Determination and regulation of hepatotoxic pyrrolizidine alkaloids in food: A critical review of recent research

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A critical review of recent research

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Abstract

Pyrrolizidine alkaloids (PAs) are secondary metabolites of plants. PAs have been reported to be hepatotoxic, mutagenic, and carcinogenic; they are a significant group of natural toxins affecting livestock, wildlife, and humans. To date, over 10,000 PAs poisoning cases have been reported worldwide. In recent years, many articles have reported the detection of PAs in various foods, including honey, milk, meat, eggs, tea and salad. This review summarized the contamination of PAs in foods, state of the art detection methods and regulations by different countries and authorities, hoping to propose effective solutions to minimize the consumption of PAs in food.

Keywords: pyrrolizidine alkaloids; food; determination; regulation
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Abstract
Pyrrolizidine alkaloids (PAs) are secondary metabolites of plants. PAs have been reported to be hepatotoxic, mutagenic, and carcinogenic; they are a significant group of natural toxins affecting livestock, wildlife, and humans. To date, over 10,000 PAs poisoning cases have been reported worldwide. In recent years, many articles have reported the detection of PAs in various foods, including honey, milk, meat, eggs, tea and salad. This review summarized the contamination of PAs in foods, state of the art detection methods and regulations by different countries and authorities, hoping to propose effective solutions to minimize the consumption of PAs in food.

Keywords: Pyrrolizidine alkaloids (PAs); Food; Determination; Regulation
1. Introduction

Pyrolizidine alkaloids (PAs) are naturally occurring heterocyclic phytotoxins that are widely distributed in about 3% of the world's flowering plants (Fu et al., 2004, EFSA, 2007). To date, more than 660 PAs and their N-oxide forms (PANOs) have been identified in over 6000 plants. Most of these plants belong to the Asteraceae, Boraginaceae, Orchidaceae, and Fabaceae families, and half of them have been reported to be hepatotoxic (Yang et al., 2001, Zhu et al., 2017, He et al., 2017). PAs are esters of three types of necine base: Retronecine type, otonecine type, and platynecine type. The former two with the necine base having a double bond at the C1 and C2 positions exhibit high levels of toxicity, while platynecine type PAs with a saturated necine base (without a double bond) are either weakly toxic or nontoxic (Figure 1) (Fu et al., 2004, Ruan et al., 2014a). The available information indicates that the adverse effects of 1, 2-dehydropyrrolizidine alkaloids (dehydroPAs) in experimental animals include hepatotoxicity, developmental toxicity, genotoxicity and carcinogenicity (EFSA, 2011a, Li et al., 2011, Lin et al., 2011, Yang et al., 2016, Fu et al., 2017a, Fu et al., 2017b, Zhu et al., 2017). Acute poisoning with PAs in humans is associated with liver damage, whereas a sub-acute or chronic onset may lead to liver cirrhosis and pulmonary arterial hypertension (Li et al., 2018, EFSA, 2011a). Compared with herbs, PAs are more widespread, more serious and more difficult to control in food. There is now increasing recognition that some widely consumed foods (e.g. grains, milk, meat, eggs, honey, pollen) are sometimes contaminated by PAs and PANOs at levels that, while insufficient to cause acute poisoning, greatly exceed maximum tolerable daily intakes and/or maximum levels determined by a number of independent risk assessment authorities (Edgar et al., 2011).

Insert Figure 1 here

In Europe, an analysis was done of a total of 1105 samples collected. These comprised milk and milk products, eggs, meat and meat products, teas, and food supplements collected in supermarkets, retail shops, and via the internet. PAs were detected in a large proportion of plant-derived foods: 60% of the food supplements and 92% of teas contained measurable amounts of PAs. As for animal-derived products, 6% of milk samples and 1% of egg samples contained PAs (Mulder et al., 2018). In Hong Kong, a total of 234 samples (48 food items) were collected randomly from a local market and analyzed. About 50% of samples were found to contain detectable amount of PAs (Chung
and Lam, 2017).

This review summarized the current global situation with regard to the presence of PAs in food, the method of detecting PAs content, and the regulation of PAs by various countries and authorities. It is hoped that more effective solutions to minimize the consumption of PAs can be developed on this information.

2. PAs in food

2.1. Bee products – honey and pollen

Apiarists in many countries regularly use a number of PA-containing plants for honey production (Edgar et al., 2002). Kempf et al. have demonstrated that honeys from many of these plants contain significant levels of PAs (Kempf et al., 2008).

It has been suggested that the PAs found in honey may have been introduced via pollen accidently dislodged into nectar, e.g. by nectar-collecting bees (Boppre et al., 2005). Pollen from PA-containing plants contains extremely high levels of PANOs (Kempf et al., 2010a). Apiarists too may accidently or deliberately introduce pollen into honey during or after harvesting (Edgar et al., 2011). The levels of PAs and PANOs found in many honeys could, according to published risk assessments, cause chronic diseases such as liver cirrhosis, pulmonary hypertension and cancer if these honeys are regularly consumed at recommended serving sizes of 15–25 g or at higher consumption levels reported by honey consumers in many countries. For example, in Australia the highest consumers of honey in the 2–4 years old age group eat 28.6 g of honey per day while older consumers, 5–65 years of age, eat 40–65 g per day (Edgar, 2011). It has been reported that a woman who consumed 20–30 mg of PAs per day, similar to those occurring in Echium honey, during her pregnancy gave birth to a child suffering fatal liver damage (Rasenack et al., 2003).

Bee pollen granules in food supplements contain nectar (used by bees as a binding agent) as well as pollen grains. A recent study that investigated PAs in bee products in Europe (Mulder et al., 2018) found that in eleven of the twelve pollen products, PAs were detected at a mean concentration of 576.0 µg/kg, while 0.6 and 15.5 µg/kg were quantified in propolis and royal jelly products.

2.2. Grain contamination

Mass intoxications have arisen from the use of contaminated grain (Tandon et al., 1976,
Schuster et al., 1993, Al-Taee et al., 2004, van Egmond et al., 2011). The earliest case termed ‘bread poisoning’ dates from 1920 (Willmot and Robertson, 1920) and more recent cases have occurred in both Afghanistan and Ethiopia in 2008 (Molyneux et al., 2011). Nowadays, episodes of acute dehydroPAs toxicity involving the contamination of bread are avoided in many countries by more effective control of weeds in crops and by strictly applying food safety standards limiting the number of foreign seeds in grain entering the human food chain, including some known to contain dehydroPAs (Edgar et al., 2015). However, it has been shown that complete removal of seeds containing PA from heavily contaminated grain still leaves readily detectable levels of PAs in the ‘cleaned’ grain and such grain has been shown to be capable of poisoning pigs (Edgar et al., 2011). Fine plant dust, generated during harvesting and adhering to the grain, is thought to be the source of the PAs (Edgar, 2003). Azadbakht et al. have found levels of PAs in wheat and flour in Iran that raise concerns for chronic toxicity (Azadbakht and Talavaki, 2003). Similar studies are needed in other countries before the contribution of PAs in grain-based products can be assessed as a potential cause of slowly progressing chronic poisoning of humans. It is normal practice in countries undertaking large scale cereal production to combine grain from many farms and from different cropping areas. Such bulking dilutes and reduces the level of dehydroPAs in the grain, but it results in a wider distribution of any dehydroPAs that were present and thus increases the population exposed to low levels of these hazardous chemicals in grain-based foods (Edgar et al., 2015). While these measures and practices are apparently sufficient to prevent the acute dehydroPAs poisoning, it is still possible that occasional, low level dietary exposure in grain-based products could be contributing worldwide to the incidence of several slowly developing, chronic diseases (Azadbakht and Talavaki, 2003).

2.3. Milk contamination

A number of experiments on milk transfer of PAs in rats and mice have been performed (Edgar et al., 2011). In the earliest of these demonstrated that on administration of the PAs lasiocarpine and retrorsine to lactating rats their suckling offspring died with distinct liver lesions, although the alkaloids had no apparent effects on the mothers. The author pointed to the possibility that various liver disorders in childhood may be the result of poisoning by PAs in milk. Although the N-oxides produced in the liver are normally rapidly excreted in the urine, in lactating animals a certain proportion may be sequestered in the aqueous phase of milk. The possibility that the ultimate toxic
metabolites, dehydroretronecine and dehydroheliotridine, are also transferred into milk should be considered too, based on their relatively high water solubility.

In recent years, PAs have been frequently detected from the real milk samples. For example, PAs were detected from the real milk samples in Europe market (Mulder et al., 2018). In 11 out of 182 (6.0%) milk samples the presence of one or two PAs could be confirmed above the limit of detection (LOD). PAs residues were found in milk from 4 different countries (Spain, Germany, Greece, and the Netherlands) and in all major types of milk regarding fat content and process of conservation. Other studies have found similar results in milk (Huybrechts and Callebaut, 2015).

2.4. Chicken egg contamination

Transfer of PAs from feed to eggs has been shown to occur (Edgar and Smith, 2000, Eroksuz et al., 2003, Diaz et al., 2014). Diaz et al. reported that the residues found in eggs were primarily of the PA free base type with only a very minor contribution of PANOs (Diaz et al., 2014). Edgar et al. reported levels of 5–168 mg/kg PAs in eggs. In this case, the layer hens had been inadvertently poisoned by Heliotropium europaeum and Echium plantagineum contamination in the grain component of their feed (Edgar and Smith, 2000).

In another study in Europe market, contamination of eggs with PAs was found in two samples out of 205 analyzed, with levels at 0.1-0.12 μg/kg. The PAs found are similar to the ones found in milk. (Mulder et al., 2016, Mulder et al., 2018).

