of the foods listed in table 2. Within the NVS II *24h-recall* data, the consumption of nettle tea, fennel tea, camomile tea and peppermint tea was not mentioned explicitly. However, it is likely that the consumption quantities for these types of tea are included in those for herbal tea (nfs). Accordingly, the exposure assessment was based on consumption data for honey, fruit tea, green tea, herbal tea (nfs), rooibos tea, black tea, iced tea, milk and spinach. In addition, the intake of 1,2-unsaturated PAs via herbs/spices, borage/'Frankfurt green sauce', oregano, lovage, flat-leaf parsley, rocket, food supplements and flour was also considered in separate scenarios for adults.

As regards consumption data for rocket, a phone-based consumer survey conducted by the BfR in 2009 was used, since no information about the consumption of rocket was available from NVS II. In this survey, 1,002 persons aged 18 and above were asked about their consumption patterns relating to rocket and related dishes as well as food containing rocket by means of a standardised questionnaire. Data for average monthly consumption and quantities consumed the previous day were collected.

3.1.5.3 Exposure assessment for all food groups considered

The exposure assessment was completed using SPSS version 21 for the short- and long-term consumption of the corresponding food groups.

[3]High-street food retailers offer ready-to-drink beverages for children that may consist of 2/3 fruit tea and 1/3 apple juice, for example.

3.1.5.3.1 Short-term (acute) exposure

The estimation of the short-term intake of 1,2-unsaturated PAs is based on the 95th percentile of the individual highest quantity consumed on a single day over the entire survey period. This consumption quantity is multiplied by the 95th percentile of the PA level for the corresponding food. The calculations also assume that 200 ml of tea infusion corresponds to a dry mass of 2 g. Short-term intake is always related to an individual foodstuff and not – as is the case for the long-term analysis – partially resultant from the overall consumption of all foodstuffs involved.

Tables 4 and 5 present values for the short-term intake from the consumption of the foods under consideration for children and adults.

Table 4: Short-term intake of 1,2-unsaturated PAs in children (6 months to under 5 years of age).

Food group	Short-term intake of 1,2-unsaturated PAs[1] [µg/kg body weight/day]
Honey	0.026
Fennel tea	0.014
Fruit tea	0.003
Green tea	0.013
Camomile tea	0.077
Herbal tea (nfs[2])	0.164
Peppermint tea	0.036
Rooibos tea	0.137
Iced tea	<0.0005
Tea with juice	0.081
Milk[3]	0.002
Spinach	0.123

[1]Based on occurrence data from the German state food monitoring programmes (sum of 21 analytes, including co-eluting isomers; 2015 to mid-2019) and the VELS study; [2]nfs = not further specified; [3]EFSA occurrence data (Mulder *et al.* 2015)

Table 5: Short-term intake of 1,2-unsaturated PAs in adolescents and adults (14 to 80 years of age).

Food group	Short-term intake of 1,2-unsaturated PAs[1] [µg/kg body weight/day]
Honey	0.016
Fruit tea	0.001
Green tea	0.009
Herbal tea (nfs[2])	0.110
Rooibos tea	0.129
Black tea	0.013
Iced tea	<0.0005
Milk[3]	<0.0005
Spinach	0.025

[1]Based on occurrence data from the German state food monitoring programmes (sum of 21 analytes, including co-eluting isomers; 2015 to mid-2019) and NVS II; [2]nfs = not further specified; [3]EFSA occurrence data (Mulder *et al.* 2015)

For all food groups considered, the short-term intake for both children and adults is less than 0.2 µg/kg body weight/day (tables 4 and 5). In children, the highest short-term intake of 1,2-unsaturated PAs results from the consumption of herbal tea (0.164 µg/kg body weight/day), followed by rooibos tea (0.137 µg/kg body weight/day) and spinach (0.123 µg/kg body weight/day). In adults, the highest short-term intake of 1,2-unsaturated PAs results from the consumption of rooibos tea (0.129 µg/kg body weight/day), followed by herbal tea (nfs) with 0.110 µg/kg body weight/day.

3.1.5.3.2 Long-term (chronic) exposure

To estimate long-term exposure, the average PA levels were linked with the quantities consumed by children and adults for all respondents at the individual level and the resulting intake quantities were then calculated. Then, for each food group, the median (normal consumers) and the 95th percentile (high consumers) of intake quantities were calculated for 1,2-unsaturated PAs (tables 6 and 7). These calculations assume that 200 ml of tea infusion corresponds to a dry mass of 2 g. Average concentrations were used in order to properly account for the fact that the various types within each food group are contaminated to a varying extent.

The intake quantities for 1,2-unsaturated PAs are listed in table 6 for children and in table 7 for adults, on the basis of all respondents (95th percentile) and on the basis of the consumers (median and 95th percentile) for the individual food groups. Actual consumers vary widely as a proportion of the total number of respondents, both between the individual food groups themselves, and between children and adults.

Intake of 1,2-unsaturated PAs via individual food groups

For children, the proportion of consumers for the individual food groups lies between 1% for green tea and 37% for fruit tea. If all respondents are considered, this produces a calculated median of zero. One exception is milk with a consumer proportion of 80%.

Table 6: Average intake[1] (monthly average) of 1,2-unsaturated PAs via various food groups in µg/kg body weight/day in children (6 months to under 5 years).

Food group	Consumer proportion [%]	All respondents	Consumers only	
		95th percentile[3]	Median[2]	95th percentile[3]
Honey	26	0.001	<0.0005	0.001
Fennel tea	16	0.001	<0.0005	0.002
Fruit tea	37	<0.0005	<0.0005	<0.0005
Green tea	1	0	<0.0005	<0.0005
Camomile tea	4	0	0.001	0.006
Herbal tea (nfs[4])	9	0.003	0.003	0.018
Peppermint tea	9	0.001	0.001	0.004
Rooibos tea	4	0	0.002	0.014
Iced tea	4	0	<0.0005	<0.0005
Tea with juice	6	0.001	0.002	0.021
Milk[5]	80	<0.0005	<0.0005	<0.0005
Spinach	19	0.004	0.002	0.007

[1]Based on occurrence data from the German state food monitoring programmes (sum of 21 analytes, including co-eluting isomers; 2015 to mid-2019) and the VELS study; [2]Median = 0 if consumer proportion <50%; [3]95th percentile = 0 if consumer proportion <5%; [4]nfs = not further specified; [5]EFSA occurrence data (Mulder *et al.* 2015)

Looking at the results for children (consumers only) shown in table 6, the highest level of exposure to 1,2-unsaturated PAs is seen to arise from the consumption of herbal tea (nfs), rooibos tea, spinach and tea with juice. The consumer proportions for these groups range from 4% to 19%.

For the adults from NVS II, a consumer proportion between 4% for rooibos tea and 22% for herbal tea (nfs) can be documented. As with the data for children, this produces a median of zero when all respondents are considered. In adults, milk again forms an exception, with a consumer proportion of 55%.

Table 7: Average intake[1] (monthly average) of 1,2-unsaturated PAs via various food groups in µg/kg body weight/day in adolescents and adults (14 to 80 years of age).