2.5. Meat contamination

Various PA-containing herbs may be present in pastures and fields where animals are foraging. They may also be present in fresh or dried products fed to animals, as shown in particular for alfalfa, a widely used feed product (EFSA, 2011a). When consumed by animals, part of the PAs will be transferred to animal-derived food products (Dickinson et al., 1976, Edgar and Smith, 2000, Hoogenboom et al., 2011). Although the transfer and levels in animal-derived products seem relatively low, exposure via such products may still be relevant. This is due to the fact that various PAs were shown to contain genotoxic and carcinogenic properties, meaning that even intake of low levels by humans may result in adverse effects (Edgar et al., 2015).

There is very limited information on the transfer of PAs to meat. Experiments have been
reported in which puppies were fed cooked meat from animals poisoned by a dehydroPA-containing species of *Trichodesma*. This resulted in death or production of irreversible pathological changes within 3–4 months and it was concluded that the meat contained toxic alkaloid residues that were not destroyed by cooking (Shevchenko and Fakhrutdinova, 1971). Recently, a study conducted PAs transfer study with eggs and meat indicate that the intake of PA-containing herbs by laying hens may result in levels in eggs and meat that could be of concern for consumers, and as such should be avoided (Mulder et al., 2016).

2.6. Salads, teas and condiments

Some leafy PA-containing plants have been, and in some cases are still being, recommended as salads. It is well known that the leaves of the common weed *Senecio vulgaris* accidentally co-occurred with salad leaves of similar appearance being sold in supermarkets in Germany (BfR, 2007). PA-containing plants are also recommended for making teas and sauces. PAs have also occurred in a cooking spice that was implicated in the death of a late-term foetus that died of liver failure (Rasenack et al., 2003).

Bodi et al. analyzed a total of 274 dry tea samples available on the German market, including, amongst others, 24 black, 23 green, 24 rooibos, 29 peppermint, 39 chamomile and 43 mixed teas, for the presence of 10 different PAs and 7 different PANOs with LC-MS/MS. The percentage of positive teas varied between 86% (peppermint teas) to 100% (rooibos teas). As in this study, rooibos tea was found to be the most highly contaminated (mean: 1856.4 μg/kg, maximum: 5647.2 μg/kg) (Bodi et al., 2014).

Schulz et al. used LC-MS/MS to analyze 169 medicinal teas, which were commercially available on the German market, for the presence of 14 different PAs and 9 different PANOs (Schulz et al., 2015).

Griffin et al. analyzed 18 herbal dry teas available from the Irish market using LC-MS/MS. The method included 10 PAs and 4 PANOs and the LOD in dry tea is 0.4-1.5 μg/kg. The mean and maximum contamination is 210 and 1733 μg/kg respectively (Griffin et al., 2014).

The study of Mathon et al. focused on the PAs content of 70 teas purchased from the Swiss market that were analyzed for 9 PAs by LC-MS/MS. Results were expressed as amount of PAs/cup of infusion (200 ml). Limit of quantification (LOQ) reported were 0.02 μg/cup, which corresponds to
approximately 10 μg/kg in dry tea. It was reported that 70% of the tea infusions contained one or more PAs above the LOQ (Mathon et al., 2014).

A recent study shows that contamination of all types of tea with PAs is very common (Mulder et al., 2018). In the majority of samples (91%) one or more PAs were detected. All types of teas appear to contain PAs, although the concentrations differed between the various types of tea. Highest contamination, with regard to maximum, mean and median concentration, was observed in rooibos tea.

Tea samples were further evaluated concerning their content of individual PAs. With respect to contribution to the mean content in tea infusions, senecionine-N-oxide is the most important compound with an average concentration of 1.74 μg/L, which makes up 28% of the total PAs concentration (6.13 μg/L) found in tea. The PAs of the senecionine group account for over 77% of the PAs content in tea, while PAs of the lycopsamine group contribute 14%, and heliotrine-type PAs contribute 8%. Approximately one third of the content of PAs in the tea samples is made up by PAs free bases and two thirds by PANOs. Summary of literature on PAs in food was shown in Table 1.

Insert Table 1 here

3. Method of analysis

Many different foods have been analyzed for PAs in the past, and most of the common analytical techniques were applied in the detection of these compounds (Crews et al., 2010). Hence, this part will focus on the most recent and most common techniques used for the trace analysis of PAs in complex matrices like foods.

3.1. Sample preparation

3.1.1. Extraction

Generally, considering the co-occurrence of PAs and corresponding PANOs, the extraction method has to ensure the efficient simultaneous extraction of both and therefore classical alkaloid extraction with semi-polar to polar organic solvents or acidified aqueous conditions are prevalent. PANOs are polar molecules that can be readily extracted by polar solvents such as methanol or diluted aqueous acids (Crews et al., 2010).
3.1.1.1 Animal-derived food products

After thoroughly shaken and homogenization, animal-derived food samples (like milk, yoghurt, reconstituted infant formula milk, eggs, meet, liver) were transferred to polypropylene tubes and internal standard solution (like epijacobine of 1000 ng/mL in methanol) was added (Mulder et al., 2016). Formic acid solution (0.2%) and hexane were added to the tubes. The samples were extracted on a rotary tumbler and then centrifuged. The hexane top layer and (most of) the solid middle layer (containing mostly fat and non-soluble proteins) were removed by suction. Concentrated ammonia (25%) was added to adjust the pH of the solution to 9-10, and then the samples were centrifuged.

Remaining aqueous extract was used for further clean-up by solid-phase extraction (SPE) over a Strata™ X. The cartridges were conditioned with methanol and ammonia solution (0.1%). The cartridges were loaded with extract, washed with ammonia solution (0.1%) and dried under vacuum (using a vacuum manifold) for 5-10 minutes. PAs were eluted from the cartridges with methanol. The eluates were dried under a nitrogen stream in a warmed water bath and reconstituted in methanol/water (10/90, v/v). The reconstituted sample extracts were filtered using filter vials.

3.1.1.2. Plant-derived food products

Tea samples were mixed with dry ice (at a mass ratio of 2:1) (Mulder et al., 2016). The mixture was allowed to stand for about 3 minutes while stirring repeatedly. The frozen sample was ground to a particle size of 500 μm using an ultra-centrifugal mill. The aggregate sample was homogenized by overhead-shaking for 2 hours. The extraction procedure was based on the protocol for the preparation of ready-to-drink products described in ISO 3103. Tea in a tea infusion bag was placed in a beaker and extracted with boiling water. Infusion was steeped for 5 minutes after which the tea bag was removed. After cooling down, the infusion was filtered through a fluted filter paper.

The SPE clean-up was carried out with reversed phase C₁₈ SPE cartridges (Discovery® DSCC₁₈ 500 mg/6 mL), which were conditioned with methanol and water. Then, the cartridges were loaded with tea infusion, washed with water and dried under vacuum (using a vacuum manifold) for 5-10 minutes. PAs were eluted from the cartridges in two steps with each of methanol or 2.5% (1.4 M) ammonia in methanol in the case of black and green tea samples.
3.1.1.3. Food supplements and bee products

For dry food supplements and food supplements containing bee pollen products, extracted with aqueous sulphuric acid solution by ultra-sonication (Mulder et al., 2018). The supernatant was decanted after centrifugation. Extraction was repeated and combined supernatants were brought to pH 6-7 with diluted ammonia solution and passed through a folded filter paper, with further clean-up by SPE.

The SPE clean-up was carried out with reversed phase C<sub>18</sub> SPE cartridges, which were conditioned with methanol and water. Then, the cartridge was loaded with sample extract, washed with water and dried under vacuum. PAs were eluted from the cartridge in two steps with methanol. The combined eluates were dried under a nitrogen stream in a heated water bath and the dried residue was reconstituted in methanol/water (5/95, v/v). The reconstituted sample extract was filtered through centrifuge filters before further analysis.

For oily food supplements, PAs were extracted with sulfuric acid in methanol by overhead shaking. The supernatant was decanted after centrifugation. Extraction was repeated, and an aliquot of combined supernatants was used for further clean-up by SPE.

3.2. Instrumental analysis

After extraction, the PA-containing food samples are detected by instruments. The methods that have become the most established ones will be briefly summarized below.

3.2.1. Spectrophotometry and thin layer chromatography (TLC)

The spectrophotometric detection for dehydroPAs and PANOs (with the exclusion of otonecine-type PAs) is based on a color reaction. The tertiary PAs are oxidized to the corresponding PANOs, dehydrogenated to the pyrroles and subsequently coupled with Ehrlich’s reagent to form a magenta colored dye which can be measured spectrophotometrically at 563 nm and shows detection limits in the mg/kg range (or 1 μg total dehydroPAs). An alternative approach is to use aqueous methyl orange, which forms a yellow complex that is very soluble in organic solvents. Methyl orange is released from the organic solution on treatment with strong acid, and measured with a spectrometer (Dwivedy et al., 2017). These methods are not able to quantify individual PAs but allow determination of the total dehydroPAs content by construction of a calibration series using the readily available standards.
TLC with both silica and aluminium oxide adsorbents has been used to separate PAs, and many procedures have used ion pairing with lithium or potassium chloride or sodium iodide to enhance separation. For detection of PAs on TLC plates Erlich’s reagent is the most specific spray reagent used (Crews et al., 2010). DehydroPAs bases are oxidized to the PANOs with hydrogen peroxide, and these are reduced to dehydropyrrolizidine by heating with acetic anhydride or o-chloranil, whereupon the pyrrole gives a violet–blue dye. Ehrlic’s reagent is used to detect dehydroPAs and Dragendorff’s reagent and nitrite were used to detect saturated PAs. High resolution is achieved by using the power of lithium chloride to act as an ion pairing reagent and reduced the analyte polarity.

3.2.2. Nuclear magnetic resonance (NMR)

NMR methods for PAs analysis have been used mainly for structural identification. They are predominantly used in the structural elucidation of purified PAs and comprehensive collections of $^1$H and $^{13}$C NMR data are available (Roeder and Pflueger, 1995). Quantitative determination of dehydroPAs is typically achieved by $^1$H NMR spectroscopy.