Food group	Consumer proportion [%]	All respondents	Consumers only	
		95th percentile[2]	Median[2]	95th percentile[3]
Honey	17	0.001	0.001	0.002
Fruit tea	10	<0.0005	<0.0005	<0.0005
Green tea	6	<0.0005	<0.0005	0.001
Herbal tea (nfs[4])	22	0.009	0.004	0.019
Rooibos tea	4	0	0.005	0.017
Black tea	13	0.001	<0.0005	0.002
Iced tea	4	0	<0.0005	<0.0005
Milk[5]	55	<0.0005	<0.0005	<0.0005
Spinach	5	0	0.002	0.003

[1]Based on occurrence data from the German state food monitoring programmes (sum of 21 analytes, including co-eluting isomers; 2015 to mid-2019) and the NVS II study; [2]Median = 0 if consumer proportion <50%; [3]95th percentile = 0 if consumer proportion <5%; [4]nfs = not further specified; [5]EFSA occurrence data (Mulder *et al.* 2015)

Looking at the individual food groups for adults (consumers), the consumption of herbal tea (nfs) and rooibos tea leads to the highest level of long-term intake of 1,2-unsaturated PAs (table 7). The consumer proportion for these two food groups lies between 4% and 22%.

Overall intake of 1,2-unsaturated PAs by the consumption of the food groups considered, on a consumer basis

If only the consumers of the various food groups are considered, it becomes clear that the numbers of consumers vary widely as a proportion of the total respondents. If overall intake were to be calculated by assuming that consumers are those individuals who have consumed at least one product from the selected food groups, then food with a high consumer proportion but having low levels of 1,2-unsaturated PAs (e.g. milk) would lead to an underestimation of the intake for consumers of food with high levels where the consumer proportion in these cases was comparatively low. This is particularly problematic because the consumer proportions in consumption studies based on only a few days are systematically underestimated. In several scenarios, the consumers of the food groups with the highest median intake values have therefore been selected as the base population and overall intake has then been calculated for this consumer group. For children, there are two scenarios with a base population of consumers of herbal tea (nfs) and rooibos tea (table 8). For adults, the two scenarios with a base population of consumers of herbal tea (nfs) and rooibos tea (table 9) are also considered.

Table 8: Average overall intake in children[1] (monthly average) of 1,2-unsaturated PAs via the food groups considered (basis: only consumers of a group).

Basis		Overall intake [µg/kg body weight/day]
Consumers of herbal tea (nfs[2])	Consumers (%)	9
	Median	0.005
	95th percentile	0.018
Consumers of Rooibos tea	Consumers (%)	4
	Median	0.004
	95th percentile	0.021

[1]6 months to under 5 years; [2]nfs = not further specified

Table 9: Average overall intake in adolescents and adults[1] (monthly average) of 1,2-unsaturated PAs via the food groups considered (basis: only consumers of a group).

Basis		Overall intake [µg/kg body weight/day]
Consumers of herbal tea (nfs[2])	Consumers (%)	22
	Median	0.005
	95th percentile	0.020
Consumers of Rooibos tea	Consumers (%)	4
	Median	0.006
	95th percentile	0.023

[1]14 to 80 years of age; [2]nfs = not further specified

3.1.5.3 Foodstuffs not included in estimates of the overall intake

In addition to the food groups considered above, the following sections discuss a number of foodstuffs that could not be included in the exposure assessments presented above for a variety of reasons. These include foods that are not reliably represented in consumption studies for methodological reasons, but which may be conspicuous by heavy contamination with PA-forming plants and can therefore make a relevant contribution to the overall intake of 1,2 unsaturated PA.

Herbs and spices

The analysis presented in this opinion includes occurrence data for 327 samples of herbs/spices. These data were taken from the data set provided by the BVL (period 2015 to 2019). Occurrence levels here are spread over a range from <LOD to 248,061 µg/kg. Table 10 presents these levels, grouped into low, medium, high and very high values.

Table 10: Distribution of levels for 1,2-unsaturated PAs (sum of 21 analytes, including co-eluting isomers) in herbs/spices over various concentration ranges.

Concentration range [µg/kg]	Number of samples	Frequency [%]
Low (<LOD to 500)	218	67
Average (500 to 2,000)	44	13
High (2,000 to 4,000)	25	8
Very high (>4,000)	40	12
Total	327	100

Looking at the overall occurrence data for herbs/spices reveals that 34% of the 327 samples have levels below the LOD. The median sample has a value of 53 µg/kg, the average is 2,906 µg/kg and the 95th percentile is 10,871 µg/kg. The high and very high levels were found in samples of the leafy herbs borage, oregano and lovage, as well as in mixed spices. A very high level of 9,565 µg/kg was found in 1 of 21 samples of thyme, although the next-highest level had a value of 382 µg/kg, which corresponds to the 95th percentile. Another very high level was observed in a sample of cumin, although data was only available for a single sample in this case. The occurrence data used for the following modelled exposure assessment are oriented on the average values of the concentration ranges presented in table 10. In the medium and high ranges, the average and the median for the concentrations measured are generally similar. For the very high concentration range, the average is considerably higher, but, as a result of the sample number and the greater robustness of the median versus outliers, the median is used for orientation here. In the group of herbs with rather lower levels, the average is used since the median is below the limit of quantification and would therefore be 0. Since this is a rough estimate, the respective values are rounded according to table 11.

The intake estimate for long-term exposure is analysed by applying the cumulative consumption for herbs (without spices) from the *24-recalls* for normal and high consumers. For normal consumers, the intake of 1,2-unsaturated PAs is calculated from the product of the median and the average value for consumption with the concentrations applied; for high consumers, the product of the 95th percentile of the consumption and the concentrations is used.

Herbs/spices have been presented separately in the present overall assessment and have not been included in the estimate of overall intake performed in section 3.1.5.3.2. One reason for this is the fact that the herbs/spices consumption data set includes several uncertainties. Often, consumption is not adequately documented in consumption studies and underestimation can therefore be assumed. Second, no consumption data were available for typical herbs/spices such as pepper, for example. Analyses nonetheless indicate that herbs/spices, despite being consumed in principle in small quantities, can contribute considerably to the intake of 1,2-unsaturated PAs. This was the reason for calculating this rough estimate.

Table 11: Long-term intake of 1,2-unsaturated PAs via consumption of herbs/spices by adolescents and adults (14 to 80 years of age).

	Quantity of herbs consumed [g/kg body weight/day]	Concentration assumed [µg/kg]	Average level [µg/kg]	Median level [µg/kg]	Intake [µg/kg body weight/day]
Adult normal consumers (median)	0.011	50 (low)	47	0	0.001
	0.011	1,000 (medium)	1,093	1,101	0.011
	0.011	3,000 (high)	3,044	3,077	0.033
	0.011	10,000 (very high)	20,394	9,737	0.110
Adult high consumers (95th percentile)	0.087	50 (low)	47	0	0.004
	0.087	1,000 (medium)	1,093	1,101	0.087
	0.087	3,000 (high)	3,044	3,077	0.261
	0.087	10,000 (very high)	20,394	9,737	0.870

In contrast to the estimate for long-term exposure, the estimate for short-term intake is not based on the consumption quantities from the *24h-recalls* from NVS II. Instead, consumption quantities were defined using selected recipes in which certain herbs/spices are typically used. Normally, a calculation of acute exposure at the individual level will take the maximum of the two consumption days from the *24-recalls* and then determine the 95th percentile of consumption quantities for consumers. The cumulative consumption quantities that were used to estimate long-term exposure, for example, would reflect the overall consumption of herbs/spices. However, taking into account an entire group is an unusual approach when estimating acute exposure. This would result in a scenario where all herbs/spices consumed in one day are assumed to be highly exposed. This assumption would be very unrealistic. Furthermore, a representation of individual cases could not be provided. In addition, even by aggregating the consumption quantities for herbs/spices overall, this would not permit conclusions to be drawn about consumption quantities for e.g. borage, as borage is not explicitly named or referenced in the consumption surveys.

In light of these facts, scenarios have been assumed for estimating acute exposure whereby the intake of 1,2-unsaturated PAs is estimated from the consumption of certain dishes containing herbs. Due to their high levels, the herbs borage, oregano, lovage, and flat-leaf parsley (dried) were included in these scenarios. The selection of recipes for dishes was made using a consumer survey conducted to ascertain the sources of information most often consulted for recipes. For these recipes, the quantities for the herbs used were taken from relevant cookbooks and from online recipes; the plausibility of these quantities was then checked by professional chefs. In doubtful cases, a conservative approach was adopted and the higher quantity therefore applied for a recommended herb.

Scenario 1: Borage

Source: https://www.chefkoch.de/rezepte/1021661207383908/Frankfurter-Gruene-Sosse.html, most clicks and best rating)

Recipe: 'Frankfurt Green Sauce'
The typical mix of herbs used consists of 25 g chives, 25 g parsley, 25 g chervil, 25 g cress, 25 g sorrel, 25 g borage, 10 g estragon, 10 g dill, 10 g savory.

To estimate short-term exposure, consumption of 25 g borage and 155 g of other herbs is assumed as a general summary of all of the ingredients in this herb mixture with the exclusion of borage. An analysis of the levels in individual herbs is not possible due to missing or inadequate sample numbers.

Scenario 2: Oregano

Source: Schulkochbuch Jubiläumsausgabe, Dr. Oetker, (Oetker 2011)

Recipe: 'Tomatensauce'
1 tbsp chopped oregano

To estimate short-term exposure, consumption of 2 g oregano is assumed.

Scenario 3: Lovage and flat-leaf parsley

Source: Schulkochbuch Jubiläumsausgabe, Dr. Oetker, (Oetker 2011)

Recipe: 'Pichelsteiner-Eintopf'
Lovage, destemmed
2 tbsp chopped parsley

To estimate short-term exposure, consumption of 2 g lovage and 4 g dried parsley is assumed (conservative approach: higher concentrations are found in dried parsley).

The consumption quantities considered for the estimate of acute exposure result from the quantities as presented above, based on a standard body weight of 70 kg for an adult (EFSA 2012). In order to take into account the fact that a meal with the amount of herbs used as shown above will result in about three servings, and that only a portion is consumed in one meal or spread over several meals, for the acute estimate it is assumes that a person will consume only a third of the total amount of the recipe, i.e. a single serving.

Table 12: Short-term exposure to 1,2-unsaturated PAs resulting from the consumption of dishes with herbs.

		Quantity of herbs consumed [g/kg body weight/day]	Level (P95) [µg/kg]	Intake [µg/kg body weight/day]
Scenario 1	Borage Other herbs	0.119 0.738	248,061 155[1]	29.53 0.11
Scenario 2	Oregano	0.010	18,772	0.18
Scenario 3	Lovage + Flat-leaf parsley	0.029	7,487	0.08

[1]Average concentration in other herbs

From the occurrence data provided by the BVL, it is not possible to determine whether the levels measured relate to dried or fresh herbs. For Scenario 1, assuming that the occurrence data relate to fresh herbs, the consumption of 'Frankfurt Green Sauce' would accordingly result in an intake of 29.64 µg/kg body weight/day (table 12). If the occurrence data relate instead to dried herbs, this would result in an intake quantity of around 8 µg/kg body weight/day, assuming a drying factor of 4. In Scenario 2, the consumption of a tomato sauce spiced with oregano would lead to a potential intake of 0.18 µg/kg body weight/day. In Scenario 3, the consumption of 'Pichelsteiner-Eintopf' would lead to a potential intake of 0.08 µg/kg body weight/day.

Rocket

As described in section 3.1.5.2, the consumption data for rocket is based on data collected in a phone-based consumer survey conducted by the BfR, since no information on the consumption of rocket is available from the NVS II study. Of these roughly 1,000 respondents, 40% were consumers of rocket. Of the 17 samples of rocket, 4 had values higher than the limit of detection (0.28 µg/kg, 26.5 µg/kg, 31.9 µg/kg and 166,384 µg/kg). The average level (*lower bound*) for PAs was 9,790 µg/kg and the 95th percentile (and maximum) was 166,384 µg/kg.

When addressing short-term consumption, the resulting intake for adults is 175.2 µg/kg body weight/day, when considering levels at the 95th percentile/maximum . If considered without the maximum value, the PA intake from rocket would be below 0.0005 µg/kg bw/day. For average long-term consumption, the intake estimate for adults for 1,2-unsaturated PAs would be an intake of 0.34 µg/kg body weight/day. If calculated while excluding the maximum value, this would result in an intake of less than 0.0005 µg/kg body weight/day.

Food supplements

High levels of 1,2-unsaturated PAs in food supplements had already been noted in an earlier opinion. The highest levels had been found in botanical food supplements using material taken from plants that form PAs (BfR 2016b).

In the current data supplied by BVL, occurrence data for 61 food supplements were analysed. Concentrations were available for food supplements (not further specified), royal jelly

preparations and plant extracts of secondary plant metabolites as well as preparations of vitamins, pollen, minerals and milk thistle oil. No PAs were detected in the oil-based preparations. Intake quantities for 1,2-unsaturated PAs could not be estimated, mainly due to a lack of data regarding the recommended daily intake as recommended by the manufacturers.

Flour

For the food group of flour, 19 samples were evaluated from various flours (spelt, rye and wheat). Values for 18 samples were below the LOD and were therefore considered to be zero. The only measurable concentration was found in a sample of rye flour. As a result of the low sample number, no statistical evaluation was possible for individual types of flour. Due to the diversity of foods in which flours of different types are used, a contribution of flour to the overall intake of 1,2 unsaturated PA appears possible.. Accordingly, it is important to collect reliable data on levels of 1,2-unsaturated PAs in flour in the future. A monitoring project is therefore planned for 2020. Participating laboratories will be asked to test flour not only for tropane alkaloids, as currently required by law, but also for its levels of 1,2-unsaturated PAs.

Other food groups not considered for exposure

In the exposure assessment, the food groups of flower pollen and mixtures of tea-like products were not considered, since no consumption data was available for these groups. An assignment to specific food groups was also not possible for a further 41 samples: as a result, no linking to consumption data was therefore performed.

In the BfR opinion published in 2016, the following food groups were also investigated, in which no 1,2-unsaturated PAs were found: yoghurt (27 samples), cheese (Gouda/Emmental, Brie/Camembert) (34 samples), infant formula (milk powder 0–6 months) (8 samples), infant formula (milk powder 6–36 months) (17 samples), beef (80 samples), pork (79 samples), poultry (83 samples), beef liver (11 samples), pork liver (10 samples), chicken liver (10 samples). In addition, 205 samples of eggs were also investigated, of which all but two samples contained no 1,2-unsaturated PAs. Eight samples of mustard were available, whose PA levels were all below the limit of quantification (BfR 2016b). These foods were not included in the exposure assessment conducted as part of the current opinion.