Signals originating from the vinylic C-2 hydrogen resonate at 6.2 ppm and 5.8 ppm for macrocyclic diesters and non-cyclic mono or diesters, respectively. Using appropriate internal standards (like paradinitrobenzene) allows the determination of the total dehydroPAs level of an extract (Molyneux et al., 1979).

Comprehensive tables of spectral data of the analysis of PAs by NMR have been published for $^{13}$C NMR spectroscopy and for $^1$H NMR (Maslennikov et al., 2010). Brief overviews of the $^1$H NMR of retrorsine, seneciphylline and senecionine has been published (Kim et al., 1993). $^1$H NMR can provide qualitative information more rapidly and from a smaller sample than $^{13}$C NMR, which gives more structural information. The shifts due to protons on the necine base are very distinctive and permit recognition of unsaturation and oxygenation. The necic acid proton shifts are less distinctive because the acids have similar structures. There is now scope for applying the much greater sensitivity available in modern high-field NMR instruments to PAs analysis.

3.2.3. Gas chromatography-mass spectrometry (GC-MS)

Free base PAs can be separated by GC and can be identified on the basis of relative retention times using different stationary phases (Crews et al., 2010). GC can be applied to most PAs except
otonecines, and it can cause thermal decomposition and the formation of diesters from monoesters (Mandic et al., 2015). The PANOs cannot be analysed by GC as they are unstable at the temperatures required for volatilisation.

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

For PAs analysis, GC is most commonly used in combination with mass spectrometric detection in electron impact (EI) mode. The mass spectra of PAs are dominated by signals unique for the necine base part of the molecule. The distinct fragmentation pattern of dehydroPAs can be used to set up dual MS detection methods (alternate switching of single ion monitoring (SIM) and scan modus: SIM/Scan) which allow fast and reliable detection of PAs in complex matrices.

To overcome volatilization, separation and peak shape difficulties of multi hydroxylated PAs, several derivatisation reagents have been used. The most common ones are the boronate derivatives for vicinal diols or trimethylsilyl ethers or the combination of both, which form bonds across vicinal diol groups (Mandic et al., 2015). Derivatisation has also been used to enable GC determination of the retornecine base, by which means the total PAs content of a sample can be measured chromatographically by comparing the signal intensity with that of a standard prepared from an available PAs such as monocrotaline or retorsine. For this purpose the heptafluorobutyrate derivative of the retornecine base has been prepared (Kowalczyk and Kwiatek, 2017).

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

Kowalczyk et al. proposed a first method for the determination of PAs in feed matrix involving GC-MS. The sum parameter method approach to determine the content of PAs in feeds (Kowalczyk and Kwiatek, 2017).

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

3.2.4. Liquid chromatography-mass spectrometry (LC-MS)

High performance liquid chromatography (HPLC) and LC separation of PAs is attracting more and more interests (Carlsson and Tornqvist, 2016). LC offers the advantage of a simultaneous detection of PAs and PANOs that means fewer steps of sample preparation and a reduced risk of alteration of the analytes (Bushway et al., 1994). Because PAs and PANOs do not show characteristic ultraviolet (UV) spectra (exhibiting only a non-specific UV-maximum of 214 nm), simple UV detection is of limited value. However, LC techniques are frequently and successfully used to purify PAs for structural identification including the enantioseparation of isomers like intermedine/lycopsamine (Narberhaus et al., 2004, Kashyap et al., 2010).

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

In 2014, sample extracts were accurately analyzed by using LC-ESI-MS/MS applying for the first time a pentafluorophenyl (PFP) core-shell column to the chromatographic separation of PAs and PANOs (Griffin et al., 2014). Furthermore, Griffin et al. also proposed a new and rapid isocratic LC-MS/MS method to purchase and analyze the targeted PAs in honey, which developed from, and is comparable with, a gradient elution method and resulted in no loss of sensitivity or reduction in chromatographic peak shape. Isocratic elution allows for significantly shorter run times (6 min), eliminates the requirement for column equilibration periods and, thus, has the advantage of facilitating a high-throughput analysis which is particularly important for regulatory testing laboratories (Griffin et al., 2015).

Yoon et al. developed a rapid and sensitive analytical method for the determination of 9 toxic PAs in popular high-lipid foodstuffs by LC-ESI-MS/MS. PAs in lipid foodstuffs were effectively purified by freezing lipid precipitation (FLP) and SCX-SPE (Yoon et al., 2015).

Betteridge et al. optimized a previously published methods (Betteridge et al., 2005), which is HPLC-MS method for the quantification of four PAs and one PANO in PA-containing plants and honey. A Waters Alliance 2965 LC system coupled to a Waters Micromass Quattro Micro triple quadrupole mass spectrometer was used (Mudge et al., 2015).

Zhu et al. proposed a rapid, selective, and sensitive ultra-high performance liquid chromatography-quadrupole time-of-flight mass spectrometry (UHPLC-QTOF–MS) method for the estimation of RET-PAs in herbs without requiring corresponding standards. This method is based on our previously established characteristic and diagnostic mass fragmentation patterns and the use of retrorsine for calibration (Zhu et al., 2016).

In another very recent study an online two dimensional liquid chromatography quadrupole time-of-flight mass spectrometry (2D-LC QTOF-MS) method was developed to resolve isomeric PAs and PANOs. In this method, a polar endcapped C_{18} column was used at pH 3 in the first dimension, and a cross-linked C_{18} column at pH 10 was used in the second dimension (van de Schans et al., 2017).
Zhang et al. used a new UPLC-Q-Orbitrap/MS method to analyse PAs in *Crotalaria sessiliflora* L. Coexisting PAs were identified by mass data of full MS-dd-MS2 based on the characteristic fragmentation pattern and necine-core structure. Moreover, quantification of PAs was conducted by parallel reaction monitoring (PRM) mode using m/z 138, m/z 120 and m/z 94 from identical necine-core structure as quantitative ions with a single monocrotaline standard for accurate calibration (Zhang et al., 2017).

Applying an UPLC-MS/MS spectrometry method, Zheng et al. *divaricata* from five different Chinese locations and their specific PAs were qualitatively characterized. Using a pre-column derivatization HPLC method, the total retronecine ester-type PAs in their alkaloids extracts were quantitatively estimated as well (Zheng et al., 2017).

For the PANOs, Ruan et al. discovered characteristic ion clusters as unique determinants to discriminate PANOs from PAs even without the availability of reference samples. The found that the mass spectra of toxic retronecine-type PA N-oxides exhibited two characteristic ion clusters at m/z 118-120 and 136-138 by HPLC-MS. Similarly, the nontoxic platynecine-type PANOs also fragmented via three similar pathways to form two characteristic ion clusters at m/z 120-122 and 138-140. Further application of using these characteristic ion clusters allowed successful and rapid identification of PAs and PANOs in two PA-containing herbal plants (Ruan et al., 2012).

### 3.3. Immunological methods

Immunology-based tests are wildly used and significant laboratory methods have been developed for rapid, selective and sensitive detection of analytes in complex matrices. It is well known that PAs are non-toxic without metabolic activation (Chen et al., 2016). All dehydroPAs are metabolically activated to the reactive pyrrolic esters, which have the same core pyrrole moiety regardless of structures of the parent PAs (Zhu et al., 2015, Zhu et al., 2016). Once formed, the pyrrolic metabolites can rapidly react with proteins via covalent binding to form pyrrole-protein adducts (Ruan et al., 2015, Gao et al., 2015, Li et al., 2015, He et al., 2017b). It has been suggested that these adducts trigger the PA-induced toxicities (Wang et al., 2016, Xia et al., 2016, Li et al., 2016). Immunoassays offer a useful tool to detect adducted protein in experimental animals and exposed humans. Using a common structural feature of many toxic PAs, the necine base retronecine, researchers have demonstrated that retronecine-protein conjugate raised antibodies can detect
retronecine but also monocrotaline in a competitive inhibition enzyme-linked immunosorbent assay (ELISA) system (Bober et al., 1989). They also developed the first compound-specific antibodies, i.e. antibodies specific the PAs monocrotaline, which have been demonstrated able to detect quaternised monocrotaline ($IC_{50} = 0.25$ ppm at pH 7.6), N-methylated monocrotaline ($IC_{50} = 5.3$ ppm at pH 7.6), and protonated monocrotaline ($IC_{50} = 6.0$ ppm at pH 6.0).

Roeder et al. reported a successful analysis of the concentrations of sencionine and integerrimine together with their N-oxides as present in zinc-dust reduced methanolic extracts of *Senecio rupestris* using antibodies to a retrorsine hemisuccinate-BSA conjugate (Roeder and Pflueger, 1995).

Lee and co-workers developed ELISA methods to detect and quantify PAs in bovine blood and plants. They developed two different systems to detect riddelliine and riddelliine N-oxide, investigated their cross reactivities to a number of other PAs (Lee et al., 2001).

Finally, a method producing monoclonal antibodies against retrorsine conjugated to bovine-thyroglobine should be mentioned, Zündorf et al. obtained antibodies with a certain affinity to 8 different PAs and no cross reaction to 12 other such compounds (Zundorf et al., 1998).

Charlermroj et al. developed and validated a rapid multiplex ELISA for the detection of a range of PAs and PANOs in feed and honey for the first time. The method is inexpensive, and thus appropriate as screening option prior to GC-MS/LC-MS confirmation for detection of PAs contaminants in food and feed (Charlermroj et al., 2013).