3.1.6 Risk characterisation

3.1.6.1 Assessment approach of the BfR

From a toxicological point of view, according to the current state of knowledge, no intake levels up to which health risks can be excluded with sufficient certainty can be derived for compounds being both genotoxic and carcinogenic. Accordingly, the genotoxic-carcinogenic effects are the most sensitive endpoint and the primary focus for the risk assessment of 1,2-unsaturated PAs.

The *margin of exposure* (MOE) approach is used in the European Union in order to prioritise the urgency of risk management measures. The MOE is the ratio calculated from a suitable toxicological reference point and the exposure to the substance in humans. For carcinogenic compounds, the $BMDL_{10}$ is usually taken as the reference point. The $BMDL_{10}$ is derived by modelling suitable data on the dose-effect relationship and corresponds to the lower bound of the confidence interval for the dose that, in the case of 1,2-unsaturated PAs, is associated with an additional cancer risk of 10% (*benchmark dose 10%,* BMD_{10}) versus the control group in animal studies. For genotoxic carcinogens, a MOE of 10,000 or higher is generally considered to be of low concern from a public health point of view, although this does not equal no concern. Accordingly, such compounds are assigned a low priority for risk management measures. However, the final decision which MOE is of concern in an individual situation and from which MOE measures should be initiated is primarily a risk management decision ((EFSA 2005).

For the risk assessment for 1,2-unsaturated PAs, the chronic toxicity studies with lasiocarpine (NCI 1978) and riddelliine (NTP 2003) described in more detail in section 3.1.3.1.2 were applied as the data basis to represent all other derivatives. In both studies, the occurrence of hepatic hemangiosarcomas in rats was identified as the most sensitive endpoint. In the present risk assessment, the BfR's calculation of MOE values is based on the $BMDL_{10}$ of 237 µg/kg body weight/day as a reference point, as derived in 2017 by EFSA on the basis of the riddelliine study (EFSA 2017b).

As has already been described in section 3.1.3.4, discussions have recently started to focus on approaches more capable of accounting for the varying carcinogenic potency of individual 1,2-unsaturated PAs and their *N*-oxides. In the BfR's opinion, however, the potency factors derived on the basis of the currently available data cannot yet be applied in order to achieve a reliable risk assessment. Accordingly, this risk assessment continues to consider the various 1,2-unsaturated PAs to be equipotent.

Regarding the non-carcinogenic risks posed by 1,2-unsaturated PAs, various case reports of poisonings in humans after ingestion of PA-containing plant material are available, as discussed in section 3.1.3.1.1. The two relatively well-documented poisoning cases in children suggest that daily intake quantities of around 1–3 mg/kg body weight can lead to severe liver damage or even prove fatal after only a few days. This dose range accordingly represents intake quantities at which the occurrence of severe effects must be expected. An intake quantity at or below which toxic effects following the short-term intake of 1,2-unsaturated PAs are no longer to be expected cannot be derived on the basis of this data, however, due to a lack of knowledge regarding the dose-effect relation. In older expert opinions published by the WHO, it was assumed that an intake of 1,2-unsaturated PAs as low as 10 µg/kg body weight/day would cause sickness in humans (IPCS/INCHEM 1988). The data set on which this statement is based is subject to serious uncertainties, however. Overall, it is therefore

not possible – even taking into account more recent case reports and newer assessment approaches – to reliably determine the intake levels at which the occurrence of non-carcinogenic effects must be expected in humans after short, medium or long-term exposure .

However, in assessments published by several scientific bodies, the occurrence of hepatocyte enlargements, which was for instance observed in the chronic study with riddelliin in rats, was considered a relevant endpoint for the assessment of non-carcinogenic effects. A NOAEL of 10 µg/kg body weight/day was identified here. While applying an extrapolation factor of 100 to account for inter- and intra-species differences, an intake quantity of 0.1 µg/kg body weight/day has been derived: at lower intake quantities, non-carcinogenic effects from 1,2-unsaturated PAs are no longer to be expected (COT 2008; BfR 2016b). In the BfR's view, this value can be applied provisionally in justified individual cases for the evaluation of non-carcinogenic risks. Accordingly, corresponding effects would not be expected to occur following an exposure to 1,2-unsaturated PAs of up to 0.1 µg/kg body weight/day. Since this value has been derived based on data from a chronic study, it is to be assumed that the value can be viewed as protective both in terms of the occurrence of non-carcinogenic effects following long-term and following short- and medium-term consumption. The application of this orientation value in the evaluation of potential effects following short- and medium-term exposure is to be considered conservative.

Overall, it should be noted that a comprehensive risk assessment must always focus on the genotoxic-carcinogenic effects, since these are the most sensitive endpoint.

3.1.6.2 Assessment of health risks following short-term intake of 1,2-unsaturated PAs

In the present overall assessment, it was possible to conduct a reliable estimate for children in the age group 6 months to under 5 years regarding short-term exposure to 1,2-unsaturated PAs by the food groups of honey, fennel tea, fruit tea, green tea, camomile tea, herbal tea (nfs), peppermint tea, rooibos tea, iced tea, tea with juice, milk and spinach. For adults, it was possible to conduct this estimate for short-term exposure by the food groups of honey, fruit tea, green tea, herbal tea (nfs), rooibos tea, black tea, iced tea, milk and spinach.

In children (consumers only), the highest exposure to 1,2-unsaturated PAs results from the consumption of herbal tea (0.164 µg/kg body weight/day), followed by rooibos tea (0.137 µg/kg body weight/day) and spinach (0.123 µg/kg body weight/day). In adults (consumers only), the highest short-term intake of 1,2-unsaturated PAs results from the consumption of rooibos tea (0.129 µg/kg body weight/day), followed by herbal tea (nfs) with 0.110 µg/kg body weight/day.

In justifiable individual cases, the BfR has provisionally used an orientation value for the evaluation of non-carcinogenic risks. Accordingly, corresponding effects would not be expected to occur following an exposure to 1,2-unsaturated PAs of up to 0.1 µg/kg body weight/day. Since this value has been derived based on data from a chronic study, it is to be assumed that the value can be viewed as protective both in terms of the occurrence of non-carcinogenic effects following long-term and following short- and medium-term consumption. The short-term intake via individual food groups identified here is slightly higher than the orientation value that was provisionally considered. This orientation value should be seen as conservative, however, since its derivation was based on a chronic study.

For all other food groups considered, the estimated intake quantities were below – sometimes clearly below – the orientation value of 0.1 µg/kg body weight/day.

3.1.6.3 Assessment of health risks following long-term intake of 1,2-unsaturated PAs via all food groups considered

In the exposure assessment, first of all, the intake quantities via the individual food groups considered were estimated (children: honey, fennel tea, fruit tea, green tea, camomile tea, herbal tea (nfs), peppermint tea, rooibos tea, iced tea, tea with juice, milk and spinach; adults: honey, fruit tea, green tea, herbal tea (nfs), rooibos tea, black tea, iced tea, milk and spinach). In children (high consumers), the highest exposure was from the consumption of herbal tea (nfs), rooibos tea and tea with juice; in adults (high consumers), the highest intake quantities were from consumption of herbal tea (nfs) and rooibos tea. The intake quantities via individual food groups were all below 0.0237 µg/kg body weight/day in each case and therefore equated to MOE values of over 10,000.

In order to evaluate the potential risks to health following long-term exposure to 1,2-unsaturated PAs, however, the overall intake across all relevant food groups must be considered. In the current assessment, the consumers of the food groups with the highest median intake values were therefore selected as the base population and the overall intake was then calculated for each of these consumer groups, calculating multiple scenarios. For children, there are two scenarios with a base population of consumers of herbal tea (nfs) and rooibos tea. For adults, the two scenarios with a base population of consumers of herbal tea (nfs) and rooibos tea are considered.

Table 13: Average overall intake (monthly average) of 1,2-unsaturated PAs across the food groups considered and the resulting MOE values, in children[1] (basis: only consumers of a group).

Basis		**Overall intake [µg/kg body weight/day]**	**MOE[2]**
Consumers of herbal tea (nfs[3])	Consumers (%)	9	
	Median	0.005	**47,365**
	95th percentile	0.018	**13,388**
Consumers of Rooibos tea	Consumers (%)	4	
	Median	0.004	**59,643**
	95th percentile	0.021	**11,275**

[1]6 months to under 5 years; [2]$BMDL_{10}$ = 237 µg/kg body weight/day (EFSA 2017b), calculation from actual intake (not rounded); [3]nfs = not further specified

Table 14: Average overall intake (monthly average) of 1,2-unsaturated PAs across the food groups considered and the resulting MOE values, in adolescents and adults[1] (basis: only consumers of a group).

Basis		Overall intake [µg/kg body weight/day]	MOE[2]
Consumers of herbal tea (nfs[3])	Consumers (%)	22	
	Median	0.005	**47,612**
	95th percentile	0.020	**11,623**
Consumers of Rooibos tea	Consumers (%)	4	
	Median	0.006	**37,436**
	95th percentile	0.023	**10,098**

[1]14 to 80 years of age; [2]$BMDL_{10}$ = 237 µg/d per kg body weight (EFSA 2017b), calculation from actual intake (not rounded); [3]nfs = not further specified

The estimated chronic overall exposure across all food groups considered leads to intake quantities for children and adults in the scenarios considered that result in MOE values of over 10,000, both for normal consumers and for high consumers. As a result, the occurrence of health risks caused by the overall exposure to 1,2-unsaturated PAs calculated in this way can be considered to be of low probability. When interpreting these results, one must take into account the fact that the MOE values determined for high consumers lie only slightly above 10,000. Consumers are also exposed to 1,2-unsaturated PAs from other food groups, which cannot yet be considered in the estimate of the overall intake as presented here. This exposure from other food groups could lead to a further decrease in the MOE values.

3.1.6.4 Foodstuffs not included in estimates of overall intake

Herbs and spices

For herbs and spices, a reliable exposure assessment is not possible, due to the lack of valid data about the consumption quantities of individual herbs/spices. The potential health risks following long-term consumption of contaminated products should therefore be estimated on the basis of model scenarios. To this end, the intake quantities that could be derived from the long-term consumption of herbs/spices with low, medium, high and very high concentrations are compared to the $BMDL_{10}$ of 237 µg/kg body weight/day, and MOE values were calculated.

Table 15: MOE values that could result from long-term exposure to 1,2-unsaturated PAs via herbs/spices in adolescents and adults (14 to 80 years of age).

	Quantity of herbs consumed [g/kg body weight/day]	Category	Level Mean [µg/kg]	Median [µg/kg]	Intake [µg/kg body weight/day]	MOE[1]
Adult normal consumers (median)	0.011	50 (low)	47	0	0.001	**430,909**
	0.011	1,000 (medium)	1,093	1,101	0.011	**21,545**
	0.011	3,000 (high)	3,044	3,077	0.033	**7,182**
	0.011	10,000 (very high)	20,394	9,737	0.110	**2,155**
Adult high consumers (95th percentile)	0.087	50 (low)	47	0	0.004	**54,483**
	0.087	1,000 (medium)	1,093	1,101	0.087	**2,724**
	0.087	3,000 (high)	3,044	3,077	0.261	**908**
	0.087	10,000 (very high)	20,394	9,737	0.870	**272**

[1]$BMDL_{10}$ = 237 µg/kg body weight/day (EFSA 2017b)

According to these model scenarios, the consumption of herbs/spices with low and medium levels (50 µg/kg and 1,000 µg/kg) results in a MOE values above 10,000 for normal adult consumers. An MOE lower than 10,000 would only result, if herbs with high levels (3,000 µg/kg) were consumed. For high consumers of herbs/spices, however, an MOE much lower than 10,000 would already result from the sole consumption of herbs/spices with medium levels (1,000 µg/kg). In interpreting these findings, it should be noted that these MOE values result from the intake of 1,2-unsaturated PAs via the sole consumption of herbs/spices: in reality, a multitude of foodstuffs contribute to a consumer's overall exposure. It should also be noted that the true consumption quantities for individual herbs are largely unknown.

To estimate the potential risks to human health following short-term consumption of herbs/spices contaminated with 1,2-unsaturated PAs, the exposure levels estimated by means of model dishes in section 3.1.5.4 were then compared with the orientation value of 0.1 µg/kg body weight/day provisionally used for the assessment of the non-carcinogenic effects.

Table 16: Short-term exposure to 1,2-unsaturated PAs from the consumption of dishes with herbs/spices, with full utilisation of the orientation value of 0,1 µg/kg body weight/day (EF) provisionally used for the assessment of non-carcinogenic effects.

		Quantity consumed (herbs) [g/kg body weight/day]	Level (P95) [µg/kg]	Intake [µg/kg body weight/day]	EF[1]
Scenario 1	Borage Other herbs	0.119 0.738	248,061 155	29.53 0.11	**296**
Scenario 2	Oregano	0.010	18,772	0.18	**1.8**
Scenario 3	Lovage + parsley[2]	0.029	7,487	0.08	**0.8**

[1]EF: exceedance factor; [2]Parsley (flat-leaf)

An analysis of the model dishes shows that, in cases where highly contaminated herbs are used for food production, even the consumption of a single portion can lead to intake quantities of 1,2-unsaturated PAs that exceed the provisionally used orientation value of 0.1 µg/kg body weight/day. Since its derivation was based on a chronic study, this orientation value should be seen as conservative: accordingly, a slight short-term exceedance of this value is probably unproblematic. However, the exceedance by a factor of almost 300 observed in Scenario 1 does give particular cause for concern. Since these scenarios each relate to the consumption of a single portion, the intake of even higher quantities appears possible.

This preliminary estimate underlines the fact that, although consumption quantities for the herbs/spices food group are presumably small, their consumption could contribute considerably to both long- and short-term exposure to 1,2-unsaturated PAs. Also noteworthy is the fact that especially high concentrations have been observed in borage, oregano, and lovage in particular, and in mixed spices. These herbs are therefore an important source of exposure. On the other hand, both the exposure assessment for long-term exposure as well as the estimated potential short-term intake of 1,2-unsaturated PAs via herbs/spices achieved by using model dishes are subject to uncertainties. At the time of writing, reliable data are lacking in particular on both the long-term and short-term consumption of various herbs.