Although the mechanisms of PA-induced toxicities have not been fully elucidated, it is well accepted that the reactive pyrrolic metabolites are associated with the toxicities of PAs (Ruan et al., 2014b). The immunochemical approach has been successfully developed to identify target proteins modified by reactive metabolites of many compounds, such as acetaminophen (Roberts et al., 1987, Bartolone et al., 1992), halothane (Vergani et al., 1980, Satoh et al., 1985), bromobenzene (Kleiner et al., 1998), naphthalene (Zheng and Hammock, 1996), trichloroethylene, and styrene (Yuan et al., 2007, Shen et al., 2009). Li et al. develop polyclonal antibodies to detect pyrrole-protein adducts modified by the reactive metabolites of PAs (Li et al., 2017). According to the authors, this approach can detect pyrrole-protein adducts resulting from exposures to a range of toxic PAs (Yang et al., 2017).
3.4. Summary of analytical methods

Colorimetric detection or TLC separation in combination with PAs detection via Ehrlich’s reagent might still be suitable methods for dehydroPAs screening, but they lack sensitivity and selectivity for the trace analysis of PAs in complex matrices. As for immunological approaches, they usually are sensitive only for a narrow range of structurally closely related PAs. The GC-MS and LC-MS have been compared in detail (Kempf et al., 2010a). In brief, compares to LC-MS, GC-MS is unlikely to miss toxic relevant PAs and not significantly dependent on standards, has true internal standard quantification, simple quantification, and can be easily adapted to different samples and backgrounds (Aguero et al., 2011). Furthermore, GC-MS can be adapted to stable isotope dilution analysis and has no need of background information for samples or PAs involved. As for LC-MS, PANOs and tertiary PAs can be determined simultaneously; the proportion of each individual structure can be known which might be necessary for further toxicological investigations; sample preparation is relatively easy and turnaround times are fast. Also, the information it produces can be used in other contexts. For example, PAs plant patterns and ratio tertiary PAs/PANOs can be linked to geographic origin. Currently, both approaches supplement each other, which are approximations to quantify PAs in complex mixtures.

The application quantity of various detection methods in PAs was shown in Figure 2. The typical application of different methods for the detection of PAs in PA-containing analytes were shown in Table 2.

4. Regulation

As PAs has been demonstrated to be a health threat for both humans and livestocks, many countries and authorities have set various limitations for PAs as summarized in Table 3.

We summarized the legal provisions of different countries and national organizations on the limitation of PAs. Our list contains all information that we were able to access but does not claim to be complete. The limited information available from human poisoning cases allowed identifying a lowest known dose of approximately 2 mg/kg bw per day associated with acute/short-term effects. This was based on a case of a 6-month-old girl who received a daily dose of approximately 0.8–1.7 mg PA/kg bw for 2 weeks and was diagnosed for hepatic veno-occlusive disease (HVOD), and a
2-month-old boy who was administered an approximate dose of 3 mg/kg bw for 4 days, with a fatal outcome (Knutsen et al., 2017).

Insert Table 3 here

4.1. Germany

Since 1992, PA-containing phytopharmaceuticals have been regulated by a Federal Pharmaceutical Ordinance. According to these regulations, only a few proven active PAs plants and preparations thereof, which are listed by name, can be marketed. After an intensive risk assessment and expert hearings, the authorities justified the regulation on the grounds that these actions would substantially reduce the risk associated with the ingestion of PAs. In addition, the package insert for orally used products needs to contain the warning notice “do not use during pregnancy or lactation” (BfR, 1992). In Switzerland the same regulations for phytopharmaceuticals as in Germany are in force.

In 2007, Bundesinstitut fur Risikobewertung (BfR) started to reevaluate and where applicable to complement the zero tolerance principle of the European Union for certain compound and compounds classes in food and feeding stuff (BfR, 2007). In this context, the zero tolerance principle should be applied if the risk cannot be calculated or if limits of exposure cannot be derived because of the lack of valid scientific data. In many cases zero tolerance should be applied if the compound possesses carcinogenic or mutagenic potential.

In 2013, the BfR began conducting a research project on the “Determination of Pyrrolizidine Alkaloids in Food and Feed”. Based on the available data, the BfR concludes that those adults and children who frequently consume of herbal tea infusions are possibly at increased cancer risk, particularly if they consume products with high PAs contamination over long periods of time (BfR, 2013).

The BfR once again recommends that the total exposure of consumers to genotoxic and carcinogenic PAs from various food sources should be kept as low as possible.

In order to avoid the marketing of contaminated batches and to protect the consumer, it is recommended that herbal tea batches that are to be brought onto the market be checked for PAs content before distribution.
4.2. UK

In the past, similar to the German regulation for herbal remedies, comfrey and preparations thereof were banned in the UK. In 2007, the UK Committee on Toxicity (COT) was asked for a statement of PAs in food, in particular in honey and milk (COT, 2007). The statement comprehensively summarized the literature data on all aspects concerning PA toxicology like hepatotoxicity, pulmonary toxicity, carcinogenicity and genotoxicity.

4.3. Austria

The legal situation in Austria affecting PAs in herbal remedies is similar to that in Germany regulations. Only a few PAs plants are authorized. These plants or preparations thereof can only be marketed if they are analyzed by a “...state of the art detection method” which proves that “... the final product does not contain pyrrolizidine alkaloids.” (Kempf et al., 2010b).

4.4. The Netherlands

It shows clearly that the regulations of the neighboring countries Austria and the Netherlands are many times more stringent than the German regulation. Recently, the Dutch National Institute for Public Health and the Environment (RIVM) suggested that the consumption of honey with elevated PAs levels should be avoided; therefore, it said that studies should be intensified to be able to further minimize the risk. It is concluded there, with no warning notice for the consumers deemed necessary (Krauterbeschluss, 2001).

4.5. Europe

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

In 2010, EFSA concluded that dehydroPAs may act as genotoxic carcinogens in humans, and there is a possible health concern for those toddlers and children who are high consumers of honey (EFSA, 2011b).

In 2016, EFSA reported that chronic and acute dietary exposure to PAs was estimated in the European population via the consumption of plant-derived foods. This resulted in the highest estimates of mean chronic dietary exposure of 34.5–48.4 ng/kg body weight bw per day in ‘Toddlers’ (LB–UB) and 154–214 ng/kg bw per day in the highly exposed population (EFSA, 2016).

In 2017, the Contaminants in the Food Chain (CONTAM) Panel updated the benchmark dose (BMD) analysis of the available long-term studies on lasiocarpine and riddelliine as determined in its previous risk assessment (Knutsen, 2017). Using model averaging, the Panel calculated the BMD confidence interval and selected the benchmark dose modelling(BMDL)10 of 237 μg/kg bw per day for increase in the incidence of liver haemangiosarcoma in female rats exposed to riddelliine as the reference point (RP) for chronic risk assessment. Considering the data on honey, tea, herbs and food supplements in 2016, the EFSA Scientific Committee concluded that, for substances that are both genotoxic and carcinogenic, a margin of exposure (MOE) of 10,000 or higher, based on a BMDL10 from an animal study, and taking into account overall uncertainties in the interpretation, would be of low concern from a public health point of view.

4.6. Australia/New Zealand

The Food Standards Australia New Zealand (FSANZ) has asked honey producers in Australia/New Zealand to mix honeys that are mainly derived from E. plantagineum (Paterson’s Curse/Salvation Jane) to blend it with other honeys to achieve the required limits (FSANZ, 2004). This recommendation seems counterproductive to the general desire to reduce the overall level of
PAs in the human food chain. Instead it would be more reasonable to take highly contaminated honeys off the market rather than to contaminate additionally PAs free honeys. This measure is also in contrast to the EFSA directive of non-dilution of contaminated food and feedstuff (EFSA, 2007).

4.7. USA

In 2001, US Food and Drug Administration (FDA) advised manufacturers of oral preparations derived from *Symphytum* spp. or products containing PAs from other sources to withdraw these from the market. Further, FDA exerted its authority to confiscate these products if necessary. According to FDA the decision is based on the clear relationship between PAs and literature reports on serious adverse health effects. Due to insufficient data, however, the authority finds itself unable to set a safe level for oral exposure.

In Canada health officials have also banned the sale of some comfrey products (Rode, 2002). For grains and pulses, the Codex Alimentarius states that toxic seeds in wheat should not be present in amounts that represent a hazard to health and mentions specifically the presence of *Crotalaria* seeds (Knutsen et al., 2017). These standards are: Maize (corn), certain pulses, sorghum grains, wheat and durum wheat, oats.

4.8. WHO

In 1988 WHO published a program on “Environmental health criteria for pyrrolizidine alkaloids” and pointed out the potential health risk from PAs contamination of the food chain (WHO, 1988). Thus, the main hazard of serious PAs intoxications is caused by grain contaminated with PAs seeds and the use of PAs plants in traditional folk remedies. Based on the observed genotoxic potential of PAs, the commission recommended reducing the contamination in the human food chain to the lowest possible degree. Therefore, the WHO advice of monitoring the PAs levels of honey and dairy products from regions with known high PA frequency could be an approach to achieve this goal. A systematic review has been evaluated by a WHO task group on environmental health criteria for PAs (coordinated by the International Programme on Chemical Safety [IPCS]) and by the International Agency for Research on Cancer (IARC) (Knutsen et al., 2017).
5. Perspectives

Though several countries and authorities have tried to establish regulations to restrict the exposure to PA-containing food and medicinal herbs, these regulations are only based on case studies and cannot be applied universally to all PAs. It is difficult to determine a toxic dosage threshold for different types of PAs as even within the same PAs type, different PAs may have varied potencies in inducing toxicity. Therefore, a systematic assessment system is needed for predicting the potency of different PAs and detecting the toxicity of PA-containing foods and medicinal herbs for establishing the appropriate regulations of the amount of PAs allowed in these PA-containing natural products. As for detection method, how to establish a fast and efficient method for a short time of a large sample of filter is still needed, such as how to quickly give the results of suspected PAs samples on the market.

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Conflict of interests

The authors declare no competing financial interest.
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Determination and regulation of hepatotoxic pyrrolizidine alkaloids in food:

A critical review of recent research

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Abstract

Pyrrolizidine alkaloids (PAs) are secondary metabolites of plants. PAs have been reported to be hepatotoxic, mutagenic, and carcinogenic; they are a significant group of natural toxins affecting livestock, wildlife, and humans. To date, over 10,000 PAs poisoning cases have been reported worldwide. In recent years, many articles have reported the detection of PAs in various foods, including honey, milk, meat, eggs, tea and salad. This review summarized the contamination of PAs in foods, state of the art detection methods and regulations by different countries and authorities, hoping to propose effective solutions to minimize the consumption of PAs in food.