Rocket

Four of 17 samples of rocket had concentrations above the limit of detection (0.28 µg/kg, 26.5 µg/kg, 31.9 µg/kg and 166,384 µg/kg): the concentration in one of these four samples was 5,000 times higher than in the other three.

In accordance with the exposure assessment described in section 3.1.5.4, and considering the concentrations in the 95th percentile (also the maximum concentration), this would result in an intake of 1,2-unsaturated PAs for short-term consumption of 175,2 µg/kg body weight/day. This value would be over 1,750 times higher than the provisionally used orientation value of 0.1 µg/kg body weight/day. If calculated while excluding the maximum value, intake from the consumption of rocket would then be below 0.0005 µg/kg body weight/day.

For average long-term consumption, the intake estimate for adults for 1,2-unsaturated PAs would be an intake of 0.34 µg/kg body weight/day. The resulting MOE would be 692. If calculated while excluding the maximum value, this would result in an intake of less than 0.0005 µg/kg body weight/day, which would equal an MOE of over 474,000.

Overall, these findings indicate that the consumption of rocket typically does not result in the intake of an appreciable quantity of 1,2-unsaturated PAs. In individual cases – presumably the result of extensive contamination with parts of plants able to form PAs – rocket can contain very high concentrations, however. At intake quantities of more than 175 µg/kg body weight/day, impairments to health appear possible even after short-term consumption.

3.2 Evaluation of the quality of the available data

3.2.1 Evaluation of toxicological data

The available toxicological data on 1,2-unsaturated PAs are very extensive. In terms of the endpoint relevant for the risk assessment, namely the genotoxic-carcinogenic effects, there are clearly positive findings from animal studies in rodents for some derivatives of this substance group. Relevant data for humans (such as epidemiological studies or follow-up studies on poisoning cases) are lacking, however. Nonetheless, based on the available data on metabolism, genotoxicity and carcinogenicity, most scientific assessment bodies – including both the BfR and EFSA – assume that 1,2-unsaturated PAs also have a genotoxic-carcinogenic activity in humans. IARC has also classified various 1,2-unsaturated PAs in category 2B, i.e. as 'possibly carcinogenic to humans' (IARC 2019).

Regarding their genotoxic-carcinogenic activity, the individual 1,2-unsaturated PAs are currently considered as a group with cumulative effects and a carcinogenic potency equivalent to that of riddelliine. Uncertainties also result from the data from this study as well as the dose-effect modelling performed. These can lead to both an overestimation and underestimation of the risk. On the basis of various experimental data, it is also to be expected that carcinogenic potency will differ between individual 1,2-unsaturated PAs and their *N*-oxides. Recent discussions have therefore focused on approaches more capable of accounting for the differing carcinogenic potency observed for individual compounds. In the BfR's opinion, however, the potency factors proposed by a number of authors on the basis of the currently available data cannot yet be applied in order to achieve a reliable risk assessment, due to various uncertainties. Accordingly, this risk assessment continues to take a conservative approach and considers the various 1,2-unsaturated PAs to have equivalent potency. This approach probably leads to an overestimation of the risk. Overall, against the background of the various uncertainties in the different areas (toxicology, analytics, exposure assessment), the consideration of potency factors derived from the current state of knowledge would imply a level of precision that has in fact not been archived, yet.

Relevant data on acute and chronic (non-carcinogenic) toxicity are available from animal studies and numerous case reports of poisonings in humans. It can be considered as an established fact that 1,2-unsaturated PAs will lead to non-carcinogenic liver damage in both humans as well as in laboratory animals and livestock following acute exposure to high doses or following medium-/long-term exposure to lower doses. Other organs, particularly the lungs, can also be affected. However, the exact dose-effect relationship following short-, medium- and long-term exposure is also subject to some uncertainty. Two relatively well-documented poisoning cases in children suggest that daily intake quantities of around 1–3 mg/kg body weight can lead to severe liver damage or prove fatal after only a few days. This dose range accordingly represents intake quantities for which the occurrence of severe effects must be expected. This information can also be used as a component when evaluating acute effects – especially in cases where intake quantities of this magnitude have been reached. Due to a lack of knowledge regarding the dose-effect relationship, however, this cannot be used to estimate the intake quantity at which toxic effects are not to be expected following short-term intake of 1,2-unsaturated PAs. Thus, for 1,2-unsaturated PA, no sufficiently safe distance between lethal doses and a safe intake quantity can be scientifically justified at present.

For the evaluation of non-carcinogenic effects potentially occurring, the BfR therefore provisionally uses an orientation value of 0.1 µg/kg body weight/day in certain cases. This value

has been derived from an NOAEL of 10 µg/kg body weight/day (endpoint: hepatocyte enlargement) obtained from a chronic study in rats and the application of an extrapolation factor of 100, to account for intra- and interspecies variabilities. Accordingly, non-carcinogenic effects would not be expected to occur following an exposure to 1,2-unsaturated PAs of up to 0.1 µg/kg body weight/day. Since this value has been derived from a chronic study, the application of this orientation value in the evaluation of potential effects following short- and medium-term exposure is to be considered conservative. Its use is therefore likely to lead to an overestimation of the risk, but takes into account the uncertainties resulting from the data set.

A further amplification of the hepatotoxic effects of 1,2-unsaturated PAs can also result from interaction with other liver-toxic substances, such as certain mycotoxins or ethanol. An increase in effect-severity must also be considered in the event of existing liver damage – such as during or as a result of viral infections. However, these factors cannot be adequately considered at present and may therefore lead to an underestimation of the risk.

3.2.2 Evaluation of analytical data

The parameters in a method that are to be validated and the performance criteria to be thereby achieved are described in a number of regulations, standards and guidelines. The 'Guidance document on analytical quality control and method validation procedures for pesticide residues and analysis in food and feed' is mentioned here as a representative document of this kind, since it provides a precise description of the method parameters and the required criteria. In combination with the pragmatic approach to execution, this document constitutes a widely-recognised international standard (SANTE 2017). Apart from the application of such guidelines, regarding the analysis of 1,2-unsaturated PA certain factors emerge that can influence the comparability of results or the validity of the methods. This state of knowledge is based in part on the results of numerous method validation studies and interlaboratory comparisons, as well as the associated tests regarding the homogeneity of the sample material (BfR 2015, 2016c).

The following points present specific factors in the analysis of 1,2-unsaturated PAs in food and feed that influence their results more strongly than is the case with other analyte-matrix combinations:

- Food and feed samples with a solid consistency that are contaminated with PA-forming plants exhibit a greater inhomogeneity than other analyte-matrix combinations.

- The analytical methods applied are reliant on reference standards that have been produced by isolation from naturally-occurring material. The quality of the reference standards may therefore influence the results.

- An improvement in mass spectrometrical quantification by compensating for so called 'matrix effects' cannot be achieved by isotope-labelled internal standards.