Keywords: Pyrrolizidine alkaloids (PAs); Food; Determination; Regulation
1. Introduction

Pyrrolizidine alkaloids (PAs) are naturally occurring heterocyclic phytotoxins that are widely distributed in about 3% of the world's flowering plants (Fu et al., 2004, EFSA, 2007). To date, more than 660 PAs and their N-oxide forms (PANOs) have been identified in over 6000 plants. Most of these plants belong to the Asteraceae, Boraginaceae, Orchidaceae, and Fabaceae families, and half of them have been reported to be hepatotoxic (Yang et al., 2001, Zhu et al., 2017, He et al., 2017). PAs are esters of three types of necine base: Retronecine type, otonecine type, and platynecine type. The former two with the necine base having a double bond at the C1 and C2 positions exhibit high levels of toxicity, while platynecine type PAs with a saturated necine base (without a double bond) are either weakly toxic or nontoxic (Figure 1) (Fu et al., 2004, Ruan et al., 2014a). The available information indicates that the adverse effects of 1, 2-dehydropyrrolizidine alkaloids (dehydroPAs) in experimental animals include hepatotoxicity, developmental toxicity, genotoxicity and carcinogenicity (EFSA, 2011a, Li et al., 2011, Lin et al., 2011, Yang et al., 2016, Fu et al., 2017a, Fu et al., 2017b, Zhu et al., 2017). Acute poisoning with PAs in humans is associated with liver damage, whereas a sub-acute or chronic onset may lead to liver cirrhosis and pulmonary arterial hypertension (Li et al., 2018, EFSA, 2011a). Compared with herbs, PAs are more widespread, more serious and more difficult to control in food. There is now increasing recognition that some widely consumed foods (e.g. grains, milk, meat, eggs, honey, pollen) are sometimes contaminated by PAs and PANOs at levels that, while insufficient to cause acute poisoning, greatly exceed maximum tolerable daily intakes and/or maximum levels determined by a number of independent risk assessment authorities (Edgar et al., 2011).

Insert Figure 1 here

In Europe, an analysis was done of a total of 1105 samples collected. These comprised milk and milk products, eggs, meat and meat products, teas, and food supplements collected in supermarkets, retail shops, and via the internet. PAs were detected in a large proportion of plant-derived foods: 60% of the food supplements and 92% of teas contained measurable amounts of PAs. As for animal-derived products, 6% of milk samples and 1% of egg samples contained PAs (Mulder et al., 2018). In Hong Kong, a total of 234 samples (48 food items) were collected randomly from a local market and analyzed. About 50% of samples were found to contain detectable amount of PAs (Chung...
This review summarized the current global situation with regard to the presence of PAs in food, the method of detecting PAs content, and the regulation of PAs by various countries and authorities. It is hoped that more effective solutions to minimize the consumption of PAs can be developed on this information.

2. PAs in food

2.1. Bee products – honey and pollen

Apiarists in many countries regularly use a number of PA-containing plants for honey production (Edgar et al., 2002). Kempf et al. have demonstrated that honeys from many of these plants contain significant levels of PAs (Kempf et al., 2008).

It has been suggested that the PAs found in honey may have been introduced via pollen accidently dislodged into nectar, e.g. by nectar-collecting bees (Boppre et al., 2005). Pollen from PA-containing plants contains extremely high levels of PANOs (Kempf et al., 2010a). Apiarists too may accidently or deliberately introduce pollen into honey during or after harvesting (Edgar et al., 2011). The levels of PAs and PANOs found in many honeys could, according to published risk assessments, cause chronic diseases such as liver cirrhosis, pulmonary hypertension and cancer if these honeys are regularly consumed at recommended serving sizes of 15–25 g or at higher consumption levels reported by honey consumers in many countries. For example, in Australia the highest consumers of honey in the 2–4 years old age group eat 28.6 g of honey per day while older consumers, 5–65 years of age, eat 40–65 g per day (Edgar, 2011). It has been reported that a woman who consumed 20–30 mg of PAs per day, similar to those occurring in Echium honey, during her pregnancy gave birth to a child suffering fatal liver damage (Rasenack et al., 2003).

Bee pollen granules in food supplements contain nectar (used by bees as a binding agent) as well as pollen grains. A recent study that investigated PAs in bee products in Europe (Mulder et al., 2018) found that in eleven of the twelve pollen products, PAs were detected at a mean concentration of 576.0 μg/kg, while 0.6 and 15.5 μg/kg were quantified in propolis and royal jelly products.

2.2. Grain contamination

Mass intoxications have arisen from the use of contaminated grain (Tandon et al., 1976,
The earliest case termed ‘bread poisoning’ dates from 1920 (Willmot and Robertson, 1920) and more recent cases have occurred in both Afghanistan and Ethiopia in 2008 (Molyneux et al., 2011). Nowadays, episodes of acute dehydroPAs toxicity involving the contamination of bread are avoided in many countries by more effective control of weeds in crops and by strictly applying food safety standards limiting the number of foreign seeds in grain entering the human food chain, including some known to contain dehydroPAs (Edgar et al., 2015). However, it has been shown that complete removal of seeds containing PA from heavily contaminated grain still leaves readily detectable levels of PAs in the ‘cleaned’ grain and such grain has been shown to be capable of poisoning pigs (Edgar et al., 2011). Fine plant dust, generated during harvesting and adhering to the grain, is thought to be the source of the PAs (Edgar, 2003). Azadbakht et al. have found levels of PAs in wheat and flour in Iran that raise concerns for chronic toxicity (Azadbakht and Talavaki, 2003). Similar studies are needed in other countries before the contribution of PAs in grain-based products can be assessed as a potential cause of slowly progressing chronic poisoning of humans. It is normal practice in countries undertaking large scale cereal production to combine grain from many farms and from different cropping areas. Such bulking dilutes and reduces the level of dehydroPAs in the grain, but it results in a wider distribution of any dehydroPAs that were present and thus increases the population exposed to low levels of these hazardous chemicals in grain-based foods (Edgar et al., 2015). While these measures and practices are apparently sufficient to prevent the acute dehydroPAs poisoning, it is still possible that occasional, low level dietary exposure in grain-based products could be contributing worldwide to the incidence of several slowly developing, chronic diseases (Azadbakht and Talavaki, 2003).

2.3. Milk contamination

A number of experiments on milk transfer of PAs in rats and mice have been performed (Edgar et al., 2011). In the earliest of these demonstrated that on administration of the PAs lasiocarpine and retrorsine to lactating rats their suckling offspring died with distinct liver lesions, although the alkaloids had no apparent effects on the mothers. The author pointed to the possibility that various liver disorders in childhood may be the result of poisoning by PAs in milk. Although the N-oxides produced in the liver are normally rapidly excreted in the urine, in lactating animals a certain proportion may be sequestered in the aqueous phase of milk. The possibility that the ultimate toxic
metabolites, dehydroretronecine and dehydroheliotridine, are also transferred into milk should be considered too, based on their relatively high water solubility.

In recent years, PAs have been frequently detected from the real milk samples. For example, PAs were detected from the real milk samples in Europe market (Mulder et al., 2018). In 11 out of 182 (6.0%) milk samples the presence of one or two PAs could be confirmed above the limit of detection (LOD). PAs residues were found in milk from 4 different countries (Spain, Germany, Greece, and the Netherlands) and in all major types of milk regarding fat content and process of conservation. Other studies have found similar results in milk (Huybrechts and Callebaut, 2015).

2.4. Chicken egg contamination

Transfer of PAs from feed to eggs has been shown to occur (Edgar and Smith, 2000, Eroksuz et al., 2003, Diaz et al., 2014). Diaz et al. reported that the residues found in eggs were primarily of the PA free base type with only a very minor contribution of PANOs (Diaz et al., 2014). Edgar et al. reported levels of 5–168 mg/kg PAs in eggs. In this case, the layer hens had been inadvertently poisoned by *Heliotropium europaeum* and *Echium plantagineum* contamination in the grain component of their feed (Edgar and Smith, 2000).

In another study in Europe market, contamination of eggs with PAs was found in two samples out of 205 analyzed, with levels at 0.1–0.12 μg/kg. The PAs found are similar to the ones found in milk. (Mulder et al., 2016, Mulder et al., 2018).

2.5. Meat contamination

Various PA-containing herbs may be present in pastures and fields where animals are foraging. They may also be present in fresh or dried products fed to animals, as shown in particular for alfalfa, a widely used feed product (EFSA, 2011a). When consumed by animals, part of the PAs will be transferred to animal-derived food products (Dickinson et al., 1976, Edgar and Smith, 2000, Hoogenboom et al., 2011). Although the transfer and levels in animal-derived products seem relatively low, exposure via such products may still be relevant. This is due to the fact that various PAs were shown to contain genotoxic and carcinogenic properties, meaning that even intake of low levels by humans may result in adverse effects (Edgar et al., 2015).

There is very limited information on the transfer of PAs to meat. Experiments have been
reported in which puppies were fed cooked meat from animals poisoned by a dehydroPA-containing species of *Trichodesma*. This resulted in death or production of irreversible pathological changes within 3–4 months and it was concluded that the meat contained toxic alkaloid residues that were not destroyed by cooking (Shevchenko and Fakhrutdinova, 1971). Recently, a study conducted PAs transfer study with eggs and meat indicate that the intake of PA-containing herbs by laying hens may result in levels in eggs and meat that could be of concern for consumers, and as such should be avoided (Mulder et al., 2016).