This means that, in analysing PAs, higher reproducibility standard deviations can occur than with other analyte-matrix combinations. This is explained, inter alia, by the reproducibility standard deviation representing the sum of the analytical measurement precision and the – despite meticulous homogenisation – variable concentrations of 1,2-unsaturated PAs in various subsamples. It is recommended that the following points be taken into account when establishing methodological performance criteria for the determination of 1,2-unsaturated PA in

food and feed, as aimed for example in the revision of SANTE/12089/2016 and Regulation (EC) No. 401/2006.

3.2.2.1 Homogeneity of samples

Currently, the sampling of retail samples for the quantification of 1,2-unsaturated PAs in food is carried out in analogy to Regulation (EC) No 401/2006, which lays down the methods of sampling and analysis for the official control of the levels of mycotoxins in foodstuffs. Food supplements are sampled to determine levels of PAs in analogy to the procedure for vegetable oils (annex I, section M), while baby foods and processed cereal-based foods for infants and young children are sampled as for tropane alkaloids as specified in section J (annex I). Sampling for tea, herbal tea and spices is conducted by following the procedure as set out for spices (annex I, section E). For the determination of levels of PAs and TAs in typical retail samples, five incremental samples, each weighing 100 g, are taken, to yield an aggregate sample having a weight of 0.5 kg (annex I, section E.4).

The first step in the subsequent analysis comprises the homogenisation of the samples, which aims to ensure that the analytical result is representative of the entire sample. In the case of samples with a solid consistency, the entire sample is ground to a uniform particle size (particle sizes of between 250 and 500 µm are typically achieved). However, it has been shown in inter-laboratory tests for the quantification of 1,2-unsaturated PAs that variable reproducibility standard deviations (RSD_R) occur, depending on whether samples with a solid consistency (such as herbal tea) or liquid/viscous samples (such as honey) are being analysed. The data shown in figure 5 illustrate the following point: where food and feed samples with a solid consistency are contaminated with PA-forming plants (category I), these have a higher inhomogeneity than artificially contaminated liquid/viscous samples (category II).

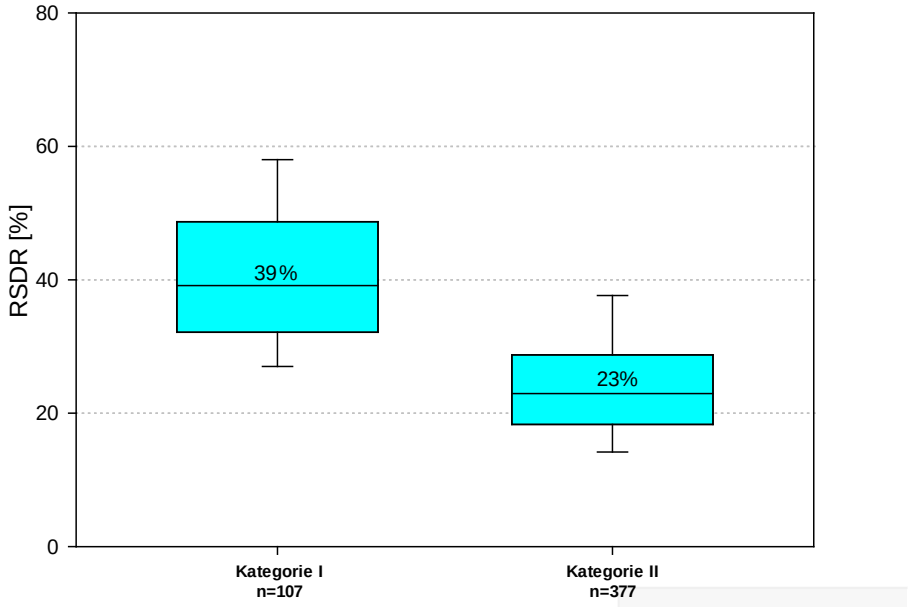

Figure 5: Medians of relative reproducibility standard deviations from various laboratories for 484 PA-matrix combinations, subdivided into naturally contaminated samples having a solid consistency (category I) and artificially contaminated, liquid/viscous samples (category II).

One difficulty in the quantification of 1,2-unsaturated PAs in food and feed with a solid consistency is the fact that a comparatively low number of PA-forming plant particles having a very high PA concentration are present in a large number of sample particles. This phenomenon is termed ‘spot contamination’. This difficulty is worsened by the fact that PA-forming plants contain different concentrations of 1,2-unsaturated PAs in different parts of the plant. This necessarily means that the particles in a sample will differ in their PA concentrations depending on whether flower, leaf or stem particles are present within the sample. Satisfactory comparability between multiple analysis results from an entire sample is therefore achievable only by ensuring that the same number and type of PA-forming plant parts are present in each subsample after homogenisation. As this is virtually impossible to achieve in practice, the necessarily heterogeneous distribution of parts of PA-forming plants in contaminated samples will influence the comparability of the analysis results from subsamples. While every effort is made to ensure effective homogenisation of food and feed samples, the concentrations of 1,2-unsaturated PAs determined will always be subject to comparatively high levels of fluctuation. These fluctuations do not reflect the capabilities of the analytical methods or the laboratories themselves but represent the different PA concentrations found in separate subsamples.

3.2.2.2 Quality of reference standards and lack of isotope-labelled standards

The 1,2-unsaturated PAs exhibit structures that can be produced – if at all – only with great effort by synthesis. Since they have no medical benefit, PA reference standards represent products that have a low sales volume, which means that chemical synthesis on an industrial scale does not currently appear practicable. From this it can be deduced that the production of isotopically labelled standards involves even more effort and costs. Isotope-labelled standards (the ‘embedding’ of heavy, stable isotopes in the molecule) have physical and chemical properties identical to those of the target analyte, and would represent ideal internal standards for improving quantification by means of mass spectrometry. Reference standards currently used in analysis are isolated with great effort as preparations from PA-forming plants and may therefore vary in their degree of purity in practice.

3.2.2.3 Matrix effects during quantification

A critical step in mass spectrometric methods is quantification, which is influenced by so-called matrix effects. Matrix effects are a term for the different efficiency of the ionization of an analyte present in a pure solvent compared to an analyte present in a matrix-rich extract from the food or feedstuff under investigation. While isotope dilution is a recognised procedure for compensating for matrix effects, it cannot be used for PA analysis since no isotope-labelled reference standards are available. The influence of the matrix on quantification must therefore be compensated for in other ways. Currently, three approaches are used in PA analysis: standard addition, external calibration via ‘matrix-matched standards’ and matrix dilution in the form of highly diluted sample solutions.

3.2.3 Evaluation of the data used for the exposure assessment

The levels of 1,2-unsaturated PAs in the various foodstuffs show a very broad distribution, with a large proportion of levels below the limit of detection. In the samples considered, 21 specific individual substances were used for summation. The samples in which not all 21 individual substances were examined were not included in the considerations. It should also be noted that there is considerable sampling uncertainty due to the presence of small sample numbers for some foodstuffs, such as rocket.

Both consumption studies (NVS II, VELS) were performed ten or more years ago. It can be assumed that, as a result of various dietary trends, preferences for certain foods have undergone changes both in general and for specific foods such as tea varieties. Today, for example, a higher consumption of rooibos tea can be assumed. In the *24h-recalls*, the consumption of nettle tea, fennel tea, camomile tea and peppermint tea is not explicitly mentioned, however, it is reasonable to assume that the consumption quantities of these tea types are included in those of herbal tea (nfs). As a result of the varying levels of 1,2-unsaturated PAs in the various types of tea, the stated methodology as applied for consumption quantities can lead to an over- or underestimation of the intake of the stated types of tea.