2.6. Salads, teas and condiments

Some leafy PA-containing plants have been, and in some cases are still being, recommended as salads. It is well known that the leaves of the common weed *Senecio vulgaris* accidentally co-occurred with salad leaves of similar appearance being sold in supermarkets in Germany (BfR, 2007). PA-containing plants are also recommended for making teas and sauces. PAs have also occurred in a cooking spice that was implicated in the death of a late-term foetus that died of liver failure (Rasenack et al., 2003).

Bodi et al. analyzed a total of 274 dry tea samples available on the German market, including, amongst others, 24 black, 23 green, 24 rooibos, 29 peppermint, 39 chamomile and 43 mixed teas, for the presence of 10 different PAs and 7 different PANOs with LC-MS/MS. The percentage of positive teas varied between 86% (peppermint teas) to 100% (rooibos teas). As in this study, rooibos tea was found to be the most highly contaminated (mean: 1856.4 μg/kg, maximum: 5647.2 μg/kg) (Bodi et al., 2014).

Schulz et al. used LC-MS/MS to analyze 169 medicinal teas, which were commercially available on the German market, for the presence of 14 different PAs and 9 different PANOs (Schulz et al., 2015).

Griffin et al. analyzed 18 herbal dry teas available from the Irish market using LC-MS/MS. The method included 10 PAs and 4 PANOs and the LOD in dry tea is 0.4-1.5 μg/kg. The mean and maximum contamination is 210 and 1733 μg/kg respectively (Griffin et al., 2014).

The study of Mathon et al. focused on the PAs content of 70 teas purchased from the Swiss market that were analyzed for 9 PAs by LC-MS/MS. Results were expressed as amount of PAs/cup of infusion (200 ml). Limit of quantification (LOQ) reported were 0.02 μg/cup, which corresponds to
approximately 10 μg/kg in dry tea. It was reported that 70% of the tea infusions contained one or more PAs above the LOQ (Mathon et al., 2014).

A recent study shows that contamination of all types of tea with PAs is very common (Mulder et al., 2018). In the majority of samples (91%) one or more PAs were detected. All types of teas appear to contain PAs, although the concentrations differed between the various types of tea. Highest contamination, with regard to maximum, mean and median concentration, was observed in rooibos tea.

Tea samples were further evaluated concerning their content of individual PAs. With respect to contribution to the mean content in tea infusions, senecionine-N-oxide is the most important compound with an average concentration of 1.74 μg/L, which makes up 28% of the total PAs concentration (6.13 μg/L) found in tea. The PAs of the senecionine group account for over 77% of the PAs content in tea, while PAs of the lycopsamine group contribute 14%, and heliotrine-type PAs contribute 8%. Approximately one third of the content of PAs in the tea samples is made up by PAs free bases and two thirds by PANOs. Summary of literature on PAs in food was shown in Table 1.

Insert Table 1 here

3. Method of analysis

Many different foods have been analyzed for PAs in the past, and most of the common analytical techniques were applied in the detection of these compounds (Crews et al., 2010). Hence, this part will focus on the most recent and most common techniques used for the trace analysis of PAs in complex matrices like foods.

3.1. Sample preparation

3.1.1. Extraction

Generally, considering the co-occurrence of PAs and corresponding PANOs, the extraction method has to ensure the efficient simultaneous extraction of both and therefore classical alkaloid extraction with semi-polar to polar organic solvents or acidified aqueous conditions are prevalent. PANOs are polar molecules that can be readily extracted by polar solvents such as methanol or diluted aqueous acids (Crews et al., 2010).
3.1.1.1 Animal-derived food products

After thoroughly shaken and homogenization, animal-derived food samples (like milk, yoghurt, reconstituted infant formula milk, eggs, meet, liver) were transferred to polypropylene tubes and internal standard solution (like epijacobine of 1000 ng/mL in methanol) was added (Mulder et al., 2016). Formic acid solution (0.2%) and hexane were added to the tubes. The samples were extracted on a rotary tumbler and then centrifuged. The hexane top layer and (most of) the solid middle layer (containing mostly fat and non-soluble proteins) were removed by suction. Concentrated ammonia (25%) was added to adjust the pH of the solution to 9-10, and then the samples were centrifuged.

Remaining aqueous extract was used for further clean-up by solid-phase extraction (SPE) over a Strata™ X. The cartridges were conditioned with methanol and ammonia solution (0.1%). The cartridges were loaded with extract, washed with ammonia solution (0.1%) and dried under vacuum (using a vacuum manifold) for 5-10 minutes. PAs were eluted from the cartridges with methanol. The eluates were dried under a nitrogen stream in a warmed water bath and reconstituted in methanol/water (10/90, v/v). The reconstituted sample extracts were filtered using filter vials.

3.1.1.2. Plant-derived food products

Tea samples were mixed with dry ice (at a mass ratio of 2:1) (Mulder et al., 2016). The mixture was allowed to stand for about 3 minutes while stirring repeatedly. The frozen sample was ground to a particle size of 500 μm using an ultra-centrifugal mill. The aggregate sample was homogenized by overhead-shaking for 2 hours. The extraction procedure was based on the protocol for the preparation of ready-to-drink products described in ISO 3103. Tea in a tea infusion bag was placed in a beaker and extracted with boiling water. Infusion was steeped for 5 minutes after which the tea bag was removed. After cooling down, the infusion was filtered through a fluted filter paper.

The SPE clean-up was carried out with reversed phase C18 SPE cartridges (Discovery® DSCC18 500 mg/6 mL), which were conditioned with methanol and water. Then, the cartridges were loaded with tea infusion, washed with water and dried under vacuum (using a vacuum manifold) for 5-10 minutes. PAs were eluted from the cartridges in two steps with each of methanol or 2.5% (1.4 M) ammonia in methanol in the case of black and green tea samples.
3.1.1.3. Food supplements and bee products

For dry food supplements and food supplements containing bee pollen products, extracted with aqueous sulphuric acid solution by ultra-sonication (Mulder et al., 2018). The supernatant was decanted after centrifugation. Extraction was repeated and combined supernatants were brought to pH 6-7 with diluted ammonia solution and passed through a folded filter paper, with further clean-up by SPE.

The SPE clean-up was carried out with reversed phase C₁₈ SPE cartridges, which were conditioned with methanol and water. Then, the cartridge was loaded with sample extract, washed with water and dried under vacuum. PAs were eluted from the cartridge in two steps with methanol. The combined eluates were dried under a nitrogen stream in a heated water bath and the dried residue was reconstituted in methanol/water (5/95, v/v). The reconstituted sample extract was filtered through centrifuge filters before further analysis.

For oily food supplements, PAs were extracted with sulfuric acid in methanol by overhead shaking. The supernatant was decanted after centrifugation. Extraction was repeated, and an aliquot of combined supernatants was used for further clean-up by SPE.

3.2. Instrumental analysis

After extraction, the PA-containing food samples are detected by instruments. The methods that have become the most established ones will be briefly summarized below.

3.2.1. Spectrophotometry and thin layer chromatography (TLC)

The spectrophotometric detection for dehydroPAs and PANOs (with the exclusion of otonecine-type PAs) is based on a color reaction. The tertiary PAs are oxidized to the corresponding PANOs, dehydrogenated to the pyrroles and subsequently coupled with Ehrlich’s reagent to form a magenta colored dye which can be measured spectrophotometrically at 563 nm and shows detection limits in the mg/kg range (or 1 μg total dehydroPAs). An alternative approach is to use aqueous methyl orange, which forms a yellow complex that is very soluble in organic solvents. Methyl orange is released from the organic solution on treatment with strong acid, and measured with a spectrometer (Dwivedy et al., 2017). These methods are not able to quantify individual PAs but allow determination of the total dehydroPAs content by construction of a calibration series using the readily available standards.
TLC with both silica and aluminium oxide adsorbents has been used to separate PAs, and many procedures have used ion pairing with lithium or potassium chloride or sodium iodide to enhance separation. For detection of PAs on TLC plates Erlich’s reagent is the most specific spray reagent used (Crews et al., 2010). DehydroPAs bases are oxidized to the PANOs with hydrogen peroxide, and these are reduced to dehydropyrrolizidine by heating with acetic anhydride or o-chloranil, whereupon the pyrrole gives a violet–blue dye. Ehrlic’s reagent is used to detect dehydroPAs and Dragendorff’s reagent and nitrite were used to detect saturated PAs. High resolution is achieved by using the power of lithium chloride to act as an ion pairing reagent and reduced the analyte polarity.

3.2.2. Nuclear magnetic resonance (NMR)

NMR methods for PAs analysis have been used mainly for structural identification. They are predominantly used in the structural elucidation of purified PAs and comprehensive collections of $^1$H and $^{13}$C NMR data are available (Roeder and Pflueger, 1995). Quantitative determination of dehydroPAs is typically achieved by $^1$H NMR spectroscopy.

Signals originating from the vinylic C-2 hydrogen resonate at 6.2 ppm and 5.8 ppm for macrocyclic diesters and non-cyclic mono or diesters, respectively. Using appropriate internal standards (like paradinitrobenzene) allows the determination of the total dehydroPAs level of an extract (Molyneux et al., 1979).

Comprehensive tables of spectral data of the analysis of PAs by NMR have been published for $^{13}$C NMR spectroscopy and for $^1$H NMR (Maslennikov et al., 2010). Brief overviews of the $^1$H NMR of retrorsine, seneciphylline and senecionine has been published (Kim et al., 1993). $^1$H NMR can provide qualitative information more rapidly and from a smaller sample than $^{13}$C NMR, which gives more structural information. The shifts due to protons on the necine base are very distinctive and permit recognition of unsaturation and oxygenation. The necic acid proton shifts are less distinctive because the acids have similar structures. There is now scope for applying the much greater sensitivity available in modern high-field NMR instruments to PAs analysis.

3.2.3. Gas chromatography-mass spectrometry (GC-MS)

Free base PAs can be separated by GC and can be identified on the basis of relative retention times using different stationary phases (Crews et al., 2010). GC can be applied to most PAs except
otonecines, and it can cause thermal decomposition and the formation of diesters from monoesters (Mandic et al., 2015). The PANOs cannot be analysed by GC as they are unstable at the temperatures required for volatilisation.