Due to the presence of at least two individual days from the *24h-recalls* in NVS II and VELS, the data are in principle suitable for use in exposure assessments considering both acute and chronic health risks. However, using consumption data that are based on only a few days to make conclusions about long-term consumption without applying extrapolation methods may lead to some bias.

Some foodstuffs could not be included in the exposure assessment or the overall calculations since requirements concerning data quality were not met. Since foods such as herbs/spices, rocket and flour are potential sources of exposure, one may assume that the overall intake of 1,2-unsaturated PAs has therefore been underestimated.

The occurrence data were calculated using a modified lower bound approach that can lead to an underestimation. Only average concentrations were included in the estimate of long-term exposure. Accordingly, overall intake can be higher for the consumption of foods with high concentrations if an individual has an increased likelihood of being exposed exclusively to foods with high concentrations over an extended period of time. This can occur in the case of brand loyalty, for example, or large single packs of foods that are then consumed by the household over a longer period. Conversely, overall intake can be lower from the long-term consumption of foods with lower concentrations.

The short-term exposure assessment may constitute an underestimation of intake, since the assessment did not consider the simultaneous intake of 1,2-unsaturated PAs from various kinds of foods on the same day.

The consumer’s overall exposure to 1,2-unsaturated PAs as presented here relates to the concentrations present in the foods for which samples were available. One should remember, however, that other foodstuffs can contain 1,2-unsaturated PAs, although these have not been the focus of investigations performed to date. It should also be noted that foodstuffs are regularly analysed in the lab only for a small proportion of individual substances: accordingly, higher overall concentrations in various foodstuffs can therefore be assumed.

3.3 Conclusions and recommended measures

In conclusion, it is clear that both the average levels of 1,2-unsaturated PAs and the levels in the 95th percentile have been clearly reduced in recent years in most of the food groups considered. This decrease is especially pronounced in green tea, black tea and peppermint tea but also in camomile tea, herbal teas and rooibos tea. As a result, the (chronic) intake of 1,2-unsaturated PAs has also declined in recent years.

The estimate of the potential short-term intake of 1,2-unsaturated PAs from the food groups investigated has shown that – from individual food groups – short-term intake levels can be obtained that are slightly higher than the value of 0.1 µg/kg body weight/day that was provisionally used as an orientation value. The short-term intake levels via individual food groups were always below 0.2 µg/kg body weight/day. It should also be noted that the orientation value provisionally used is to be considered as conservative, since its derivation was based on a chronic study. Furthermore, the estimated intake levels for most of the food groups considered were below – sometimes clearly below – the orientation value.

The estimated chronic overall exposure across all food groups considered leads in all scenarios considered to intake levels for both children and adults that result in MOE values of over 10,000, both for normal consumers and for high consumers. As a result, the occurrence of health risks caused by the overall exposure to 1,2-unsaturated PAs calculated in this way can be considered to be of low probability. When interpreting these results, one must take into account the fact that the MOE values determined for high consumers lie only slightly above 10,000.

Consumers are also exposed to 1,2-unsaturated PAs from other foods, which cannot yet be considered in the estimate of overall intake as presented here. This exposure from other food groups could lead to a further decrease in the MOE values stated. Examples of such foods include herbs/spices and food supplements. A preliminary estimate for herbs/spices, for example, indicates that, although consumption quantities for this food group are small, it could nonetheless make a considerable contribution to both long- and short-term exposure to 1,2-unsaturated PAs. As examples, model calculations made using practical assumptions show that an MOE could be clearly lower than 10,000, even if considering only the consumption of herbs/spices with high levels (3,000 µg/kg) for adult normal consumers or when considering the consumption of herbs/spices with average levels (1,000 µg/kg) for adult high consumers. In interpreting these findings, it should be noted that these MOE values result from the sole intake of 1,2-unsaturated PAs via the consumption of herbs/spices: in reality, a multitude of foodstuffs contribute to a consumer's overall exposure. Especially high levels were found in borage, oregano and lovage, and in mixed spices. These herbs therefore constitute a relevant additional source of exposure. However, reliable data for PA levels and real-world consumption quantities for individual herbs are not available for these food groups.As a result, it has not been possible to include these in the estimate of overall exposure to 1,2-unsaturated PAs to date.

The effects of 1,2-unsaturated PAs as genotoxic carcinogens form the primary cause for concern for a risk assessment. In the BfR's opinion, risk management measures should be oriented on the avoidance of such effects. The general recommendation made in the European Union is to minimise exposure to substances that are both genotoxic and carcinogenic as far as it is reasonably possible (ALARA principle: *as low as reasonably achievable*). This is because even small quantities of these substances can be associated with an increase in health risks – especially if consumed on a regular basis.

The BfR therefore recommends to continue efforts to reduce the levels of 1,2 unsaturated PAs to the lowest level technically possible across all foods groups by improving cultivation, harvesting and washing methods. This applies in particular to food groups such as herbs/spices, whose data still occasionally show abnormally high levels.

Further information on the topic of pyrrolizidine alkaloids in food from the BfR website

https://www.bfr.bund.de/en/pyrrolizidine_alkaloids__pa_-192924.html

https://www.bfr.bund.de/en/frequently_asked_questions_on_pyrrolizidine_alkaloids_in_foods-187360.html

https://www.bfr.bund.de/cm/349/pyrrolizidine-alkaloid-levels-in-dried-and-deep-frozen-spices-and-herbs-too-high.pdf

BfR "Opinions app"

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Annex

Table S1: Levels of 1,2-unsaturated PAs (sum of 21 analytes, including co-eluting isomers) in herbs/spices

	N[1]	<LOD[2] [%]	Mean [µg/kg]	Median [µg/kg]	95th percentile [µg/kg]
Mugwort	13	15	53.7	21.9	231.0
Borage	8	0	50,493.1	14,546.0	248,060.9
Various[3]	24	79	72.4	0	237.1
Lovage	22	18	1,710.1	1,073.7	6,255.6
Oregano, marjoram	95	9	4,465.1	1,484.0	18,771.5
Flat-leaf parsley	41	54	222.3	0	1,230.8
Parsley leaves	13	69	1.5	0	14.4
Rosemary	21	90	3.2	0	6.8
Thyme	21	48	514.3	0.2	381.8[4]
Cumin	1		17,900.0		
Mixtures	68	24	648.0	85.5	3,818.3
Total	327	34	2,905.7	53.3	10,871.1

[1]N = number of samples (occurrence data from German state food monitoring programmes, 2015 to mid-2019); [2]LOD = limit of detection; [3]Basil, savory, dill, chervil, coriander, caraway, chives; [4]One sample had a concentration of 9,565.2 µg/kg, the next-lower concentration was 381.8 µg/kg

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The German Federal Institute for Risk Assessment (BfR) is a scientifically independent institution within the portfolio of the Federal Ministry of Food and Agriculture (BMEL) in Germany. It advises the German federal government and German federal states ("Laender") on questions of food, chemical and product safety. The BfR conducts its own research on topics that are closely linked to its assessment tasks.

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