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

For PAs analysis, GC is most commonly used in combination with mass spectrometric detection in electron impact (EI) mode. The mass spectra of PAs are dominated by signals unique for the necine base part of the molecule. The distinct fragmentation pattern of dehydroPAs can be used to set up dual MS detection methods (alternate switching of single ion monitoring (SIM) and scan modus: SIM/Scan) which allow fast and reliable detection of PAs in complex matrices.

To overcome volatilization, separation and peak shape difficulties of multi hydroxylated PAs, several derivatisation reagents have been used. The most common ones are the boronate derivatives for vicinal diols or trimethylsilyl ethers or the combination of both, which form bonds across vicinal diol groups (Mandic et al., 2015). Derivatisation has also been used to enable GC determination of the retronecine base, by which means the total PAs content of a sample can be measured chromatographically by comparing the signal intensity with that of a standard prepared from an available PAs such as monocrotaline or retrorsine. For this purpose the heptafluorobutyrate derivative of the retronecine base has been prepared (Kowalczyk and Kwiatek, 2017).

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

Kowalczyk et al. proposed a first method for the determination of PAs in feed matrix involving GC-MS. The sum parameter method approach to determine the content of PAs in feeds (Kowalczyk and Kwiatek, 2017).

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

3.2.4. Liquid chromatography-mass spectrometry (LC-MS)

High performance liquid chromatography (HPLC) and LC separation of PAs is attracting more and more interests (Carlsson and Tornqvist, 2016). LC offers the advantage of a simultaneous detection of PAs and PANOs that means fewer steps of sample preparation and a reduced risk of alteration of the analytes (Bushway et al., 1994). Because PAs and PANOs do not show characteristic ultraviolet (UV) spectra (exhibiting only a non-specific UV-maximum of 214 nm), simple UV detection is of limited value. However, LC techniques are frequently and successfully used to purify PAs for structural identification including the enantioseparation of isomers like intermedine/lycopsamine (Narberhaus et al., 2004, Kashyap et al., 2010).

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

In 2014, sample extracts were accurately analyzed by using LC-ESI-MS/MS applying for the first time a pentafluorophenyl (PFP) core-shell column to the chromatographic separation of PAs and PANOs (Griffin et al., 2014). Furthermore, Griffin et al. also proposed a new and rapid isocratic LC-MS/MS method to purchase and analyze the targeted PAs in honey, which developed from, and is comparable with, a gradient elution method and resulted in no loss of sensitivity or reduction in chromatographic peak shape. Isocratic elution allows for significantly shorter run times (6 min), eliminates the requirement for column equilibration periods and, thus, has the advantage of facilitating a high-throughput analysis which is particularly important for regulatory testing laboratories (Griffin et al., 2015).

Yoon et al. developed a rapid and sensitive analytical method for the determination of 9 toxic PAs in popular high-lipid foodstuffs by LC-ESI-MS/MS. PAs in lipid foodstuffs were effectively purified by freezing lipid precipitation (FLP) and SCX-SPE (Yoon et al., 2015).

Betteridge et al. optimized a previously published methods (Betteridge et al., 2005), which is HPLC-MS method for the quantification of four PAs and one PANO in PA-containing plants and honey. A Waters Alliance 2965 LC system coupled to a Waters Micromass Quattro Micro triple quadrupole mass spectrometer was used (Mudge et al., 2015).

Zhu et al. proposed a rapid, selective, and sensitive ultra-high performance liquid chromatography-quadrupole time-of-flight mass spectrometry (UHPLC-QTOF–MS) method for the estimation of RET-PAs in herbs without requiring corresponding standards. This method is based on our previously established characteristic and diagnostic mass fragmentation patterns and the use of retrorsine for calibration (Zhu et al., 2016).

In another very recent study an online two dimensional liquid chromatography quadrupole time-of-flight mass spectrometry (2D-LC QTOF-MS) method was developed to resolve isomeric PAs and PANOs. In this method, a polar endcapped C_{18} column was used at pH 3 in the first dimension, and a cross-linked C_{18} column at pH 10 was used in the second dimension (van de Schans et al., 2017).
Zhang et al. used a new UPLC-Q-Orbitrap/MS method to analyse PAs in *Crotalaria sessiliflora* L. Coexisting PAs were identified by mass data of full MS-dd-MS2 based on the characteristic fragmentation pattern and necine-core structure. Moreover, quantification of PAs was conducted by parallel reaction monitoring (PRM) mode using m/z 138, m/z 120 and m/z 94 from identical necine-core structure as quantitative ions with a single monocrotaline standard for accurate calibration (Zhang et al., 2017).

Applying an UPLC-MS/MS spectrometry method, Zheng et al. *divaricata* from five different Chinese locations and their specific PAs were qualitatively characterized. Using a pre-column derivatization HPLC method, the total retronecine ester-type PAs in their alkaloids extracts were quantitatively estimated as well (Zheng et al., 2017).

For the PANOs, Ruan et al. discovered characteristic ion clusters as unique determinants to discriminate PANOs from PAs even without the availability of reference samples. The found that the mass spectra of toxic retronecine-type PA N-oxides exhibited two characteristic ion clusters at m/z 118-120 and 136-138 by HPLC-MS. Similarly, the nontoxic platynecine-type PANOs also fragmented via three similar pathways to form two characteristic ion clusters at m/z 120-122 and 138-140. Further application of using these characteristic ion clusters allowed successful and rapid identification of PAs and PANOs in two PA-containing herbal plants (Ruan et al., 2012).

### 3.3. Immunological methods

Immunology-based tests are wildly used and significant laboratory methods have been developed for rapid, selective and sensitive detection of analytes in complex matrices. It is well known that PAs are non-toxic without metabolic activation (Chen et al., 2016). All dehydroPAs are metabolically activated to the reactive pyrrolic esters, which have the same core pyrrole moiety regardless of structures of the parent PAs (Zhu et al., 2015, Zhu et al., 2016). Once formed, the pyrrolic metabolites can rapidly react with proteins via covalent binding to form pyrrole-protein adducts (Ruan et al., 2015, Gao et al., 2015, Li et al., 2015, He et al., 2017b). It has been suggested that these adducts trigger the PA-induced toxicities (Wang et al., 2016, Xia et al., 2016, Li et al., 2016). Immunoassays offer a useful tool to detect adducted protein in experimental animals and exposed humans. Using a common structural feature of many toxic PAs, the necine base retronecine, researchers have demonstrated that retronecine-protein conjugate raised antibodies can detect
retronecine but also monocrotaline in a competitive inhibition enzyme-linked immunosorbent assay (ELISA) system (Bober et al., 1989). They also developed the first compound-specific antibodies, i.e. antibodies specific the PAs monocrotaline, which have been demonstrated able to detect quaternised monocrotaline (IC$_{50}$ = 0.25 ppm at pH 7.6), N-methylated monocrotaline (IC$_{50}$ = 5.3 ppm at pH 7.6), and protonated monocrotaline (IC$_{50}$ = 6.0 ppm at pH 6.0).

Roeder et al. reported a successful analysis of the concentrations of sencionine and integerrimine together with their N-oxides as present in zinc-dust reduced methanolic extracts of Senecio rupestris using antibodies to a retrorsine hemisuccinate-BSA conjugate (Roeder and Pflueger, 1995).

Lee and co-workers developed ELISA methods to detect and quantify PAs in bovine blood and plants. They developed two different systems to detect riddelliine and riddelliine N-oxide, investigated their cross reactivities to a number of other PAs (Lee et al., 2001).

Finally, a method producing monoclonal antibodies against retrorsine conjugated to bovine-thyroglobine should be mentioned, Zündorf et al. obtained antibodies with a certain affinity to 8 different PAs and no cross reaction to 12 other such compounds (Zundorf et al., 1998).

Charlemroj et al. developed and validated a rapid multiplex ELISA for the detection of a range of PAs and PANOs in feed and honey for the first time. The method is inexpensive, and thus appropriate as a screening option prior to GC-MS/LC-MS confirmation for detection of PAs contaminants in food and feed (Charlemroj et al., 2013).

Although the mechanisms of PA-induced toxicities have not been fully elucidated, it is well accepted that the reactive pyrrolic metabolites are associated with the toxicities of PAs (Ruan et al., 2014b). The immunochemical approach has been successfully developed to identify target proteins modified by reactive metabolites of many compounds, such as acetaminophen (Roberts et al., 1987, Bartolone et al., 1992), halothane (Vergani et al., 1980, Satoh et al., 1985), bromobenzene (Kleiner et al., 1998), naphthalene (Zheng and Hammock, 1996), trichloroethylene, and styrene (Yuan et al., 2007, Shen et al., 2009). Li et al. develop polyclonal antibodies to detect pyrrole-protein adducts modified by the reactive metabolites of PAs (Li et al., 2017). According to the authors, this approach can detect pyrrole-protein adducts resulting from exposures to a range of toxic PAs (Yang et al., 2017).
3.4. Summary of analytical methods

Colorimetric detection or TLC separation in combination with PAs detection via Ehrlich’s reagent might still be suitable methods for dehydroPAs screening, but they lack sensitivity and selectivity for the trace analysis of PAs in complex matrices. As for immunological approaches, they usually are sensitive only for a narrow range of structurally closely related PAs. The GC-MS and LC-MS have been compared in detail (Kempf et al., 2010a). In brief, compares to LC-MS, GC-MS is unlikely to miss toxic relevant PAs and not significantly dependent on standards, has true internal standard quantification, simple quantification, and can be easily adapted to different samples and backgrounds (Aguero et al., 2011). Furthermore, GC-MS can be adapted to stable isotope dilution analysis and has no need of background information for samples or PAs involved. As for LC-MS, PANOs and tertiary PAs can be determined simultaneously; the proportion of each individual structure can be known which might be necessary for further toxicological investigations; sample preparation is relatively easy and turnaround times are fast. Also, the information it produces can be used in other contexts. For example, PAs plant patterns and ratio tertiary PAs/PANOs can be linked to geographic origin. Currently, both approaches supplement each other, which are approximations to quantify PAs in complex mixtures.

The application quantity of various detection methods in PAs was shown in Figure 2. The typical application of different methods for the detection of PAs in PA-containing analytes were shown in Table 2.

Insert Figure 2 here
Insert Table 2 here

4. Regulation

As PAs has been demonstrated to be a health threat for both humans and livestocks, many countries and authorities have set various limitations for PAs as summarized in Table 3.

We summarized the legal provisions of different countries and national organizations on the limitation of PAs. Our list contains all information that we were able to access but does not claim to be complete. The limited information available from human poisoning cases allowed identifying a lowest known dose of approximately 2 mg/kg bw per day associated with acute/short-term effects. This was based on a case of a 6-month-old girl who received a daily dose of approximately 0.8–1.7 mg PA/kg bw for 2 weeks and was diagnosed for hepatic veno-occlusive disease (HVOD), and a
2-month-old boy who was administered an approximate dose of 3 mg/kg bw for 4 days, with a fatal outcome (Knutsen et al., 2017).

Insert Table 3 here

4.1. Germany

Since 1992, PA-containing phytopharmaceuticals have been regulated by a Federal Pharmaceutical Ordinance. According to these regulations, only a few proven active PAs plants and preparations thereof, which are listed by name, can be marketed. After an intensive risk assessment and expert hearings, the authorities justified the regulation on the grounds that these actions would substantially reduce the risk associated with the ingestion of PAs. In addition, the package insert for orally used products needs to contain the warning notice “do not use during pregnancy or lactation” (BfR, 1992). In Switzerland the same regulations for phytopharmaceuticals as in Germany are in force.

In 2007, Bundesinstitut fur Risikobewertung (BfR) started to reevaluate and where applicable to complement the zero tolerance principle of the European Union for certain compound and compounds classes in food and feeding stuff (BfR, 2007). In this context, the zero tolerance principle should be applied if the risk cannot be calculated or if limits of exposure cannot be derived because of the lack of valid scientific data. In many cases zero tolerance should be applied if the compound possesses carcinogenic or mutagenic potential.

In 2013, the BfR began conducting a research project on the “Determination of Pyrrolizidine Alkaloids in Food and Feed”. Based on the available data, the BfR concludes that those adults and children who frequently consume of herbal tea infusions are possibly at increased cancer risk, particularly if they consume products with high PAs contamination over long periods of time (BfR, 2013).

The BfR once again recommends that the total exposure of consumers to genotoxic and carcinogenic PAs from various food sources should be kept as low as possible.

In order to avoid the marketing of contaminated batches and to protect the consumer, it is recommended that herbal tea batches that are to be brought onto the market be checked for PAs content before distribution.
4.2. UK

In the past, similar to the German regulation for herbal remedies, comfrey and preparations thereof were banned in the UK. In 2007, the UK Committee on Toxicity (COT) was asked for a statement of PAs in food, in particular in honey and milk (COT, 2007). The statement comprehensively summarized the literature data on all aspects concerning PA toxicology like hepatotoxicity, pulmonary toxicity, carcinogenicity and genotoxicity.

4.3. Austria

The legal situation in Austria affecting PAs in herbal remedies is similar to that in Germany regulations. Only a few PAs plants are authorized. These plants or preparations thereof can only be marketed if they are analyzed by a “...state of the art detection method” which proves that “... the final product does not contain pyrrolizidine alkaloids.” (Kempf et al., 2010b).

4.4. The Netherlands

It shows clearly that the regulations of the neighboring countries Austria and the Netherlands are many times more stringent than the German regulation. Recently, the Dutch National Institute for Public Health and the Environment (RIVM) suggested that the consumption of honey with elevated PAs levels should be avoided; therefore, it said that studies should be intensified to be able to further minimize the risk. It is concluded there, with no warning notice for the consumers deemed necessary (Krauterbeschluss, 2001).

4.5. Europe

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

In 2010, EFSA concluded that dehydroPAs may act as genotoxic carcinogens in humans, and there is a possible health concern for those toddlers and children who are high consumers of honey (EFSA, 2011b).

In 2016, EFSA reported that chronic and acute dietary exposure to PAs was estimated in the European population via the consumption of plant-derived foods. This resulted in the highest estimates of mean chronic dietary exposure of 34.5–48.4 ng/kg body weight bw per day in ‘Toddlers’ (LB–UB) and 154–214 ng/kg bw per day in the highly exposed population (EFSA, 2016).

In 2017, the Contaminants in the Food Chain (CONTAM) Panel updated the benchmark dose (BMD) analysis of the available long-term studies on lasiocarpine and riddelliine as determined in its previous risk assessment (Knutsen, 2017). Using model averaging, the Panel calculated the BMD confidence interval and selected the benchmark dose modelling(BMDL)10 of 237 μg/kg bw per day for increase in the incidence of liver haemangiosarcoma in female rats exposed to riddelliine as the reference point (RP) for chronic risk assessment. Considering the data on honey, tea, herbs and food supplements in 2016, the EFSA Scientific Committee concluded that, for substances that are both genotoxic and carcinogenic, a margin of exposure (MOE) of 10,000 or higher, based on a BMDL10 from an animal study, and taking into account overall uncertainties in the interpretation, would be of low concern from a public health point of view.

4.6. Australia/New Zealand

The Food Standards Australia New Zealand (FSANZ) has asked honey producers in Australia/New Zealand to mix honeys that are mainly derived from *E. plantagineum* (Paterson’s Curse/Salvation Jane) to blend it with other honeys to achieve the required limits (FSANZ, 2004). This recommendation seems counterproductive to the general desire to reduce the overall level of
PAs in the human food chain. Instead it would be more reasonable to take highly contaminated honeys off the market rather than to contaminate additionally PAs free honeys. This measure is also in contrast to the EFSA directive of non-dilution of contaminated food and feedstuff (EFSA, 2007).

4.7. USA

In 2001, US Food and Drug Administration (FDA) advised manufacturers of oral preparations derived from Symphytum spp. or products containing PAs from other sources to withdraw these from the market. Further, FDA exerted its authority to confiscate these products if necessary. According to FDA the decision is based on the clear relationship between PAs and literature reports on serious adverse health effects. Due to insufficient data, however, the authority finds itself unable to set a safe level for oral exposure.

In Canada health officials have also banned the sale of some comfrey products (Rode, 2002). For grains and pulses, the Codex Alimentarius states that toxic seeds in wheat should not be present in amounts that represent a hazard to health and mentions specifically the presence of Crotalaria seeds (Knutsen et al., 2017). These standards are: Maize (corn), certain pulses, sorghum grains, wheat and durum wheat, oats.

4.8. WHO

In 1988 WHO published a program on “Environmental health criteria for pyrrolizidine alkaloids” and pointed out the potential health risk from PAs contamination of the food chain (WHO, 1988). Thus, the main hazard of serious PAs intoxications is caused by grain contaminated with PAs seeds and the use of PAs plants in traditional folk remedies. Based on the observed genotoxic potential of PAs, the commission recommended reducing the contamination in the human food chain to the lowest possible degree. Therefore, the WHO advice of monitoring the PAs levels of honey and dairy products from regions with known high PA frequency could be an approach to achieve this goal. A systematic review has been evaluated by a WHO task group on environmental health criteria for PAs (coordinated by the International Programme on Chemical Safety [IPCS]) and by the International Agency for Research on Cancer (IARC) (Knutsen et al., 2017).
5. Perspectives

Though several countries and authorities have tried to establish regulations to restrict the exposure to PA-containing food and medicinal herbs, these regulations are only based on case studies and cannot be applied universally to all PAs. It is difficult to determine a toxic dosage threshold for different types of PAs as even within the same PAs type, different PAs may have varied potencies in inducing toxicity. Therefore, a systematic assessment system is needed for predicting the potency of different PAs and detecting the toxicity of PA-containing foods and medicinal herbs for establishing the appropriate regulations of the amount of PAs allowed in these PA-containing natural products. As for detection method, how to establish a fast and efficient method for a short time of a large sample of filter is still needed, such as how to quickly give the results of suspected PAs samples on the market.

Acknowledgement

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Conflict of interests

The authors declare no competing financial interest.
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FDA FDA advises dietary supplement manufacturers to remove comfrey products from the market.

US food and drug administration, center for food safety and applied nutrition.

Food standards Australia New Zealand.


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<table>
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<th>Class</th>
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Note:
<sup>a</sup>LB: Lower bond
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Notes:

- For a maximum of 6 weeks year;  
- If longer than 6 weeks year;  
- Non-cancer effects unlikely;  
- Cancer unlikely;  
- a virtually safe dose for PAs leading to an increased risk of at most one person in a million developing cancer;  
- Based on virtual safe dose of 0.43ng/kg body weight/day;  
- TDI based on avoidance of VOD, cancer risk considered not proven;  
- Zero exposure for pregnant and lactating women.

FPO: Federal Pharmaceutical Ordinance; BfR: Bundesinstitut für Risikobewertung; COC: The committee on the Carcinogenicity of Chemical in Food, Consumer Products and the Environment; COT: The UK Committee on Toxicity; RIVM: Rijksinstituut voor Volksgezondheid en Milieu; FSANZ: Food Standards Australia New Zealand; EC: European Commission; EFSA: European Food Safety Authority; ANZFA: Australia New Zealand Food Authority; FDA: US Food and Drug Administration
Figure legends

**Figure 1** The names and structures of the representative retronecine-type, otonecine-type, and platynecine-type pyrrolizidine alkaloids.

**Figure 2** The quantities of research articles on PAs determination by different methods (Data by PubMed and Google scholarship from 1977 to 2018).
Figure 1
Figure 2